Molecular causes of aneuploidy in mammalian eggs

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Summary

Mammalian oocytes are particularly error prone in segregating their chromosomes during their two meiotic divisions. This results in the creation of an embryo that has inherited the wrong number of chromosomes: it is aneuploid. The incidence of aneuploidy rises significantly with maternal age and so there is much interest in understanding this association and the underlying causes of aneuploidy. The spindle assembly checkpoint, a surveillance mechanism that operates in all cells to prevent chromosome mis-segregation, and the cohesive ties that hold those chromosomes together, have thus both been the subject of intensive investigation in oocytes. It is possible that a lowered sensitivity of the spindle assembly checkpoint to certain types of chromosome attachment error may endow oocytes with an innate susceptibility to aneuploidy, which is made worse by an age-related loss in the factors that hold the chromosomes together.

Key words: Aneuploidy, Cell cycle, Chromosomes, Meiosis, Oocyte

Introduction

The oocyte and sperm, following their respective pre-meiotic S phases, undergo two meiotic divisions of their chromosomes to generate the haploid (see Glossary, Box 1) gametes. In women, and especially with increasing age (Fig. 1), these meiotic divisions (MI and MII, see Glossary, Box 1) are error prone, which results in whole chromosomes being either in excess or missing from the embryo following fertilization. Such embryos, which do not contain the diploid (see Glossary, Box 1) number and have lost or gained discrete chromosomes, are referred to as aneuploid (see Box 2). Most aneuploid embryos are non-viable, probably as a result of a gene dose effect that causes too much or too little of a crucial gene(s) to be expressed. Loss of autosomes (see Glossary, Box 1) appears to be especially lethal, and trisomy (see Glossary, Box 1), rather than monosomy (see Glossary, Box 1), is the principal aneuploidy detected in clinically recognized pregnancies. Although all autosomal trisomies show a high mortality rate *in utero*, some babies with trisomy 21, 18 and 13 (Down, Edward and Patau syndromes respectively, Fig. 1A) can survive to term.

Some adult tissues appear to tolerate high rates of somatic cell aneuploidy; up to 50% aneuploidy is found in human hepatocytes (Duncan et al., 2012a). However, the functionality of these distinct aneuploid cells within a predominantly diploid cell environment is difficult to assess, and generally somatic cell aneuploidy is regarded as both a driver and an indicator of abnormal cell function. It is also important to appreciate that reported aneuploidy rates can vary between studies and between species, depending on precisely how aneuploidy is being measured and the assumptions that have been made (e.g. if the rate of aneuploidy is measured for one

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chromosome and extrapolated to all chromosomes). Whole-chromosome losses or gains, which are the most relevant type of aneuploidy in oocytes, have been reported to occur at a rate of 4-5% in human sperm (Templado et al., 2011b; Lu et al., 2012). Interestingly, when chromosomal errors are seen with advanced paternal age, it is generally structural rearrangements rather than numerical changes that are observed – this has been suggested to be due to errors arising during spermatogonial stem cell division in the adult testis (Templado et al., 2011a). By contrast, aneuploidy rates in human eggs can be about 60% or higher (Fragouli et al., 2011; Kuliev et al., 2011) (Fig. 1B). Furthermore, although older studies report low aneuploidy rates in mice, similar rates up to this value have recently been reported in the eggs of aged mice (Fig. 1C). For example, recent independent studies have shown increases in mice: (1) from 4% to 25% in 8-week-old versus 70-week-old mice, by measuring only rates of hyperploidy (one can assume that this would be doubled to account for hypoploidy) (Pan et al., 2008); (2) from 12% to 30% in 3-month-old versus 12-month-old mice (Selesniemi et al., 2011); and (3) from 3% to 60% in 1-month-old versus 15-month-old mice (Merriman et al., 2012) (Fig. 1C). It is intriguing that much older studies failed to find any age-related effects in mice, even when using the same superovulation procedures used in the more recent studies (Golbus, 1981). We can only speculate that maternal age-related aneuploidy is influenced by factors such as mouse strain, a topic that lies outside the scope of this Primer. However, one can conclude that at least some recent independent studies have demonstrated a maternal age effect in mice.

