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Krakauer and Mira reply — Many of the issues raised by Perez *et al.* concern molecular mechanism rather than evolutionary function. Thus 'cause' does not carry the same meaning in evolution as in physiology. The evolutionary cause or function of sexual reproduction or two-step meiosis can be seen as a means of fending off parasites or selfish genetic elements: the developmental or mechanistic causes of these are different and involve precise chemical and genetic processes. We propose that the evolutionary function of atresia has been to eliminate mildly deleterious mutations from mitochondria, thereby retarding Müller's ratchet[1]. It is not necessary for all of the oocytes undergoing apoptosis in each generation to contain defective mitochondrial genomes, much as individuals do not need to be teeming with parasites or sister-killer mutants to engage in sex, or for cells to undergo meiosis. Once these adaptations arise, organisms are often committed to their implementation[2].

We suggest that the long-term evolutionary persistence of atresia requires that germ cells with healthy mitochondria represent statistically superior competitors in the ovary and are thus more likely to escape cell death to seed successive generations. The result of this competition is to eliminate the worst cells (when present) and retain a sufficiently large population of cells to produce the maximum number of offspring. This is rather like a game of musical chairs, where the worst competitors are eliminated early on and there is only one winner among many equally matched contestants. Thus many egg cells compete for survival factors through metabolism, but only a few will be successful. The mechanism remains unclear, although oocyte elimination must involve initiation of apoptosis by some somatically derived signal.

A key part of our theory is that competition for survival factors be promoted among germ cells carrying a small number of mitochondrial genomes. Cells with large numbers of defective mitochondria can metabolize at almost 100% efficiency, providing at least 10% of wild-type mitochondrial genomes are present[3] (the 'threshold effect'), so selective differences among cells only become apparent when wild-type genomes are rare in the germ cell. This is the function of the bottleneck — it decreases mitochondria to a level that makes the functional variance among cells selectively detectable (Fig. 1b of ref. 1).

The results of Perez *et al.* agree with our hypothesis and match our comparative data, showing that the number of mitochondria present during the bottleneck can predict the level of atresia. Both experimental and comparative data indicate that small numbers of healthy mitochondria can inhibit atresia, which in our view is evidence that mitochondria can indirectly influence oocyte fate. Mitochondrial genome-deletion experiments confirm the importance of genome quality for mitochondrial persistence.

Perez *et al.* suggest that atresia in the next generation is redundant, as the founder population of oocyte mitochondria have previously experienced selection. What is important selectively is the stage in development when mitochondria are sequestered into the oocytes and the reproductive lifespan of the mother. Assuming that all mitochondria in the oocyte at reproductive maturity are a subset of those present in pre-atretic fetal development (not the case in all species), then mutations could accumulate (through division or the effects of environmental mutagens) from the time of atresia up until the formation of the next-generation fetus. This can be between 13 and 40 years in humans. There is good evidence that ontogenetic mutations accumulate in mitochondria to reconstitute polymorphism[4], which might contribute by a similar mechanism towards the ongoing postnatal atresia discussed by Perez *et al.* In the absence of a germline bottleneck, ontogenetic ageing of mitochondria should proceed more rapidly.

As Perez *et al.* point out, many more cells are eliminated during early atresia than might plausibly contain defective mitochondrial genomes. This is surprising, but selection may incur some continuous or facultative response proportional to the mutational load per generation. As we noted earlier, however, this is not a feature of mammalian meiosis or sex that is obligate for development. The adaptively salient outcome of atresia is that it retains sufficient eggs for the female's reproductive lifetime. This might simply be a matter of insurance: evolution cannot always discover the globally optimal solution.

We have offered a purely functional theory of atresia, one that has the merit of addressing an evolutionary puzzle, Müller's ratchet in mitochondria. We believe that evolution (function) and molecular biology (mechanism) must work together in this area.

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Embryogenesis

Demethylation of the zygotic paternal genome

In mammals, both parental genomes undergo dramatic epigenetic changes after fertilization to form the diploid somatic genome. Here we show that the paternal genome in the mouse is significantly and actively demethylated within 6–8 hours of fertilization, before the onset of DNA replication, whereas the maternal genome is demethylated after several cleavage divisions. This active demethylation of the paternal genome may be associated with epigenetic remodelling of sperm chromatin, in order to establish parent-specific developmental programmes during early embryogenesis.

