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Amphibian interorder nuclear transfer embryos reveal conserved embryonic gene transcription, but deficient DNA replication or chromosome segregation

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ABSTRACT Early interspecies nuclear transfer (iNT) experiments suggested that a foreign nucleus may become permanently damaged after a few rounds of cell division in the cytoplasm of another species. That is, in some distant species combinations, nucleocytoplasmic hybrid (cybrid) blastula nuclei can no longer support development, even if they are back-transferred into their own kind of egg cytoplasm. We monitored foreign DNA amplification and RNA production by quantitative PCR (qPCR) and RT-qPCR in interorder amphibian hybrids and cybrids formed by the transfer of newt *(Pleurodeles waltl)* embryonic nuclei into intact and enucleated frog *(Xenopus laevis)* eggs. We found a dramatic reduction in the expansion of foreign DNA and cell numbers in developing cybrid embryos that correlated with reduced gene transcription. Interestingly, expansion in cell numbers was rescued by the recipient species *(Xenopus)* maternal genome in iNT hybrids, but it did not improve *P. waltl* DNA expansion or gene transcription. Also, foreign gene transcripts, normalized to DNA copy numbers, were mostly normal in both iNT hybrids and cybrids. Thus, incomplete foreign DNA replication and/or chromosome segregation during cell division may be the major form of nuclear damage occurring as a result of nuclear replication in a foreign cytoplasmic environment. It also shows that the mechanisms of embryonic gene transcription are highly conserved across amphibians and may not be a major cause of cybrid lethality.

KEY WORDS: *nucleocytoplasmic hybrid (cybrid), hybrid, interspecies nuclear transfer (iNT), nucleocytoplasmic incompatibility, triploid nuclear transfer embryos*

Introduction

Shortly after the introduction of the technique of nuclear transfer (NT) in *Rana pipiens* frogs (Briggs and King, 1952), Moore was the first to report interspecies nuclear transfer (iNT) experiments, between *R. pipiens* and *R. sylvatica* (Moore, 1958). His major observations were that cybrid development proceeded normally until the late blastula stage, but that the embryos then arrested and expired, similar to what happened when enucleated eggs of one species were cross-fertilized with sperm of the other species (Moore and Moore, 1953). Moreover, after performing back-transfer experiments in which cybrid blastomere nuclei were transferred back into enucleated eggs of the nuclear species, he observed that these were no longer able to develop beyond the late blastula stage, suggesting that the nuclei were damaged by a few rounds of division in the foreign species cytoplasm (Moore, 1958). Similar results were later obtained using two *Xenopus* species, *X. laevis* and *X. tropicalis* (Gurdon, 1962). These early studies indicated that nuclear damage during cell division in a foreign cytoplasm could be a cause of cybrid developmental arrest, although the type and

Abbreviations used in this paper: Cybrid, nucleocytoplasmic hybrid; EGA, embryonic genome activation; iNT, interspecies nuclear transfer; iSCNT, interspecies somatic cell nuclear transfer; mtDNA, mitochondrial DNA; MY, million years; NT, nuclear transfer; qPCR, quantitative PCR; SCNT, somatic cell nuclear transfer; UV, ultraviolet.

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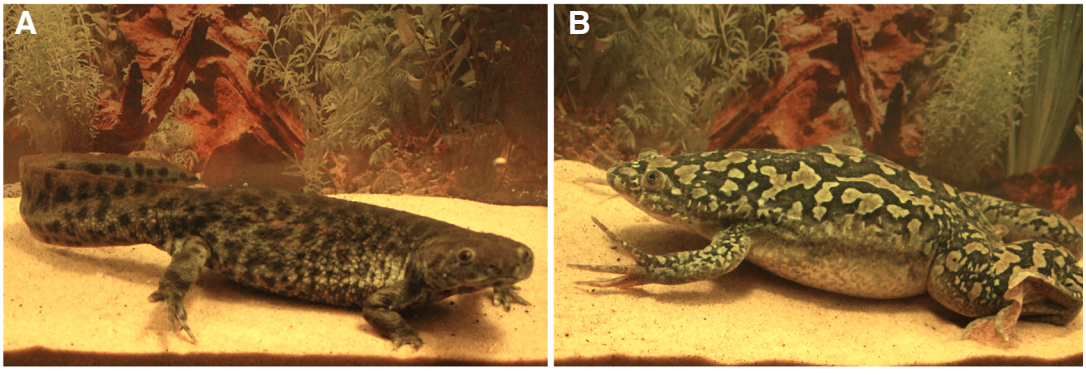
extent of damage remained unclear. It was later demonstrated that such kinds of irreversible nuclear damage does not occur in all lethal interspecific cybrids (Hennen, 1974), thereby indicating that other kinds of fatal nucleocytoplasmic incompatibilities exist between distantly related species. Indeed, there is now increasing evidence that a whole range of nucleocytoplasmic incompatibilities exists across species, and that these may include reprogramming, cellular and/or developmental incompatibilities (Narbonne *et al.,* 2012a).

The possibility of an interspecific reprogramming incompatibility that would affect cybrid survival so far remains theoretical as the major reprogramming mechanisms and pluripotency factors appear to be highly conserved, at least across the class mammalia (Narbonne *et al.,* 2012a; Rajarajan *et al.*, 2012). Indeed, even if treatment with a deacetylase inhibitor (trichostatin A) improves some aspects of reprogramming in interspecies somatic cell nuclear transfer (iSCNT), much like it does in same-species somatic cell nuclear transfer (SCNT), it does not improve survival (Shi *et al.*, 2008; Srirattana *et al.*, 2008; Lee *et al.*, 2010; Gómez *et al.*, 2011). Reprogramming defects therefore occur in most iSCNT embryos, but these defects may not be different from those occurring in same-species SCNT embryos, hence they may not be due to an interspecies incompatibility. It nonetheless remains possible that some potential reprogramming incompatibilities in cybrids may be hidden by other fatal defects. Also, certain cybrid combinations may be more susceptible to suffer from reprogramming incompatibilities than others, for example due to a divergence in the way eggs demethylate sperm (or somatic nuclei) among some mammalian orders, such as between sheep and mice (Beaujean *et al.*, 2004).

Cellular nucleocytoplasmic incompatibilities, such as those that generate permanent nuclear damage in some frog cybrid combinations (Moore, 1958; Gurdon, 1962), may exist in many cybrid cells and embryos. Indeed, in experiments where cells were isolated from cybrid embryos and grown in culture, in most cases they had a reduced proliferation and expansion capacity (Tecirlioglu *et al.*, 2006; Sha *et al.*, 2009; Narbonne *et al.*, 2012b). Also, experiments involving cultured cybrid cells generated by cell fusion suggest that there may be a nucleo-mitochondrial incompatibility between species that leads to oxidative respiration defects in cybrid cells, the severity of which correlates with evolutionary distance (McKenzie *et al.*, 2003). For example, it was found that mitochondrial DNA (mtDNA) from closely related non-human primates, including the chimpanzee and the gorilla, can rescue oxidative phosphorylation in mtDNA-less human cells, but not mtDNA from more distant primates, such as the orangutan (Kenyon and Moraes, 1997). The *in vivo* relevance of this phenomenon however remains unclear (Cannon *et al.*, 2011).

Cybrid developmental incompatibilities may include defects in embryonic genome activation (EGA), if the recipient egg cytoplasm is unable to activate embryonic transcription in the foreign nucleus (Beyhan *et al.*, 2007). Recent evidence however suggests that EGA is at least partially successful in several relatively distant and lethal cybrid embryos, in that the transcription of many embryonic genes is initiated normally (Wang *et al.*, 2009, 2011; Fujimoto *et al.*,



Summary of interclass and non-mammalian interorder nuclear transfer experiments

Donor nucleus species	Enucleated egg species	Estimated evolutionary distance (MY)	% late blastocyst or blastula	% early gastrula	% larval stage	Reference
G. domesticus (chicken)	*O. cuniculus* (rabbit)	312	9.7	0	0	Liu *et al.* 2004
G. domesticus (chicken)	*B. Taurus* (cattle)	312	3	0	0	Kim *et al.* 2004
P. waltl (newt)	*X. laevis* (frog)	320	35.8	0	0	This paper
O. nilotica (tilapia)	*C. auratus* (goldfish)	260	53.7	0.15	0	Yan *et al.* 1990
O. nilotica (tilapia)	*P. dabryanus* (loach)	260	75.5	2.07	0.05	Yan *et al.* 1991

Fig. 1. Cybrid embryos formed by *P. waltl* nuclei and *X. laevis* egg cytoplasm are possibly the most evolutionary distant ones ever reported. *Adult* P. waltl **(A)** *and* X. laevis **(B)** *females are shown approximately at the same magnification.* **(C)** *A simplified evolutionary tree encompassing selected vertebrate superclasses (Osteichthyes, Tetrapoda, Amniota), classes (Actinopterygii, Amphibia, Reptilia, Aves, Mammalia) and orders (Perciformes, Cypriniformes, Anura, Caudata). Approximate divergence times (MY) are based on minimal divergence times estimated from fossil records (Janvier, 2006; Benton and Donoghue, 2007), except for the radiation of Actinopterygii (~260 MY), which is the mean of estimates based on mtDNA and nuclear DNA divergence (Hurley* et al., *2007), and for the separation of Anura and Caudata (~320 MY), which occurred within 10 MY following the appearance of Amphibia (Roelants* et al., *2007). Adapted from (Nomiyama* et al., *2011).* **(D)** *The developmental potential of the most distant inter-class and non-mammalian interorder cybrids reported.*

2010; Narbonne *et al.*, 2011). Defects in gene expression however become apparent if the embryos are cultivated for longer durations (Fujimoto *et al.*, 2010), but it is unclear whether this occurs secondarily to other reprogramming, cellular and/or developmental defects, or because of a genuine inability to maintain embryonic transcription.