Using three main approaches, a number of recent studies have shed some light both on what causes aneuploidy in young oocytes and the reasons behind age-related aneuploidy. The first approach involved very detailed imaging of the movements of the bivalents (also known as homologous chromosome pairs, see Glossary, Box 1) of young oocytes during MI – no mean feat for a process lasting several hours. These bivalent structures are assembled during foetal life following homologous recombination (see Glossary, Box 1; Fig. 2) and stay associated until anaphase I. Second, studies have been undertaken that test the ability of the spindle assembly checkpoint (SAC) in young oocytes to respond to either naturally occurring chromosome attachment errors or to chromosome attachment errors caused by various perturbations of the spindle or kinetochores (see Glossary, Box 1). Third, the decline of chromosome-associated factors controlling cell division has been examined during the aging process. Imaging shows that bivalents often form incorrect attachments to the spindle microtubules (Kitajima et al., 2011) (see Box 3) that need to be corrected to ensure faithful chromosome division at anaphase onset. However, in addition to this, the SAC, a process normally thought to monitor attachments and stall cell division until all chromosomes achieve bi-orientation (see Glossary, Box 1), appears to be less sensitive in oocytes: at least it appears not to be activated by a small number of attachment errors (Gui and Homer, 2012; Kolano et al., 2012; Lane et al., 2012; Sebestova et al., 2012). The conclusions borne

Box 1. Glossary

Autosomes. All the chromosomes that are not the sex chromosomes (X and Y, in mammals).

Bi-orientation. The correct attachment of chromosomes to the spindle; in meiosis I, one pair of sister kinetochores are attached to each spindle pole, resulting in tension across the bivalent.

Bivalent. The structure that results from the association of a pair of homologous chromosomes during meiosis. It is normally present in MI.

Centromere. The region of the chromosomes upon which kinetochores are assembled.

Cohesin. A protein ring responsible for tethering chromosome arms together following DNA replication in S phase. In oocytes, cohesin is also responsible for holding homologous chromosomes together in the bivalent structure during meiosis I.

Diploid. The state a cell is in if it has pairs of all its homologous chromosomes and sex chromosomes. This would be considered normal.

Dyad. Chromosome structure normally present in MII as a result of a bivalent separation in MI. It is identical in structure to a 'sister chromatid pair' or 'univalent'.

Haploid. The state in which a cell has only one of each pair of homologous chromosomes and one sex chromosome. It is only ever achieved in gametes.

Homologous chromosomes. Chromosomes in a cell that differ only in their parental origin. Diploid human cells have 22 homologous chromosomes (autosomes) and one pair of sex chromosomes.

Homologous recombination. Exchange of genetic material between the two homologous chromosomes. It is a normal feature of meiosis occurring in females during foetal life.

K-fibre (kinetochore-fibre). A microtubule bundle that promotes chromosome movement at anaphase. One end of the bundle associates with the spindle pole and the other attaches to the kinetochore in an end-on manner.

Kinetochore. A protein-based structure on the chromosome that allows docking with spindle microtubules.

MI. The first meiotic division, during which bivalents separate.

MII. The second meiotic division, during which the univalents separate.

Monosomy. A state in which a cell that has lost one or more chromosomes.

Polar body. A small portion of oocyte cytoplasm, containing half the chromosomes, that plays no further role in meiosis. Both meiotic divisions in the oocyte are highly asymmetrical, resulting the extrusion of the first polar body (at completion of MI) and the second polar body (at completion of MII).

Trisomy. A state in which a cell has gained a single copy of one chromosome.

Univalent. One member of the pair of the homologous chromosomes. If, during MI, the bivalent disassociates into its two pairs of homologous chromosomes, each pair would be known as a univalent. Identical in structure to a 'dyad' or 'sister chromatid pair'; sometimes used specifically to refer to separation of a bivalent in MI.

Fig. 1. The effects of maternal age on aneuploidy rates. (**A**) Live birth incidence of the three most viable human trisomies: 13, 18 and 21. Incidence is predicted in the absence of any termination of pregnancy. Data are taken from Savva et al. (Savva et al., 2010) and constitute pre- and postnatal records from 4.5 million births in the UK and Australia. (**B**) Incidence of aneuploidy for chromosomes 13, 16, 18, 21 and 22 detected between 1997 and 2009 in a single Chicago clinic that screened both polar bodies in over 20,000 human metaphase II eggs. Data are taken from Kuliev et al. (Kuliev et al., 2011). (**C**) Incidence of total aneuploidy rates measured in ovulated eggs from Swiss CD1 mice (all chromosomes); analysis was made from chromosome and kinetochore counts remaining in the egg after first polar body extrusion. Data are taken from Merriman et al. (Merriman et al., 2012).

Here, we review recent data on factors that determine successful segregation in female meiosis and explain how this might be related to an age-related decline in female segregation accuracy. Furthermore, we present our ideas for future developments in this field. We will not discuss male meiosis in detail, primarily because these segregation errors do not appear to contribute as significantly to aneuploidy in embryos. This is probably related to a fundamental difference in the timing of male meiosis: post-puberty, waves of spermatogenesis produce mature sperm; hence, the extreme time delay between meiosis entry and completion, which is relevant in females, is not a feature in males.