Previous molecular analysis[1–3] of digested genomic DNA from mature germ cells indicated that ovulated mouse oocytes are globally undermethylated compared with the sperm genome. A gradual genome-wide demethylation seems to occur during preimplantation development, leading to indistinguishable alleles at most gene loci, but not at those that are imprinted[1,2].

Intermediate amounts of methylation could be caused by a combination of undermethylated maternal and methylated paternal DNA[3], although they could be the result of highly dynamic and sometimes opposing demethylation or *de novo* methylation processes in parental genomes[4]. To investigate this, we analysed the global methylation of the paternal and maternal genomes in mouse pre-implantation embryos by using immunofluorescence against 5-methylcytosine (MeC). Because most MeC is found in various repeat-DNA families[4,5], antibody staining mainly reflects the density of MeC in interspersed repetitive sequences.

At 3 hours (Fig. 1a) and 6 hours (Fig. 1b) after fertilization, both the paternal and maternal chromosomes stained equally intensely with anti-MeC antibody. Because the ovulated oocytes are globally undermethylated, this finding suggests that there is rapid *de novo* methylation of egg chromatin shortly after fertilization[1]. After 8 hours, the paternal pronuclei were decondensed and so were much larger than the maternal pronuclei. Unexpectedly, all

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paternal pronuclei analysed after 8 hours showed very little methylation (Fig. 1c).

To prevent DNA replication, one-cell embryos were collected after 6 hours and cultured for a further 10 hours in the presence of aphidicolin (at 2 μg ml^{-1})[6]. The unreplicated paternal pronuclei also became MeC-negative (Fig. 1d). This demethylation may be associated with transient hyperacetylation of histone H4 (ref. 7), because both replication and transcription are initiated earlier in the male pronucleus[8], which is less condensed.

At first metaphase, we observed two differentially methylated and spatially separated chromosome sets[9] (Fig. 1e). To exclude the possibility that differential MeC staining was due to changes in the accessibility of paternal DNA, we stained mouse embryos with anti-DNA antibody[10]: male and female pronuclei produced equally intense immunofluorescence (Fig. 1f–j). Two-cell embryos in phases G1 and G2 of the cell cycle were prepared at 22 and 32 hours. Most interphase nuclei displayed highly localized MeC staining (Fig. 1k,l), reflecting the compartmentalization of the two genomes, which may bring about the differential epigenetic reprogramming of the two genomes.

The amount of global methylation of the maternal genome was largely maintained from the early pronuclear to the two-cell stage, but four-cell embryos 45 hours after fertilization had a much lower MeC density over the maternal half of the nucleus (Fig. 1m). Interphase nuclei of 16- and 32-cell embryos had equivalently low methylation of paternal and maternal DNA (data not shown). Thus, in contrast to the very rapid and active demethylation of the paternal pronucleus, gradual demethylation of the maternal genome occurred passively during the second and third cleavage stages by a replication-dependent mechanism[9], which may involve the loss of maintenance methylase activity. The second polar body remained methylated throughout preimplantation development.

Our results provide a dramatic demonstration of the loss of DNA modification after fertilization. Active zygotic demethylation of the paternal genome has important implications for the understanding of genomic imprinting, X-chromosome inactivation, mammalian cloning and *in vitro* fertilization.