A new form of developmental nucleocytoplasmic incompatibility has also recently been identified, and may occur when a gene's orthologs have different expression levels or patterns, or have different regulatory mechanisms, in related species. Examples of this come from studies in *X. laevis* and *X. tropicalis*, two frog species separated by approximately 50-65 million years (MY) (Evans *et al.*, 2004; Roelants *et al.*, 2007). Incubation of nuclei from one species in egg extracts from the other species results in nuclear and spindle expansion, the magnitude of which depends on the extract (cytoplasmic) species. This difference arises because a relatively small number of protein orthologs diverge in their concentrations or in their regulation in the two species. Indeed, the difference in nuclear expansion is dependent on the concentrations of two nuclear transport factors (Importin α and Ntf2), while spindle scaling is affected by the presence of an inhibitory phosphorylation site on a microtubule severing factor (Katanin) ortholog (Levy and Heald, 2010; Loughlin *et al.*, 2011). It is however uncertain whether the aforementioned differences affect development in cybrid embryos made from these two species. It would nonetheless appear that an interspecific difference in the concentrations of a key embryonic transcription factor (Xbra) that is important in driving gastrulation movements might be detrimental to cybrid development. This occurs because the regulation of protein concentration in the early cybrid embryo is under maternal, cytoplasmic (rather than nuclear) control, which leaves cybrids with inappropriate protein concentrations to sustain development that could be promoted by their foreign nuclei (Narbonne *et al.*, 2011). It is uncertain whether similar kinds of problems may occur in other cybrid combinations.

A large number of relatively distant cybrids formed by the combination of interspecific, intergeneric, interfamily and interorder placental mammal nuclei and egg cytoplasm have been described (Beyhan *et al.*, 2007; Loi *et al.*, 2011). In most combinations, blastocysts have been obtained, but embryo survival and development potential generally correlates inversely with evolutionary separation. The entire placental mammal lineage evolved over about 160 MY (Luo *et al.*, 2011); very few highly divergent (>250 MY) non-mammalian interorder, or interclass cybrid embryos have been generated, and they remain poorly described (Fig. 1; Yan *et al.*, 1990, 1991; Kim *et al.*, 2004; Liu *et al.*, 2004). Moreover, in these studies it was not demonstrated that the few animals that developed further were indeed genuine cybrids (without any nuclear DNA contribution from the recipient species). As the most subtle interspecies incompatibilities may become obvious in increasingly distant combinations, we have examined sequence-specific nucleic acid contents in an amphibian interorder cybrid, generated by the transfer of embryonic *Pleurodeles waltl* (Iberian ribbed newt) nuclei into enucleated and non-enucleated *Xenopus laevis* (African clawed frog) eggs. This is possibly the most evolutionarily divergent (~320 MY) interspecific cybrid so far reported (Fig. 1).

Results

P. waltl *to* X. laevis *nuclear transfer interorder cybrids form late blastulae*

To determine the extent with which the egg cytoplasm of one amphibian species can support the development that is promoted by the nucleus of a different order, we have transferred endoderm nuclei from *P. waltl* embryos into *X. laevis* eggs whose nuclei had been previously inactivated using ultraviolet (UV) irradiation (Elsdale *et al.*, 1960). These particular two species were chosen based on availability and their similar culture conditions and optimal growth temperature range. *X. laevis* eggs were chosen as recipients because of the well established NT methodology using this species' eggs (Elsdale *et al.*, 1960). Embryonic endoderm is an efficient nuclear donor tissue for same-species NT in *X. laevis* and 30 to 45% of total nuclear transferred embryos commonly reach a late blastula stage (Gurdon, 1960). We presumed that tissue differentiation would follow similar rules in *Pleurodeles* (Picheral, 1962; Gallien *et al.*, 1973), and thus we used this tissue as a nuclear donor in our experiments in order to minimize the effect of potential reprogramming incompatibilities between the two species. A relatively high percentage (~35%) of the resulting *P*->[*X*] (where the first and second capital italic letters represent the genus of the nuclear donor and recipient egg, respectively; the arrow (->) indicates that the procedure for NT was carried out; and square brackets indicate that a component's nucleus had been inactivated) cybrids could develop until the late blastula stage, albeit they all had varying degrees of abnormality (Table 1; Fig. 2). No cybrid embryos were observed to begin gastrulation movements, and they instead arrested and died as late blastulae (Figs. 1-2). Interestingly, although we have not quantified this defect, the early cleavages in *P*->[*X*] NT embryos were frequently delayed and abnormal or irregular, resulting in misshaped or unequal blastomeres (Fig. 2), which may be linked to defects in spindle

TABLE 1

GRADE◆ OF NUCLEAR TRANSFER LATE BLASTULAE

NT (N)	n	1	2	RCB§ (%)	3- (%*)	3 (%*)	3+ (%*)	4 (%*)	5 (%*)
X -> [*X*]^{a} (3)	114	48	17	49 (43.0)	5 (10.2)	3 (6.1)	7 (14.3)	20 (40.8)	14 (28.6)
P -> [*X*]^{b} (5)	288	104	81	103 (35.8)	46 (44.7)	39 (37.9)	15 (14.6)	3 (2.9)	0 (0)
P -> *X*^{c} (2)	106	31	21	54 (50.9)	1 (1.9)	1 (1.9)	8 (14.8)	11 (20.4)	33 (61.1)

◆, Nuclear transfer embryos were graded when they reached (or should have reached) a late blastula stage (equivalent to *X. laevis* stage 8-9) on a scale of 1 to 5. Grade 1: No apparent division occurred. Grade 2: Signs of division, but no regular cells. Grade 3-: Presence of very few regular cells. Grade 3: Presence of a small patche(s) of regular cells, covering less than ½ of total area. Grade 3+: Presence of a large patche(s) of regular cells, covering ½ - ¾ of total area. Grade 4: Regular cells covering more than ¾ of the total area, but not completely. Grade 5: Wholly regular late blastulae. A relationship exists between NT kind and developmental outcome, both in terms of showing signs of division and forming regular cells (P = 0.007), and in terms of the surface covered by regular cells, amongst the blastulae that contained regular cells (P < 0.001). Chi Square analysis.

N, Number of different *X. laevis* egg female donors used. n, Total number of NTs. §, RCB: Regular Cell containing late NT Blastulae. Includes grades 3- to 5. *, Percent of RCB. $^{a-c}$, In terms of their potential to demonstrate signs of division and form regular cells, rows a-b and b-c differ significantly (P = 0.02 each), but rows a-c do not differ significantly (P = 0.13) in pairwise Chi Square comparisons. In terms of the surface covered by regular cells, amongst the blastulae that had regular cells, rows a-b (P < 0.001), b-c (P < 0.001) and a-c (P = 0.008) differ significantly in pairwise Chi Square comparisons.

assembly and/or chromosome segregation (Wheatley *et al.*, 1998). This also happened in same-species NT (*X*->[*X*]), but to a much lesser extent and magnitude (Fig. 2). These results indicate that the *X. laevis* egg cytoplasm is unable to sustain the full development that is promoted by a *P. waltl* nucleus. On the other hand, it suggests that the *X. laevis* egg cytoplasm can recognize this very distantly related nucleus, such that a relatively high percentage of the resulting cybrid embryos can undergo multiple rounds of cell division and develop until a late blastula stage.

Inherent inability of X. laevis *egg cytoplasm to replicate and/or segregate* P. waltl *DNA*

To approximate the expansion in cell numbers in cybrid embryos, at the time where controls had reach the late blastula stage, we classified all the iNT embryos into one of five grades (Table 1). The results indicated that *P*->[*X*] cybrid embryos showed a significantly reduced ability to undergo cleavage or form regular cells, when compared to *X*->[*X*] controls (Table1). Similarly, amongst the cybrid embryos which formed regular cells, they covered a significantly smaller area of the late blastulae in *P*->[*X*] than in *X*->[*X*] controls (Table 1; Fig. 2). Therefore, we conclude that expansion in cell numbers is impaired in interorder cybrid embryos.

The abnormal cell divisions and reduced expansion in cell numbers in cybrid embryos could either arise as a result of the foreign nuclei or chromosomes interfering with the process of cell division in *X. laevis* cytoplasm, or because of a genuine incapacity of the *X. laevis* cytoplasm to efficiently replicate and/or divide cells carrying *P. waltl* DNA or chromosomes. To test this, we have transferred endoderm nuclei from *P. waltl* embryos into intact (non-enucleated) *X. laevis* eggs. Interestingly, when compared to *P*->[*X*], the resulting *P*->*X* embryos formed significantly more blastulae that either showed signs of cleavage or had regular cells, and amongst blastulae that had regular cells, the area covered was significantly increased (Table 1; Fig. 2). The developmental success of *P*->*X* hybrid embryos, in terms of the area of blastula stage embryos covered by regular cells, was significantly improved even compared to *X*->[*X*] controls (Table 1; Fig. 2). These observations argue that *P. waltl* chromosomes do not interfere with cell division promoted by the *X. laevis* egg cytoplasm.