Timelines of oocyte development

In mammals, the female, once born, is endowed with her full complement of oocytes, and these have to last her entire reproductive lifespan (Fig. 3, top panel). Following puberty, cyclical recruitment of ovarian follicles into the growing pool culminates in the formation of fully grown follicles, the number of which depends on the species, that contain a prophase I-arrested oocyte. Follicle growth does not cause any progression in the meiotic status of the oocyte, because this arrest has been

from these imaging studies reveal a vulnerability of oocytes to segregation errors. Added to this is the observation that with increasing female age there is an associated decline in protein factors, such as Rec8 and Sgo2 (shugoshin-like 2; Sgol2 – Mouse Genome Informatics), which are normally viewed as being necessary to maintain bivalent cohesion (Duncan et al., 2009; Chiang et al., 2010; Lister et al., 2010). Such findings do not point to a single factor governing the rise in age-related aneuploidy, but do aid our understanding of how such errors can occur.
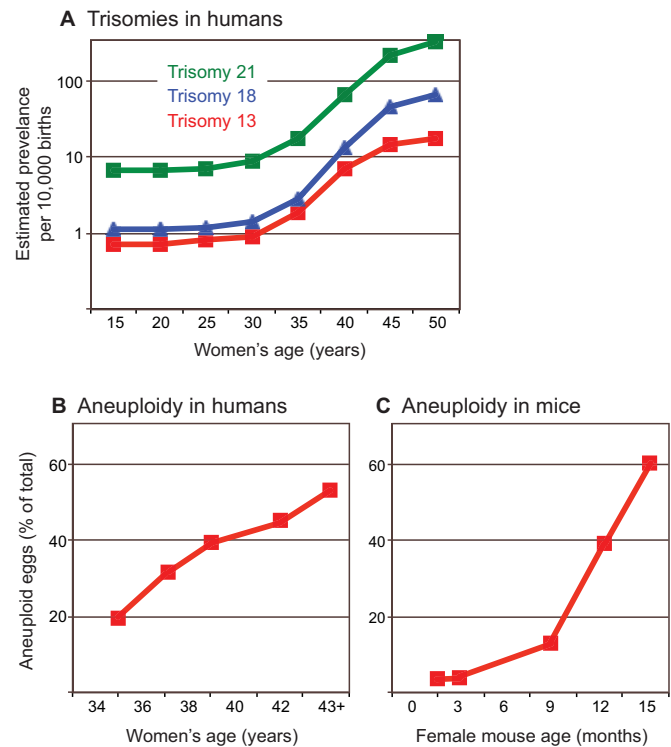
maintained since the initiation of meiosis in the foetal ovary. Instead, it is a surge of luteinizing hormone that triggers both ovulation and the release from prophase I arrest, and in so doing promotes the completion of MI and extrusion of the first polar body (see Glossary, Box 1; Fig. 3, part ii). By the time the oocyte has been ovulated, the now fully mature oocyte (or 'egg') has become re-arrested at metaphase of the second meiotic division (met II; Fig. 3, top panel). It is the sperm at fertilization that triggers release

from this arrest, completion of MII and entry into the subsequent embryonic mitotic cell cycles (Fig. 3, top panel). This sperm, by contrast, is formed only after puberty and from a continuous supply, arresting neither in prophase I nor in metaphase II. Its meiotic divisions are symmetrical, giving rise to four mature gametes per progenitor cell (Fig. 3, bottom panel).

Bivalents exist in a prophase I-arrested oocyte and are formed from the tethering together of homologous chromosomes during recombination. This tethering happens shortly after commitment to meiosis in the foetal ovary (Fig. 2B,C). It is the segregation of these bivalents, which is often called a 'reductional division', that occurs in MI. This division generates two pairs of sister chromatids, one of which ends up in the first polar body. The sister chromatid pair remaining in the egg cytoplasm is called a dyad (see Glossary, Box 1). The sperm at fertilization triggers the segregation of the dyad into two single chromatids; this is often called an 'equational division' (Fig. 4A). This second division resembles the mitotic division of somatic cells because, in both, sister chromatid pairs are dividing. By contrast, MI is a division unique to sperm and oocytes because a bivalent is a meiosis-specific structure.

When do the errors in meiotic chromosome segregation occur?

The loss or gain of chromosomes in an embryo can occur because of a mis-segregation event in: (1) the mitotic divisions of the primordial germ cell from which the gametes are created; (2) the meiotic divisions of an oocyte or sperm; or (3) the mitotic divisions of the embryo following fertilization. Indeed, errors at all these points have been reported (Hultén et al., 2008; Vanneste et al., 2009; Fragouli et al., 2011). However, the available evidence clearly shows that the most segregation errors arise during the maternal meiotic divisions (Hassold and Hunt, 2009; Nagaoka et al., 2012).