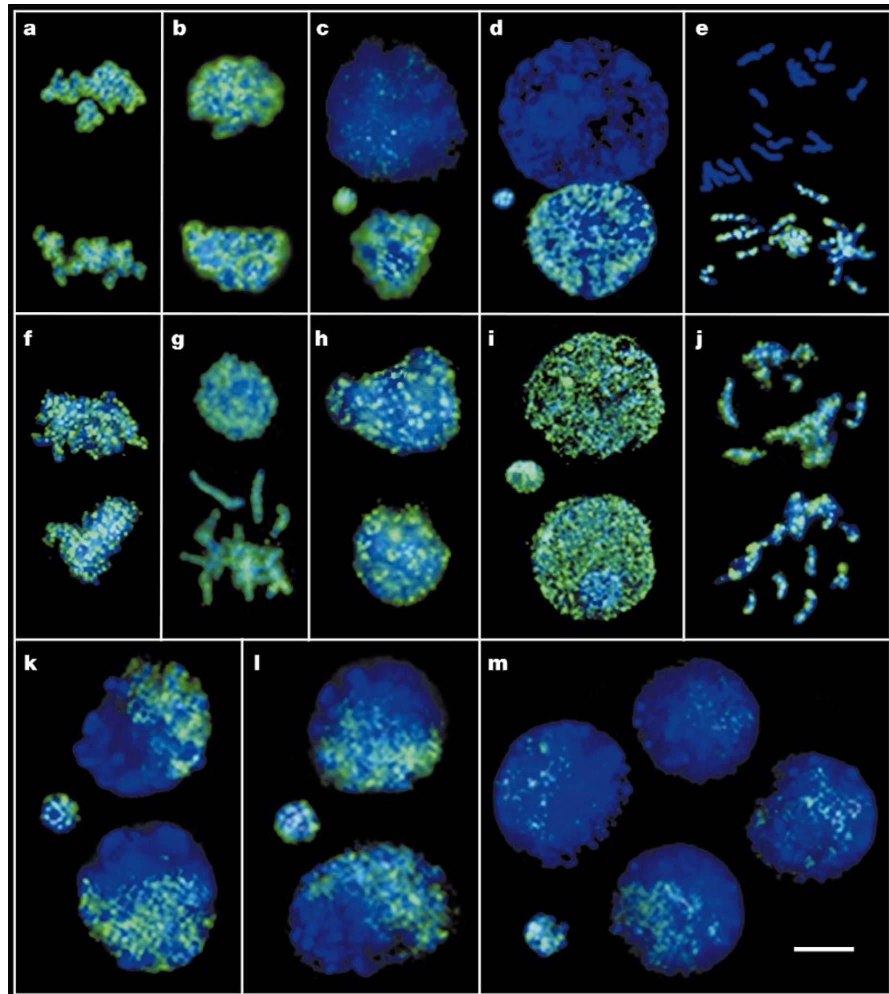

Figure 1 Differential demethylation of parental chromatin in the early mouse embryo. **a–e,** Anti-5-methylcytosine (MeC) immunofluorescence of mouse one-cell embryos. **a,** Zygote 3 h after fertilization with intense MeC labelling of both pronuclei (>10). Numbers in parentheses indicate the number of embryos analysed. **b,** Paternal and maternal pronuclei at 6 h (>10). **c,** Undermethylated paternal pronucleus at 8 h (>20). The smaller female pronucleus remains methylated. **d,** Aphidicolin-treated one-cell embryo displaying demethylation of the male pronucleus (>20). **e,** First metaphase (>5). **f–j,** Controls. Anti-DNA immunofluorescence of one-cell embryos demonstrates that both chromatin sets are accessible to antibody molecules. **f,** 3 h (>5). **g,** 6 h (>5). **h,** 8 h (>5). **i,** Aphidicolin treatment (>5). **j,** First metaphase (2). **k,l,** MeC staining of two-cell embryos at 22 h (>20) (**k**) and 32 h (>20) (**l**) shows that the paternal and maternal compartments have different methylation levels. **m,** Four-cell embryo at 45 h (>10). The MeC-staining intensity of the maternal half of the nucleus is weaker than in two-cell embryos. Scale bar, 10 μm.

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Correction

Fluid 'rope trick' investigated

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We wish to amend a small mistake in our calculations, although this does not affect the basic idea in our paper, particularly by comparison with experiment. As the longitudinal viscous stress σ in the filament scaled as $\sim \mu Ur/R^2$ varies linearly across the cross-section, the integrated stress resultant $\int \sigma dA$ vanishes. However, the net bending torque due to this viscous stress does not vanish and scales as $\int \sigma r dA \sim \mu Ur^4/R^2$. The force per unit volume on the fluid due to centripetal and Coriolis accelerations scales as $f \sim \rho \Omega^2 R$, so that the bending torque on the whirling filament in the vicinity of the coil scales as $fr^2R^2 \sim \rho \Omega^2 r^2 R^3$. Torque balance, together with the ancillary continuity relations, leads to a scaling law for the coiling frequency

$$\Omega \sim Q^{4/3} r^{-10/3} \nu^{-1/3} \qquad (1)$$

which is slightly different from the result given in our paper, where an erroneous argument confuses the transverse and longitudinal timescales in the filament. Equation (1) can be derived directly using an analogy to the coiling of an elastic rope by simply replacing the elastic bending modulus Er^4 in ref. 1 with the 'viscous bending modulus' $\mu r^4 U/R$. A reconsideration of the experimental results leads to data collapse with a power law $\Omega/Q^{1.33} \sim r^{-3.45 \pm 0.10}$, in agreement with our argument.

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