We allowed several of the most regular *P*->*X* late blastulae obtained to develop further, and to our surprise most of them survived through gastrulation and neurulation (Table 2), and formed

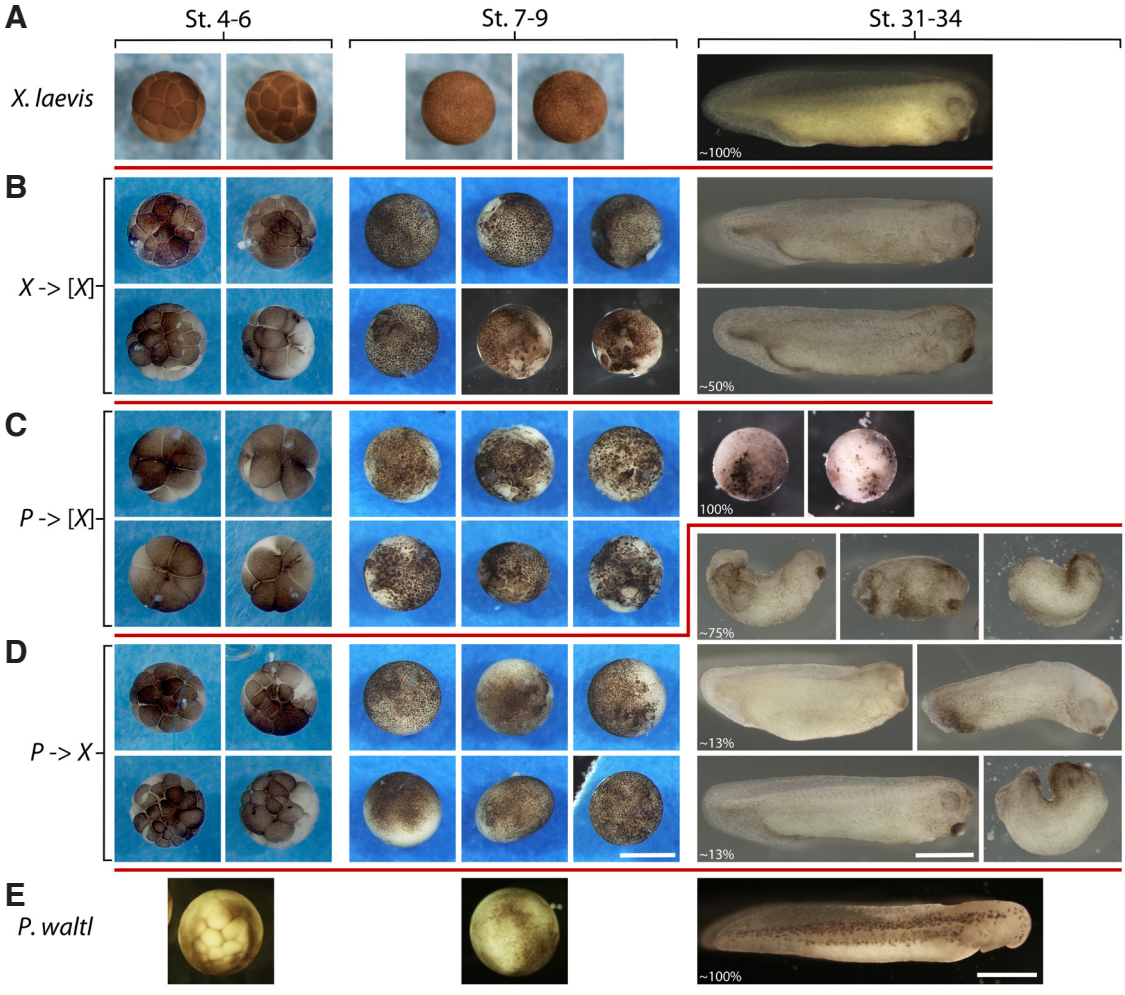


Fig. 2. Development of amphibian interorder nuclear transfer cybrids and hybrids. (A) X. laevis *diploid control embryos.* **(B)** X->[X] *Diploid NT control embryos. About 50% of Grade 5 St. 9 late blastulae (see Table 2) give rise to normal late tailbud stage embryos.* **(C)** P->[X] *interorder cybrids can form late blastulae of varied appearances, but they all expire before beginning gastrulation. Cleavages were often irregular and/or asynchronous between blastomeres, and development was slightly delayed compared to* X. laevis *and* X->[X] *controls.* **(D)** P->X *interorder hybrids can develop into normal,* X. laevis*-looking, late tailbud-stage embryos (lower left panel in the St. 31-34 column; ~13% of Grade 5 St. 9 late blastulae). Early cleavages were often irregular and/or asynchronous between blastomeres, but these defects disappeared in many embryos by the late blastula stage. They however often form abnormal embryos, including haploid-looking (middle left panel in the St. 31-34 column; ~13% of Grade 5 St. 9 late blastulae) and various poorly-developed post neurulae (top and right-most panels in the St. 31-34 column; ~75% of Grade 5 St. 9 late blastulae). No obvious* P. waltl*-like morphological features were observed.* **(E)** P. waltl *diploid control naturally fertilized embryos. In each section, a range of 1-7 representative embryos is shown. Scale bars for all blastulae and* X. laevis *egg-based tailbud stage embryos: 1 mm; and* P. waltl *tailbud stage embryo: 2 mm.*

abnormal post-neurula stage embryos that ranged from poorly elongated, microcephalic post-neurulae, to haploid syndrome-looking (Hamilton, 1963), or even perfectly normal, diploid-looking *X. laevis* late tailbud stage embryos (Fig. 2). Unlike all known hybrids, which show a phenotype that is intermediate between the two parental genomes, none of the *P->X* embryos showed any obvious features that would make them resemble *P. waltl* embryos. These observation led us to suspect that *P->X* embryos may either not contain *P. waltl* DNA, and/or may not express *P. waltl* genes. Thus we considered the possibility that the *X. laevis* egg cytoplasm may not be very efficient at replicating and/or segregating *P. waltl* chromosomes. If this was the case, it would suggest that the poor capacity of the *X. laevis* egg cytoplasm to promote the normal cell division and expansion of cells carrying *P. waltl* nuclei in *P->*[*X*] cybrids arises as a result of a genuine incapacity of the *X. laevis* cytoplasm to efficiently replicate and/or segregate *P. waltl* DNA or chromosomes.

To test this hypothesis, in a first instance, we looked at the chromosomal and nucleolar content in a small number of late neurula/early tailbud stage *P->X* embryos that were sacrificed. Chromosomal spread analysis indicated that some cells in *P->X* embryos unambiguously contained more than 18 chromosomes, the haploid *X. laevis* chromosomal complement, but no cells had 42 distinguishable chromosomes, the full complement that would be expected in this hybrid NT combination (Fig. 3). In *Xenopus*, nuclei contain up to one nucleolus per haploid genome, thus up to two nucleoli per diploid nucleus. In *P. waltl*, we observed up to four nucleoli per nucleus in diploid individuals (Fig. 4). In *P->X* hybrids, there were up to three nucleoli/nucleus, albeit three occurred only in a very small proportion (~0.4%) of the nuclei, and most had either one (65.5%) or two (34%) nucleoli (Fig. 4). Since haploid *X. laevis* embryos have only one nucleolus per nucleus (Narbonne *et al.*, 2011), this suggests that at least some, but likely not all of the nucleolar organizing centres within the diploid *P. waltl* genome initially transferred are still present and functionally recognized in many cells of late neurula/early tailbud stage *P->X* hybrid embryos. These data together suggest that several cells in late neurula/early tailbud stage *P->X* hybrid embryos likely contain an incomplete fraction of the *P. waltl* diploid genome initially transferred.

To unambiguously assess the fate of the transferred *P. waltl* genome in *P->*[*X*] cybrid and *P->X* hybrid embryos, we performed qPCR using both *X. laevis* and *P. waltl* specific primers for several genes with known nucleotide sequences, which we found on the NCBI nucleotide database. Amongst the selected *P. waltl* genes, we chose three Hox genes located in different clusters, each of which are generally located on a different chromosome in vertebrates (Bailey *et al.*, 1997). Thus, our analysis is expected to be representative of the behaviour of at least a few different chromosomes. To control and validate our approach, we first quantified gene copy

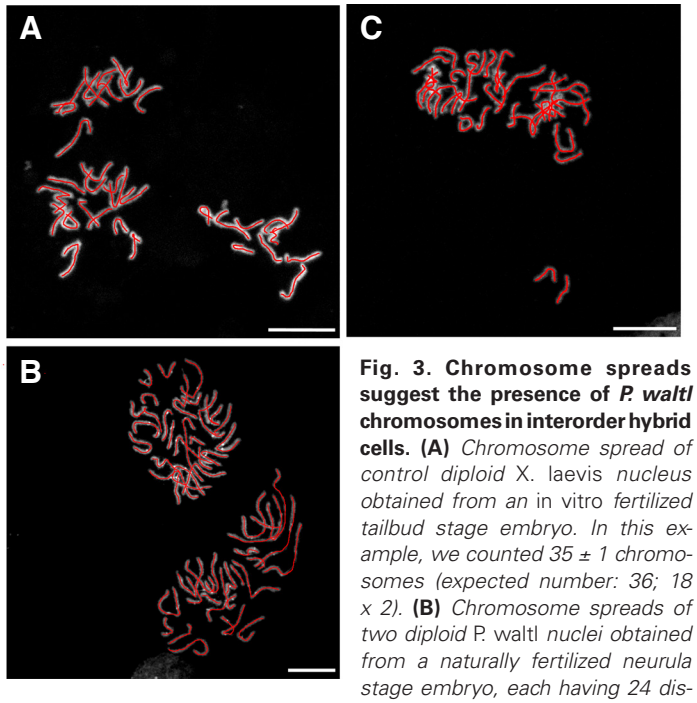


Fig. 3. Chromosome spreads suggest the presence of *P. waltl* chromosomes in interorder hybrid cells. (A) *Chromosome spread of control diploid* X. laevis *nucleus obtained from an* in vitro *fertilized tailbud stage embryo. In this example, we counted 35 ± 1 chromosomes (expected number: 36; 18 x 2).* **(B)** *Chromosome spreads of two diploid* P. waltl *nuclei obtained from a naturally fertilized neurula stage embryo, each having 24 distinct chromosomes (expected number: 24; 12 x 2).* **(C)** *One example of a nucleus isolated from a neurula stage* P -> X *NT embryo showed 30 ± 3 chromosomes (expected number: 42; 18 + (12 x 2)), suggesting that a number of* P. waltl *chromosomes were retained until this stage in triploid hybrids. Presumed chromosomes were manually highlighted in red. Scale bars in A, C: 10 μm, and B: 20 μm.*

numbers of a few *X. laevis* genes in the different kinds of embryos analysed here. The results were homogenous across all genes tested, and indicated an expansion in *X. laevis* gene copy number in the order of 10 000-fold in all stage 9 embryos that contained at least one copy of the *X. laevis* genome, but not in *P. waltl* or *P->*[*X*] embryos, confirming successful nuclear inactivation of *X. laevis* eggs (Fig. 5). The magnitude of DNA amplification is also consistent with published data (Dawid, 1965). We expected *P->X* stage 9 embryos to contain only half as many gene copies as *X. laevis* stage 9 diploid controls, but our assay did not detect a significant difference (Fig.5). This is likely due to a limitation in the sensitivity of the assay, which may not have detected a two-fold difference in this case. It is also conceivable that a number of *P->X* embryos contained a diploid *X. laevis* genome complement, possibly due either from a failed extrusion of the polar body or to endoreplication before the first cleavage. This possibility however appears unlikely to fully explain the result as such abnormal genome duplications only occur in about 10% of the cases in *X. laevis* NT embryos (Gurdon, 1959). When we performed the qPCR analysis with *P.*

TABLE 2

DEVELOPMENT AND SURVIVAL OF POST-BLASTULA STAGE NUCLEAR TRANSFER HYBRIDS

			Late gastrulae			Late neurulae*	
NT	**Number of grade 5 late blastulae***	**Died during gastrulation**	**Abnormal**	**Normal**	**Died during neurulation**	**Abnormal**	**Normal**
X -> [*X*]	4	0	2	2	1	1	2
P -> *X*	16	0	2	14	0	8	8

*, Many of the late blastulae indicated in Table 1, were sacrificed for analysis, and hence not included in this table. Some of the abnormal and normal late neurulae were also sacrificed for analysis either at this stage, or at the early tailbud stage. Most of the animals that were allowed to develop further are shown in Fig. 2 as examples. Post-blastula development of *P->X* was not significantly different from *X->*[*X*] controls (P = 0.5; Chi Square analysis).

waltl gene specific primers, we again found homogenous results across all genes tested. As expected, *P. waltl* late blastulae (stage 7) and early gastrulae (stage 8) respectively had about 7000-fold and 14000-fold more copies of each genes than one-cell *P->[X]* and other controls (Fig. 6). These numbers are consistent with cell count estimates in *A. mexicanum* embryos (Hara, 1977), a related Caudata species. Interestingly, we found that in both *P->[X]* and *P->X*, *P. waltl* genes were amplified on average only ~200-fold by the time control *X. laevis* embryos reached a late blastulae stage (Fig. 6). These results confirm that the *X. laevis* egg cytoplasm is unable to properly expand *P. waltl* DNA during development, but that it significantly expands it, and rather homogeneously, at least across the genes/chromosomes tested. Moreover, leaving the maternal genome in the recipient egg rescued the expansion in cell numbers (surface of late blastulae covered by regular cells; Table 1; Fig. 2), but had no significant effect (P = 0.3) on *P. waltl* DNA expansion (similar gene copy numbers in *P->[X]* and *P->X* late blastulae; Fig. 6). This indicates that the reduced *P. waltl* DNA copy numbers present in iNT late cybrid blastulae does not result from a reduced rate/success of cell division *per se*, but from a genuine inability of the *X. laevis* cytoplasm to properly replicate and/or to segregate *Pleurodeles* DNA/chromosomes into daughters cells during cell division.

Embryonic gene transcription occurs in cybrid embryos

Embryonic gene transcription has been demonstrated to occur, at least at a global scale, in some lethal cybrids (Fujimoto *et al.*, 2010; Narbonne *et al.*, 2011; Wang *et al.*, 2011), but we do not know whether EGA failure could explain lethality in the most distant cybrid combinations. We have used RT-qPCR to assess gene transcription in interorder hybrid and cybrid NT embryos, using *P. waltl*-specific primers. A limited number of genes were investigated for transcription due to the poor availability of known *P. waltl* gene sequences that are transcribed in late blastula stage embryos. We detected significant transcript levels for *GAPDH*, *Shh*, *Otx2*, and *larval* α*-Globin* in stage 7 *P. waltl* embryos (Fig. 7) and thus pursued the analysis with these four genes in cybrids. Although some iNT embryo samples did not contain detectable levels of transcript for some of the genes tested, transcription from these *P. waltl* genes was detected in both *P->[X]* (4/4 genes) and *P->X* (3/4 genes) late blastula stage embryos (equivalent to *P. waltl* St. 7 or *X. laevis* St. 9). Expression levels were however generally reduced (~10-1000 fold) as compared to *P. waltl* late blastula stage embryos (Fig. 7). For *GAPDH*, we have sequenced the amplified RT-qPCR product directly and confirmed a *P. waltl*-specific amplicon origin in 2/2 cybrid embryos (Fig. 8). Since preventing *X. laevis* embryo transcription by the use of α-amanitin results in 100% late blastula stage arrest (Narbonne *et al.*, 2011), EGA was undoubtedly initiated and successful in many of the *P->X* embryos (Fig. 2; Table 2). As *P. waltl* gene expression was not

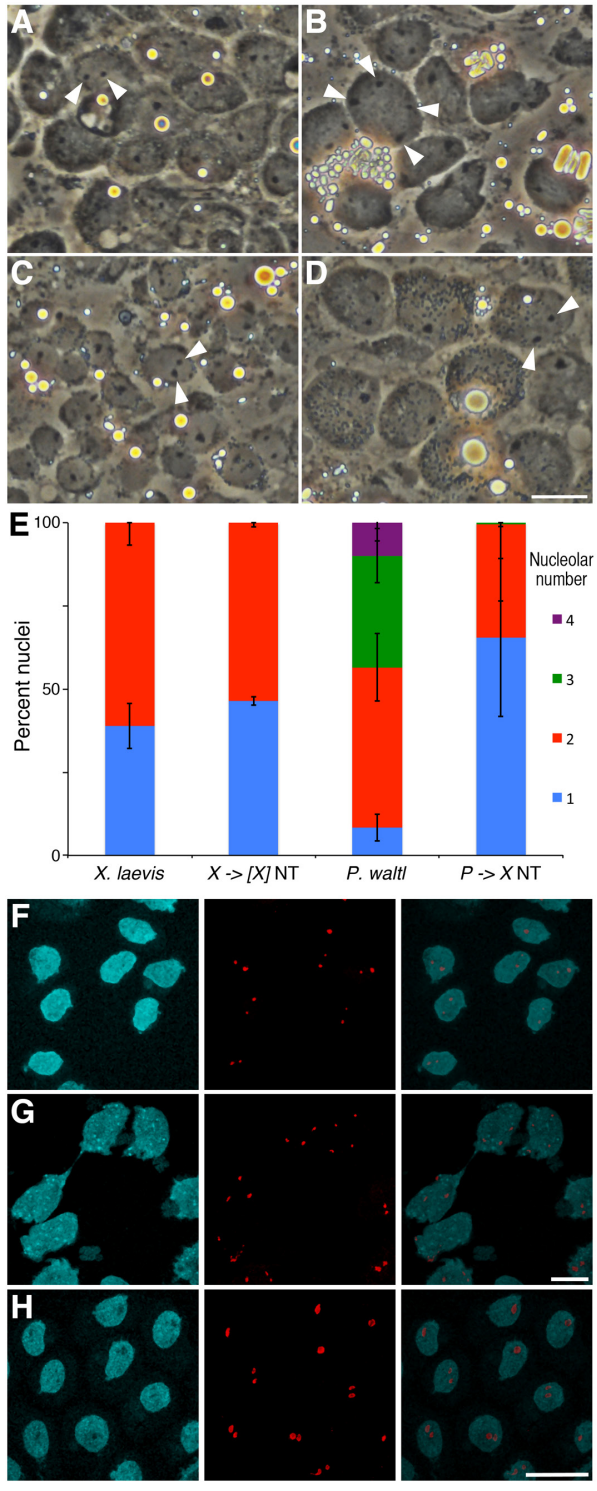


Fig. 4. Nucleolar numbers suggest the presence of *P. waltl* nucleolar organizing centers in interorder hybrid cells. (A-D) *Phase contrast microscopy revealed nucleolar numbers in neurula/tailbud stage nuclei. Arrowheads point at the nucleoli (darker spots) present in one nucleus for each panel as an example.* **(A)** *Diploid* X. laevis *nuclei have 1 or 2 nucleoli per nucleus.* **(B)** *Diploid* P. waltl *nuclei have up to 4 nucleoli per nucleus.* **(C)** X -> *[X] nuclei are similar to diploid* X. laevis *and have up to 2 nucleoli per nucleus.* **(D)** *Cells in* P -> X *triploid hybrids generally have 1 or 2 nucleoli per nucleus, and rarely (0.4%) 3 nucleoli (not shown).* **(E)** *Quantitative representation of phase contrast observations. Nucleolar numbers were averaged over ten embryos for* X. laevis *and* P. waltl *diploids, 7 for* P -> X, *and 2 for* X -> *[X]. (F-H) Monoclonal anti-fibrillarin antibodies (red) were used to confirm nucleolar numbers and integrity in* **(F)** *diploid* X. laevis, **(G)** *diploid* P. waltl, *and* **(H)** P -> X *embryos. DNA (blue) was counterstained with Hoechst. A few nuclei having three distinct fibrillarin-marked spots were also observed in* P -> X *embryos (not shown), confirming the existence of rare nuclei having three nucleoli per nucleus in these iNT hybrids. Scale bars in (A-D), (F-G) and (H)m 20* μm.

increased in *P*->*X*, compared to *P*->[*X*] embryos (Fig. 7), it suggests that the activation of *P. waltl* gene transcription by the *X. laevis* egg cytoplasm is independent of the presence of a *X. laevis* genome, or of the global transcriptional activity of the embryo, and that EGA (on *P. waltl* genes) occurred equally well in *P*->[*X*] and *P*->*X* iNT embryos.

Although *P. waltl* transcript numbers were dramatically reduced in iNT embryos as compared to *P. waltl* diploid late blastulae (Fig.7), *P. waltl* DNA levels were also dramatically reduced (Fig. 6). We therefore divided the quantity of transcripts obtained for each gene by the number of *P. waltl* diploid genome equivalents (based on the qPCR assays) found in each kind of late blastula stage embryos (Fig. 7), in order to determine the level of *P. waltl* gene transcription per gene copy. Remarkably, the average number of transcripts per gene copy was, in most cases, comparable in *P*->[*X*] and *P. waltl* diploid late blastula stage embryos (Fig. 7). Thus, the results overall suggest that the *X. laevis* egg cytoplasm has an inherent inability to efficiently replicate and/or segregate the foreign *P. waltl* genome, but that it is able to appropriately activate and transcribe genes from the *P. waltl* genome.

Discussion

Despite an extensive exploration of the potential of one species' eggs to reprogram and promote the development that is supported by another species nucleus, little is known about the nature of interspecific nucleocytoplasmic incompatibilities (Beyhan *et al.*, 2007; Loi *et al.*, 2011). One certainty that arose from early back-transfer experiments in frogs is that the foreign nucleus may be permanently damaged after undergoing a few rounds of cell division in some, but not all, other species' egg cytoplasm (Moore, 1958; Gurdon, 1962; Hennen, 1974). We have shown here that the genes, and hence presumably also the chromosomes that carry them, of *P. waltl* are inefficiently replicated and/or segregated during cell division in embryos with *X. laevis* cytoplasm, in a rather uniform manner. DNA/chromosome loss during cell division in a foreign species cytoplasm is therefore a form of nuclear damage that occurs during the development of cybrid embryos, and may therefore explain some of their lethality and the failure of back-transfer experiments. Which of DNA replication or chromosome segregation defects are responsible for the interspecific nucleocytoplasmic incompatibility

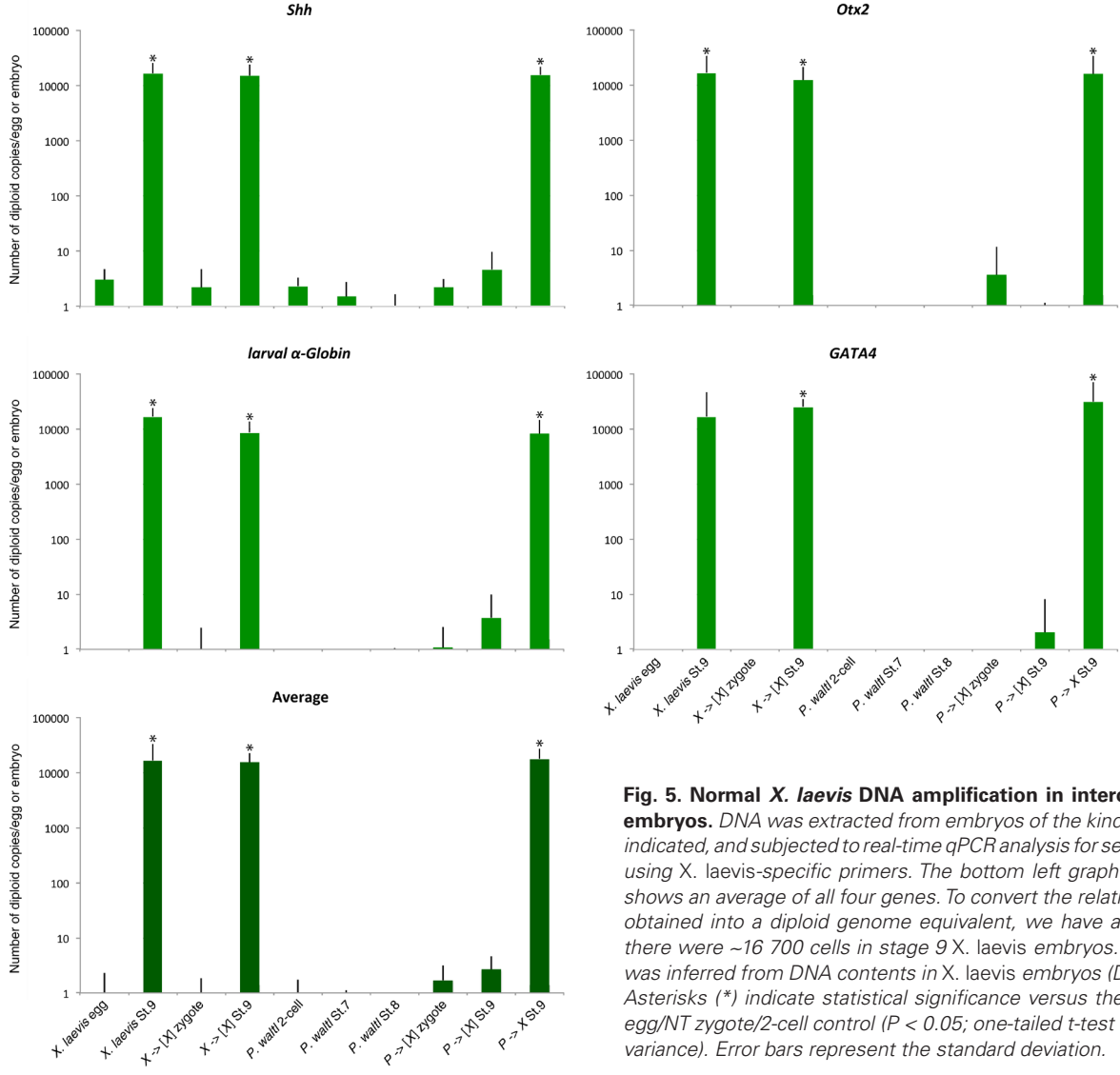


Fig. 5. Normal *X. laevis* DNA amplification in interorder hybrid embryos. *DNA was extracted from embryos of the kinds and stages indicated, and subjected to real-time qPCR analysis for selected genes using* X. laevis*-specific primers. The bottom left graph (dark green) shows an average of all four genes. To convert the relative quantities obtained into a diploid genome equivalent, we have assumed that there were ~16 700 cells in stage 9* X. laevis *embryos. This number was inferred from DNA contents in* X. laevis *embryos (Dawid, 1965). Asterisks (*) indicate statistical significance versus their respective egg/NT zygote/2-cell control ($P < 0.05$; one-tailed t-test with unequal variance). Error bars represent the standard deviation.*

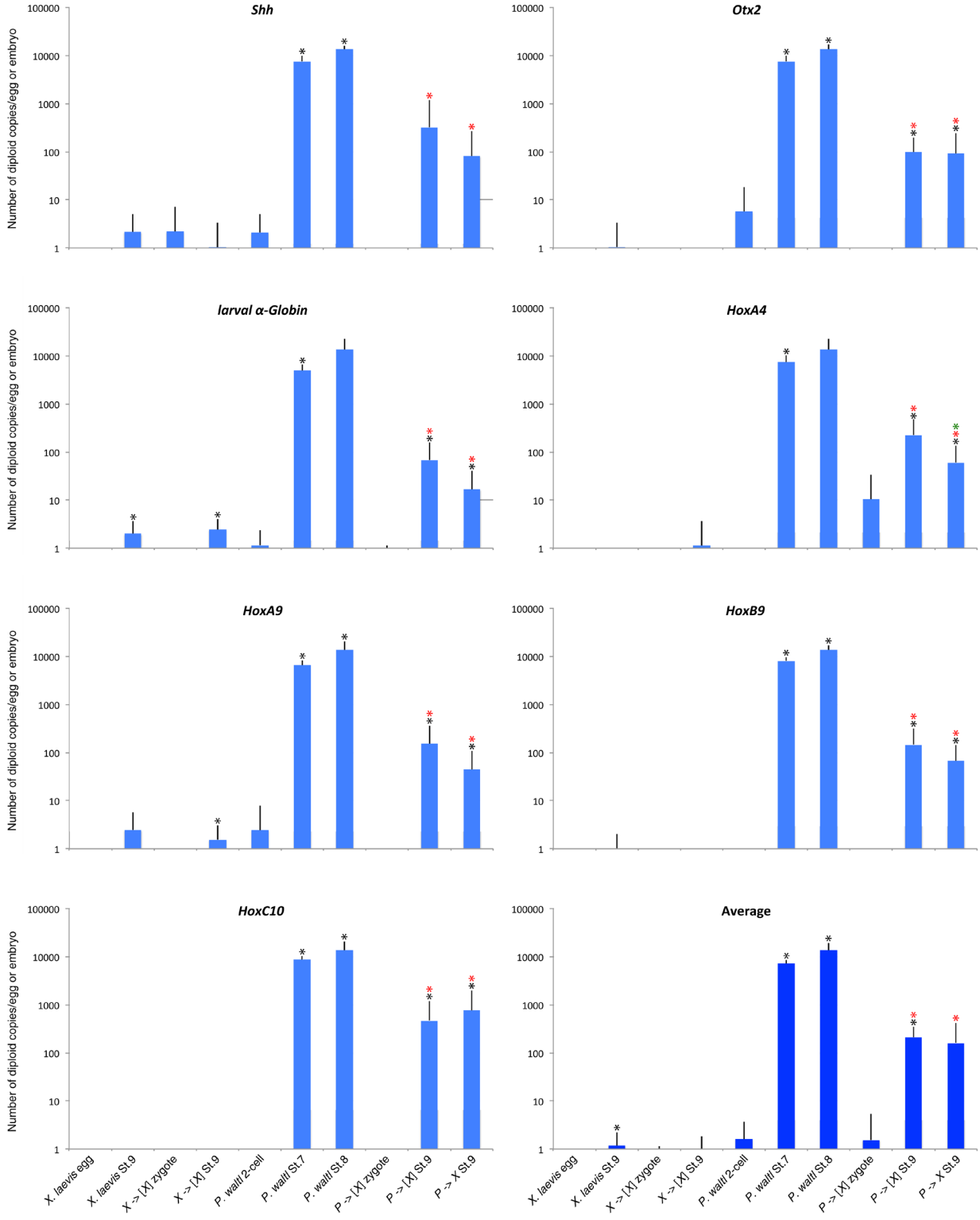


Fig. 6. Poor *Pleurodeles* DNA amplification in interorder embryos. *DNA was extracted from embryos of the kinds and stages indicated, and subjected to real-time qPCR analysis for selected genes using* P. waltl*-specific primers. The bottom right graph (dark blue) shows an average of all seven genes. To convert the relative quantities obtained into diploid genome equivalents, we assumed that there were ~13 677 cells in stage 8* P. waltl *embryos. We could not find a cell number estimate for* Pleurodeles*, but (Hara, 1977) estimated that there were approximately 13 677 cells in axolotl embryos when they begin gastrulation. We then adjusted our real-time PCR data such that stage 8 DNA quantities for each gene corresponded to 13 677 diploid genome copies. Black asterisks indicate statistical significance versus their respective egg/NT zygote/2-cell control; red asterisks, versus* P. waltl *St.7; green asterisks, versus* P->[X] St.9 *($P < 0.05$; one-tailed t-test with unequal variance). Error bars represent the standard deviation.*

remains unclear, yet the latter appears more likely. Indeed, DNA replication in early *X. laevis* embryos does not depend on specific sequences (Hyrien and Méchali, 1993), and *X. laevis* egg extracts can replicate mammalian DNA almost as efficiently as *X. laevis* sperm DNA (Dimitrova and Gilbert, 1998; Ganier *et al.*, 2011). Thus, it appears unlikely that *P. waltl* DNA would not be efficiently replicated in *X. laevis* early embryos. Faithful chromosome segregation, on the other hand, requires the recognition of centromeres by kinetochore proteins, and yet the DNA and protein components specific to centromeric chromatin show rapid evolution (Henikoff *et al.*, 2001). Indeed, centromeric DNA sequences have little or no resemblance to each other over relatively short evolutionary times (Birchler *et al.*, 2009). Thus, we would favour the hypothesis that centromeric sequence divergence between *X. laevis* and *P. waltl* has led to the consequence that centromeric *P. waltl* DNA is not efficiently recognized by centromeric proteins in the *X. laevis* cytoplasm, leading to an unfaithful segregation of *P. waltl* chromosomes. This idea is supported by two analyses of interspecific lethal fish hybrids in which uniparental chromosome elimination has been shown to occur during early embryogenesis, although the chromosomes were not always lost from the foreign species (paternal) nucleus (Fujiwara *et al.*, 1997; Sakai *et al.*, 2007). Also, since aberrant chromosome segregation frequently occurs (in >90% of the embryos) during early cleavages in same-species SCNT mouse embryos (Mizutani *et al.*, 2012), even a small interspecific incompatibility that would impair foreign species chromosome segregation in cybrid and hybrid cells may be exacerbated in NT embryos.

Defects in EGA have long been thought to be a major cause of interspecies nucleocytoplasmic incompatibilities

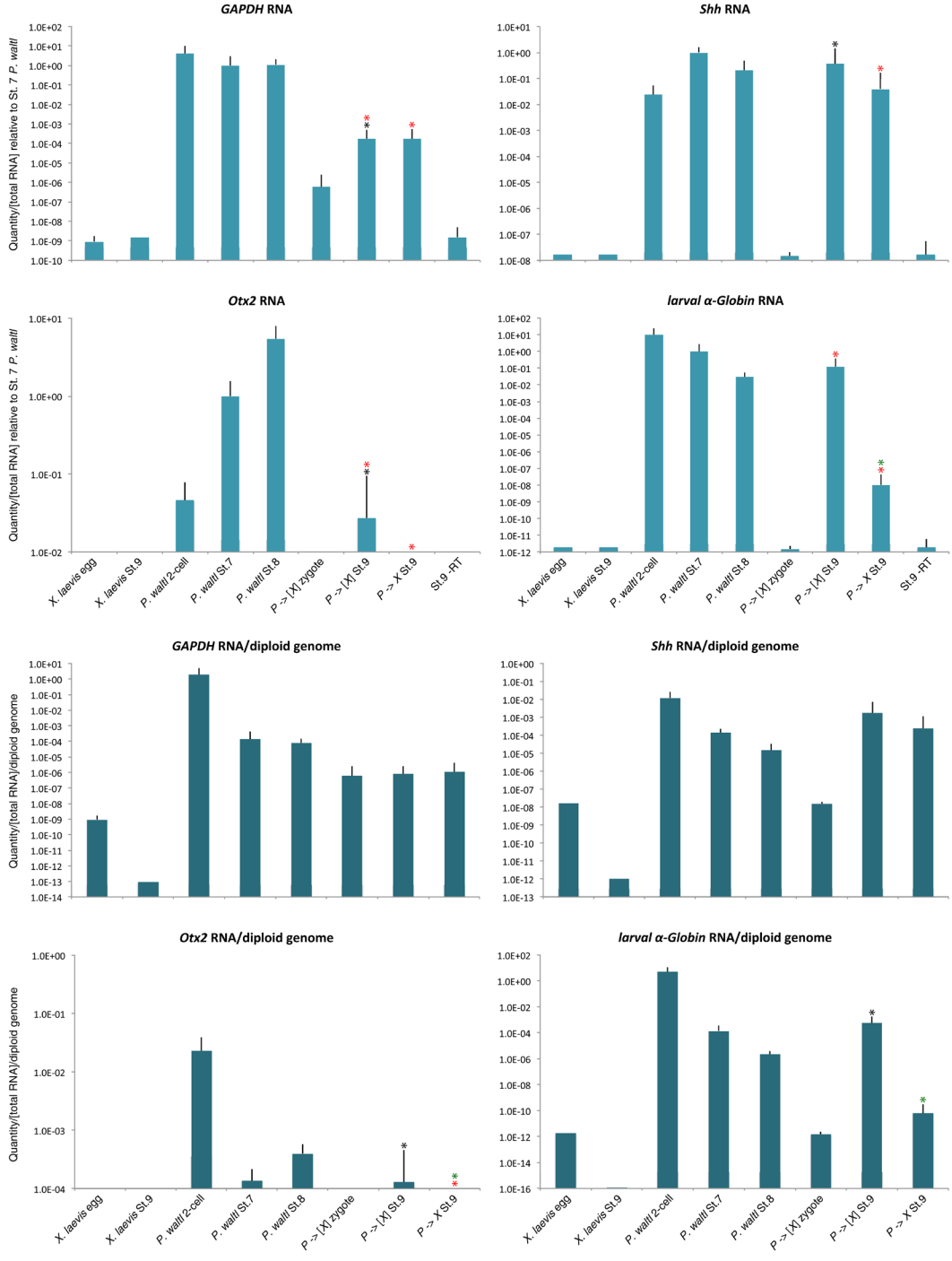


Fig. 7. Embryonic gene transcription in interorder cybrid embryos. *RNA was extracted from embryos of the kinds and stages indicated, and subjected to RT-qPCR analysis for selected genes using* P. waltl*-specific primers. The top four graphs (turquoise) indicate the amount of RNA relative to the total RNA concentration in each egg or embryo, relative to a stage 7* P. waltl *embryo (late blastula). The bottom four graphs (dark turquoise) represent the same amounts, except they have been divided by the number of* X. laevis *(*X. laevis *egg, St.9) or* P. waltl *(*P. waltl *2-cell, St.7, St.8;* P->[X] *zygote, St.9;* P->X *St.9) diploid genome equivalents present in these embryos (from Figures 5-6). Black asterisks indicate statistical significance versus its* P->[X] *zygote control; red asterisks, versus* P. waltl *St.7; green asterisks, versus* P->[X] *St.9 (*$P < 0.05$*; one-tailed Mann-Whitney test for the top four graphs, and one-tailed t-test with unequal variance for the lower four). Error bars represent the standard deviation.*

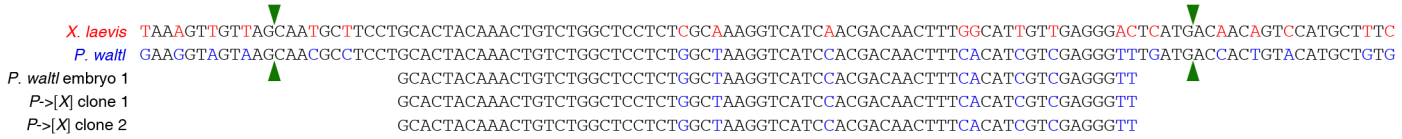


Fig. 8. *P. waltl* gene transcription by the *X. laevis* egg cytoplasm. *RT-qPCR amplicons from one* P. waltl *and two* P->[X] *cloned embryo were sent for sequencing using the same P. waltl-specific primers as for the qPCR reaction (shown at each ends of the target sequence in the second row). Sequences perfectly matched with the* P. waltl GAPDH *cDNA sequence found in the NCBI database (second row), but not with* X. laevis GAPDH *cDNA (top row). Nucleotides specific to* X. laevis *are shown in red and those specific to* P. waltl*, in blue. Predicted exon/exon boundaries are indicated by green arrowheads, assuming they are conserved between* X. tropicalis, X. laevis *and* P. waltl*, and that splicing follows the rules established by (Breathnach and Chambon, 1981).*

and cybrid lethality, yet in most studies in which embryonic gene transcription has been assayed, transcript levels were normal for at least some of the genes that were investigated (Wang *et al.*, 2009, 2011; Fujimoto *et al.*, 2010; Narbonne *et al.*, 2011). This contrasts with some earlier studies in which mRNA production was globally reduced in cybrids (Woodland and Gurdon, 1969; Chung *et al.*, 2009), but cell numbers or DNA content of cybrid embryos were not investigated. Our results are thus key in that they provide evidence that gene transcription in early embryos is a highly conserved process, at least in amphibia, and may not therefore be one of the critical limiting factors for cybrid development.

The efficiency of same-species SCNT is low, especially in primates, and a few groups have explored the potential of non-enucleated eggs as NT recipients, as opposed to enucleated eggs. In all cases, the development of the resulting NT triploid or tetraploid embryos was dramatically improved when non-enucleated eggs were used as recipients when compared to enucleated eggs (Sotomaru *et al.*, 2009; Yang *et al.*, 2010; Noggle *et al.*, 2011). Because the maternal nucleus can support early embryonic development on its own to form haploid larvae/blastocysts, it is conceivable that the transplanted somatic nucleus does not fully participate in development during the early steps, although it is eventually completely reprogrammed after culture of the ES cells extracted from the resulting triploid NT blastocyst stage embryos in human (Noggle *et al.*, 2011). A different interpretation is that some key reprogramming factors are so tightly associated with the maternal chromosomes, even during mitosis, that they are inevitably removed from the egg along with the maternal genome, at least with the current enucleation methods (Egli and Eggan, 2010). Here we have compared the developmental potential of iNT embryos using intact and enucleated recipient eggs. Our results showed that the presence of the maternal genome did not improve the replication and/or segregation of the foreign DNA. Also, because the transcription of *P. waltl* genes was not improved when the maternal genome was left intact and EGA evidently occurred normally, it proves that embryonic gene transcription initiated normally in cybrids. It is however possible that the transcription of a certain number of genes would remain aberrant in cybrids, due to incompatibilities in their regulatory elements.

Materials and Methods

Eggs and embryos

X. laevis adults were maintained and eggs were obtained as previously described (Narbonne *et al.*, 2011). *P. waltl* adults were maintained as described elsewhere (Gallien *et al.*, 1973). All embryos were maintained at 16-18°C. *X. laevis* egg-based embryos were staged according to (Nieuwkoop and Faber, 1956) and *P. waltl* embryos, according to (Shi and Boucaut, 1995).

Nuclear inactivation and nuclear transfer

Nuclear inactivation and NT were performed essentially as previously described (Elsdale *et al.*, 1960), using endodermal cells from gastrula, neurula or early tailbud stage *X. laevis* or *P. waltl* dissociated embryos (depending on availability) as nuclear donors. For NT to non-enucleated *X. laevis* eggs, the jelly was permeabilized by placing the eggs sideways for the ~4 seconds UV (Hanovia) treatment, ensuring that the white germinal vesicle breakdown spot was not irradiated.

Nucleic acid isolation and qPCR/RT-qPCR

DNA and RNA were simultaneously isolated from 1 to 3 eggs/embryos at the time, using the AllPrep® DNA/RNA kit (Qiagen), according to the manufacturer's recommendation. In cases where only RNA was isolated, the RNeasy® kit (Qiagen) was instead used. Total RNA concentrations were calculated from their optical density at 260 nm using a NanoDrop®. Reverse transcription and/or qPCR reactions were performed using SYBR Green as previously described (Halley-Stott *et al.*, 2010), with the following primer pairs (5'->3': Forward - Reverse) for *P. waltl GAPDH*: see (Bascove and Frippiat, 2010); *Shh*: TCAGCGCCCAGAGAGCTT - CCATTCGCCGGTCCCTAT; *Otx2*: GCGGGAAGTGAGCTCAGAAA - TGTGGTAGGCGGTGGTGTAA; *HoxA4*: CGGCGCATGAAATGGAA - GGAGCGCATCTTGGTGTTG; *HoxA9*: CCGTCCGCCAACTGGTT - GGTGTAAGGGCAGCGTTTTTT; *HoxB9*: CCCCATGTAGTGTGTCCACAAC - GCGCGCTCCTGGAAGA; *HoxC10*: TGAATCGCCCCCTTACGAT - CACACAAGACCCACTACAAAACG; larval α-*Globin*: AAACGACATGGAGGCAAGGT - TGCATGTGCTTGGCTGCTT, and for *X. laevis GATA4*: see (Narbonne *et al.*, 2011); *Shh*: GACCGCG-GCCCATCTAC - ACGACCTGGTGCCGTTGA; *Otx2*: GCGACCCCCAG-GAAACA - CAGTTGGGCCCTGGTAAAAG; larval α-*Globin*: TGTCCCA-CACTATCCAGGTGACT - GGCTGTGGCATCAAATTCATC. Dissociation curves were checked for each reaction, and when there was no clear peak present at the appropriate melting temperature (+/- 0.5°C) for an RT-PCR amplification product (as in the case of unspecific amplification products), the relative quantity was adjusted to the –RT value, in order to facilitate data interpretation. Data were averaged across biological replicates (3 to 21 samples per egg/embryo kind), and in cases where there were technical replicates, the highest quantity obtained for each sample was selected for the analysis. Only grade 3 (or better) blastulae (see Table 1) were used for this analysis.

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References

BAILEY, W.J., KIM, J., WAGNER, G.P., RUDDLE, F.H. (1997). Phylogenetic Reconstruction of vertebrate Hox cluster duplications. *Mol Biol Evol* 14: 843–853.

BASCOVE, M., FRIPPIAT, J.-P. (2010). Molecular characterization of *Pleurodeles waltl* activation-induced cytidine deaminase. *Mol Immunol* 47: 1640–1649.

BEAUJEAN, N., TAYLOR, J.E., MCGARRY, M., GARDNER, J.O., WILMUT, I., LOI, P., PTAK, G., GALLI, C., LAZZARI, G., BIRD, A., *et al.,* (2004). The effect of interspecific oocytes on demethylation of sperm DNA. *Proc Natl Acad Sci USA* 101: 7636–7640.

BENTON, M.J., DONOGHUE, P.C.J. (2007). Paleontological evidence to date the tree of life. *Mol Biol Evol* 24: 26 –53.

BEYHAN, Z., IAGER, A.E., CIBELLI, J.B. (2007). Interspecies nuclear transfer: implications for embryonic stem cell biology. *Cell Stem Cell* 1: 502–512.

BIRCHLER, J.A., GAO, Z., HAN, F. (2009). A tale of two centromeres-diversity of structure but conservation of function in plants and animals. *Funct Integr Genomics* 9: 7–13.

BREATHNACH, R., CHAMBON, P. (1981). Organization and expression of eucaryotic split genes coding for proteins. *Annu Rev Biochem* 50: 349–383.

BRIGGS, R., KING, T.J. (1952). Transplantation of living nuclei from blastula cells into enucleated frogs' eggs. *Proc Natl Acad Sci USA* 38: 455–463.

CANNON, M.V., DUNN, D.A., IRWIN, M.H., BROOKS, A.I., BARTOL, F.F., TROUNCE, I.A., PINKERT, C.A. (2011). Xenomitochondrial mice: investigation into mitochondrial compensatory mechanisms. *Mitochondrion* 11: 33–39.

CHUNG, Y., BISHOP, C.E., TREFF, N.R., WALKER, S.J., SANDLER, V.M., BECKER, S., KLIMANSKAYA, I., WUN, W.-S., DUNN, R., HALL, R.M., *et al.,* (2009). Reprogramming of human somatic cells using human and animal oocytes. *Cloning Stem Cells* 11: 213–223.

DAWID, I.B. (1965). Deoxyribonucleic acid in amphibian eggs. *J Mol Biol* 12: 581–599.

DIMITROVA, D.S., GILBERT, D.M. (1998). Regulation of mammalian replication origin usage in *Xenopus* egg extract. *J Cell Sci* 111: 2989–2998.

EGLI, D., EGGAN, K. (2010). Recipient cell nuclear factors are required for reprogramming by nuclear transfer. *Development* 137: 1953–1963.

ELSDALE, T.R., GURDON, J.B., FISCHBERG, M. (1960). A description of the technique for nuclear transplantation in *Xenopus laevis*. *J Embryol Exp Morphol* 8: 437–444.

EVANS, B.J., KELLEY, D.B., TINSLEY, R.C., MELNICK, D.J., CANNATELLA, D.C. (2004). A mitochondrial DNA phylogeny of African clawed frogs: phylogeography and implications for polyploid evolution. *Mol Phylogenet Evol* 33: 197–213.

FUJIMOTO, T., SAITO, T., SAKAO, S., ARAI, K., YAMAHA, E. (2010). Developmental potential of embryonic cells in a nucleocytoplasmic hybrid formed using a goldfish haploid nucleus and loach egg cytoplasm. *Int J Dev Biol* 54: 827–835.

FUJIWARA, A., ABE, S., YAMAHA, E., YAMAZAKI, F., YOSHIDA, M.C. (1997). Uniparental chromosome elimination in the early embryogenesis of the inviable salmonid hybrids between masu salmon female and rainbow trout male. *Chromosoma* 106: 44–52.

GALLIEN, C.L., AIMAR, C., GUILLET, F. (1973). Nucleocytoplasmic interactions during ontogenesis in individuals obtained by intra- and interspecific nuclear transplantation in the genus *Pleurodeles* (urodele amphibian). *Dev Biol* 33: 154–170.

GANIER, O., BOCQUET, S., PEIFFER, I., BROCHARD, V., ARNAUD, P., PUY, A., JOUNEAU, A., FEIL, R., RENARD, J.-P., MÉCHALI, M. (2011). Synergic reprogramming of mammalian cells by combined exposure to mitotic *Xenopus* egg extracts and transcription factors. *Proc Natl Acad Sci USA* 108: 17331 –17336.

GÓMEZ, M.C., POPE, C.E., BIANCARDI, M.N., DUMAS, C., GALIGUIS, J., MORRIS, A.C., WANG, G., DRESSER, B.L. (2011). Trichostatin A modified histone covalent pattern and enhanced expression of pluripotent genes in interspecies black-footed cat cloned embryos but did not improve *in vitro* and *in vivo* viability. *Cell Reprogram* 13: 315–329.

GURDON, J.B. (1959). Tetraploid frogs. *J Exp Zool* 141: 519–543.

GURDON, J.B. (1960). The developmental capacity of nuclei taken from differentiating endoderm cells of *Xenopus laevis*. *J Embryol Exp Morphol* 8: 505–526.

GURDON, J.B. (1962). The transplantation of nuclei between two species of *Xenopus*. *Dev Biol* 5: 68–83.

HALLEY-STOTT, R.P., PASQUE, V., ASTRAND, C., MIYAMOTO, K., SIMEONI, I., JULLIEN, J., GURDON, J.B. (2010). Mammalian nuclear transplantation to Germinal Vesicle stage *Xenopus* oocytes - a method for quantitative transcriptional reprogramming. *Methods* 51: 56–65.

HAMILTON, L. (1963). An experimental analysis of the development of the haploid syndrome in embryos of *Xenopus laevis*. *J Embryol Exp Morphol* 11: 267–278.

HARA, K. (1977). The cleavage pattern of the axolotl egg studied by cinematography and cell counting. *Roux Arch Dev Biol* 181: 73–87.

HENIKOFF, S., AHMAD, K., MALIK, H.S. (2001). The centromere paradox: stable inheritance with rapidly evolving DNA. *Science* 293: 1098–1102.

HENNEN, S. (1974). Back-transfer of late gastrula nuclei of nucleocytoplasmic hybrids. *Dev Biol* 36: 447–451.

HURLEY, I.A., MUELLER, R.L., DUNN, K.A., SCHMIDT, E.J., FRIEDMAN, M., HO, R.K., PRINCE, V.E., YANG, Z., THOMAS, M.G., COATES, M.I. (2007). A new time-scale for ray-finned fish evolution. *Proc R Soc B* 274: 489–498.

HYRIEN, O., MÉCHALI, M. (1993). Chromosomal replication initiates and terminates at random sequences but at regular intervals in the ribosomal DNA of *Xenopus* early embryos. *EMBO J* 12: 4511–4520.

JANVIER, P. (2006). Palaeontology: modern look for ancient lamprey. *Nature* 443: 921–924.

KENYON, L., MORAES, C.T. (1997). Expanding the functional human mitochondrial DNA database by the establishment of primate xenomitochondrial cybrids. *Proc Natl Acad Sci USA* 94: 9131–9135.

KIM, T.M., PARK, T.S., SHIN, S.S., HAN, J.Y., MOON, S.Y., LIM, J.M. (2004). An interclass nuclear transfer between fowl and mammal: *In vitro* development of chicken-to-cattle interclass embryos and the detection of chicken genetic complements. *Fertil Steril* 82: 957–959.

LEE, H.-S., YU, X.-F., BANG, J.-I., CHO, S.-J., DEB, G.K., KIM, B.-W., KONG, I.-K. (2010). Enhanced histone acetylation in somatic cells induced by a histone deacetylase inhibitor improved inter-generic cloned leopard cat blastocysts. *Theriogenology* 74: 1439–1449.

LEVY, D.L., HEALD, R. (2010). Nuclear size is regulated by Importin α and Ntf2 in *Xenopus*. *Cell* 143: 288–298.

LIU, S., ZHOU, Z., CHEN, T., ZHANG, Y., WEN, D., KOU, Z., LI, Z., SUN, Q., CHEN, D. (2004). Blastocysts produced by nuclear transfer between chicken blastodermal cells and rabbit oocytes. *Mol Reprod Dev* 69: 296–302.

LOI, P., MODLINSKI, J.A., PTAK, G. (2011). Interspecies somatic cell nuclear transfer: a salvage tool seeking first aid. *Theriogenology* 76: 217–228.

LOUGHLIN, R., WILBUR, J.D., MCNALLY, F.J., NÉDÉLEC, F.J., HEALD, R. (2011). Katanin contributes to interspecies spindle length scaling in *Xenopus*. *Cell* 147: 1397–1407.

LUO, Z.-X., YUAN, C.-X., MENG, Q.-J., JI, Q. (2011). A Jurassic eutherian mammal and divergence of marsupials and placentals. *Nature* 476: 442–445.

MCKENZIE, M., CHIOTIS, M., PINKERT, C.A., TROUNCE, I.A. (2003). Functional respiratory chain analyses in murid xenomitochondrial cybrids expose coevolutionary constraints of cytochrome b and nuclear subunits of complex III. *Mol Biol Evol* 20: 1117–1124.

MIZUTANI, E., YAMAGATA, K., ONO, T., AKAGI, S., GESHI, M., WAKAYAMA, T. (2012). Abnormal chromosome segregation at early cleavage is a major cause of the full-term developmental failure of mouse clones. *Dev Biol* 364: 56–65.

MOORE, J.A. (1958). Transplantation of nuclei between *Rana pipiens* and *Rana sylvatica*. *Exp Cell Res* 14: 532–540.

MOORE, J.A., MOORE, B.C. (1953). Studies in the development of frog hybrids. Iv. Competence of gastrula ectoderm in androgenetic hybrids. *Biol Bull* 104: 68–74.

NARBONNE, P., MIYAMOTO, K., GURDON, J.B. (2012a). Reprogramming and development in nuclear transfer embryos and in interspecific systems. *Curr Opin Genet Dev* 22: 450-458.

NARBONNE, P., HALLEY-STOTT, R.P., GURDON, J.B. (2012b). On the cellular and developmental lethality of a Xenopus nucleocytoplasmic hybrid. *Commun Integr Biol* 5: 329-333.

NARBONNE, P., SIMPSON, D.E., GURDON, J.B. (2011). Deficient induction response in a *Xenopus* nucleocytoplasmic hybrid. *PLoS Biol* 9: e1001197.

NIEUWKOOP, P.D., FABER, J. (Ed.) (1956). *Normal table of Xenopus laevis (Daudin): a systematical and chronological survey of the development from the fertilized egg till the end of metamorphosis.* North-Holland Pub Co, Amsterdam.

NOGGLE, S., FUNG, H.-L., GORE, A., MARTINEZ, H., SATRIANI, K.C., PROSSER, R., OUM, K., PAULL, D., DRUCKENMILLER, S., FREEBY, M., *et al.* (2011). Human oocytes reprogram somatic cells to a pluripotent state. *Nature* 478: 70–75.

NOMIYAMA, H., OSADA, N., YOSHIE, O. (2011). A family tree of vertebrate chemokine receptors for a unified nomenclature. *Dev Comp Immunol* 35: 705–715.

PICHERAL, B. (1962). Capacities of the nuclei of embryonic endodermal cells to organize a viable germ in the urodele, *Pleurodeles waltlii Michah*. *C R Hebd*

Seances Acad Sci 255: 2509–2511.

RAJARAJAN, K., ENGELS, M.C., WU, S.M. (2012). Reprogramming of mouse, rat, pig, and human fibroblasts into iPS cells. *Curr Protoc Mol Biol* 97: 23.15.1–23.15.32.

ROELANTS, K., GOWER, D.J., WILKINSON, M., LOADER, S.P., BIJU, S.D., GUILLAUME, K., MORIAU, L., BOSSUYT, F. (2007). Global patterns of diversification in the history of modern amphibians. *Proc Natl Acad Sci USA* 104: 887–892.

SAKAI, C., KONNO, F., NAKANO, O., IWAI, T., YOKOTA, T., LEE, J., NISHIDA-UMEHARA, C., KUROIWA, A., MATSUDA, Y., YAMASHITA, M. (2007). Chromosome elimination in the interspecific hybrid medaka between Oryzias latipes and O. hubbsi. *Chromosome Res* 15: 697–709.

SHA, H., CHEN, J., CHEN, J., ZHANG, P., WANG, P., CHEN, L., CHENG, G., ZHU, J. (2009). Fates of donor and recipient mitochondrial DNA during generation of interspecies SCNT-derived human ES-like cells. *Cloning Stem Cells* 11: 497–507.

SHI, D.L., BOUCAUT, J.C. (1995). The chronological development of the urodele amphibian *Pleurodeles waltl* (*Michah*). *Int J Dev Biol* 39: 427–441.

SHI, L.-H., MIAO, Y.-L., OUYANG, Y.-C., HUANG, J.-C., LEI, Z.-L., YANG, J.-W., HAN, Z.-M., SONG, X.-F., SUN, Q.-Y., CHEN, D.-Y. (2008). Trichostatin A (TSA) improves the development of rabbit-rabbit intraspecies cloned embryos, but not rabbit-human interspecies cloned embryos. *Dev Dyn* 237: 640–648.

SOTOMARU, Y., HIRAKAWA, R., SHIMADA, A., SHIOZAWA, S., SUGAWARA, A., OIWA, R., NOBUKIYO, A., OKANO, H., TAMAOKI, N., NOMURA, T., *et al.* (2009). Preimplantation development of somatic cell cloned embryos in the common marmoset (*Callithrix jacchus*). *Cloning Stem Cells* 11: 575–583.

SRIRATTANA, K., LAOWTAMMATHRON, C., DEVAHUDI, R., IMSOONTHORN-RUKSA, S., SANGMALEE, A., TUNWATTANA, W., LORTHONGPANICH, C., SRIPUNYA, N., KEAWMUNGKUN, K., PHEWSOI, W., *et al.* (2008). Effect of trichostatin A on developmental potential of inter-species cloned gaur (Bos gaurus) embryos. *Reprod Fertil Dev* 21: 126–126.

TECIRLIOGLU, R.T., GUO, J., TROUNSON, A.O. (2006). Interspecies somatic cell nuclear transfer and preliminary data for horse-cow/mouse iSCNT. *Stem Cell Rev* 2: 277–287.

WANG, K., BEYHAN, Z., RODRIGUEZ, R.M., ROSS, P.J., IAGER, A.E., KAISER, G.G., CHEN, Y., CIBELLI, J.B. (2009). Bovine ooplasm partially remodels primate somatic nuclei following somatic cell nuclear transfer. *Cloning Stem Cells* 11: 187–202.

WANG, K., OTU, H.H., CHEN, Y., LEE, Y., LATHAM, K., CIBELLI, J.B. (2011). Reprogrammed transcriptome in rhesus-bovine interspecies somatic cell nuclear transfer embryos. *PLoS ONE* 6: e22197.

WHEATLEY, S.P., O'CONNELL, C.B., WANG, Y.-L. (1998). Inhibition of chromosomal separation provides insights into cleavage furrow stimulation in cultured epithelial cells. *Mol Biol Cell* 9: 2173–2184.

WOODLAND, H.R., GURDON, J.B. (1969). RNA synthesis in an amphibian nuclear-transplant hybrid. *Dev Biol* 20: 89–104.

YAN, S.Y., MAO, Z.R., YANG, H.Y., TU, M.A., LI, S.H., HUANG, G.P., LI, G.S., GUO, L., JIN, G.Q., HE, R.F. (1991). Further investigation on nuclear transplantation in different orders of teleost: the combination of the nucleus of Tilapia (*Oreochromis nilotica*) and the cytoplasm of Loach (*Paramisgurnus dabryanus*). *Int J Dev Biol* 35: 429–435.

YAN, S.Y., TU, M., YANG, H.Y., MAO, Z.G., ZHAO, Z.Y., FU, L.J., LI, G.S., HUANG, G.P., LI, S.H., JIN, G.Q. (1990). Developmental incompatibility between cell nucleus and cytoplasm as revealed by nuclear transplantation experiments in teleost of different families and orders. *Int J Dev Biol* 34: 255–266.

YANG, H., SHI, L., ZHANG, S., LING, J., JIANG, J., LI, J. (2010). High-efficiency somatic reprogramming induced by intact MII oocytes. *Cell Res* 20: 1034–1042.

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