During the two divisions in the oocyte, there are many possible routes by which aneuploidy can be generated. The extra chromosome present in trisomies, for example, may have arisen through the lack of segregation of a bivalent between the oocyte and first polar body during MI. This is described as 'MI', 'homologue', 'true' or 'classic' non-disjunction (Fig. 4B, part i). Alternatively, the trisomy may have been caused by a lack of segregation of the sister chromatid pair between the egg and the

Fig. 2. The generation of a bivalent in meiosis I. (**A,B**) During pre-meiotic S-phase, the sister chromatids (red and blue indicate different parental origin) become tethered together by cohesin rings (green hoops). (**C**) During MI prophase, the homologous chromosomes become paired and joined by the process of homologous recombination to form a bivalent. (**D**) During the MI division, the sister kinetochore pairs (grey) act as a single unit; they will each make attachments to a single spindle pole via k-fibres (green) in a process termed bi-orientation. The k-fibres are not able to pull apart the bivalent because of the cohesin located distal to the points of crossover on the chromosome arms. At anaphase onset, separase acting on these distal cohesin units permits dissolution of the bivalent.

Box 3. What is correct attachment in MI?

Meiosis I is unique in that it contains the bivalent. The bivalent comprises four kinetochores; however, the two kinetochores of each sister chromatid pair act as a single unit, creating only two functional kinetochores.

Amphitelic attachment (see figure) is a correct attachment – it requires that the two kinetochore pairs are connected to opposite spindle poles by k-fibres; each kinetochore pair has a monopolar attachment. This results in equal tension generation across the bivalent, coming from pulling forces towards the two poles, and this should lead to faithful segregation of the chromosomes at anaphase I. Syntelic attachment is where both pairs of sister kinetochores form attachments to the same spindle pole. This does not result in the generation of tension, and would lead to movement of the bivalent towards the attached pole. Merotelic attachment occurs when one of the kinetochore pairs attaches to both spindle poles simultaneously. It is likely to happen following loss of cohesin, which allows the two sister kinetochores of a pair more flexibility, and so allows them to act independently of each other. Merotelic attachment does not necessarily affect the position of the chromosome on the spindle. Instead, merotelic attachments are only likely to be revealed at anaphase, when the balanced pulling forces on the kinetochores result in the chromosome 'lagging' at the furrow cleavage, instead of being pulled towards one pole. Loss of cohesion is thought to influence the ability of the two sister kinetochores to act as a single unit. With age, increases in the distance between sister kinetochores in MII correlate with aneuploidy. This may be because the increased distance allows independent behaviour of the two kinetochores in MI, potentially allowing formation of merotelic attachments.

second polar body during MII: MII-non-disjunction (Fig. 4B, part ii). Finally, at some point in MI or MII, before or during the segregation event, the normal pairing of chromosomes may have broken down. In MI, this could lead to the generation of two pairs of sister chromatids, called univalent (see Glossary, Box 1), formed by the breaking down of a bivalent (Fig. 4B, part iii). These two univalents may also lose their individual integrity and dissolve down further into the two single chromatids that make up the univalent. Alternatively, the dyad could be prematurely resolved into two single chromatids in MII (Fig. 4B, part iv). These events depicted in Fig. 4B, parts iii and iv are often referred to as 'pre-division' or 'premature separation of sister chromatids' (PSSC), and

Fig. 3. The many divisions involved in the formation of an embryo. Divisions occurring in the female (top panel) and male (bottom panel) germ cell lineage are shown, from the pre-meiotic germ cell progenitor divisions (i, iv), through male and female meiosis (ii, v), including fertilization, to the early mitotic divisions of the embryo (iii). The chromosome complement is indicated, such that c represents the haploid number for an organism (humans, c=23; mouse, c=20). One pair of chromosomes is shown (maternal in red/green and paternal in blue/cyan). LH, luteinizing hormone; MI, first meiotic division I; MII, second meiotic division 2; met II, metaphase of the second meiotic division; PB, polar body; pro I, prophase I.

Fig. 4. Possible routes to aneuploidy during the female meiotic divisions. The division of a single set of chromosomes during the two meiotic divisions in the case of normal divisions (A) or abnormal divisions that lead to aneuploidy (B). (**A**) A normal set of meiotic divisions involves the division of a bivalent in meiosis I and the equational division of the remaining dyad in meiosis II. Addition of a single chromatid (pale blue and dark blue) from the sperm results in the correct chromosome complement (two chromatids) in the zygote. (**B**) Aneuploidy can arise in several ways. In classic non-disjunction (i), both homologous chromosomes remain in the oocyte following meiosis I. Following fertilization and an equational division in meiosis II, the resulting zygote has an extra single chromatid. Failure of the two chromatids within a dyad to segregate 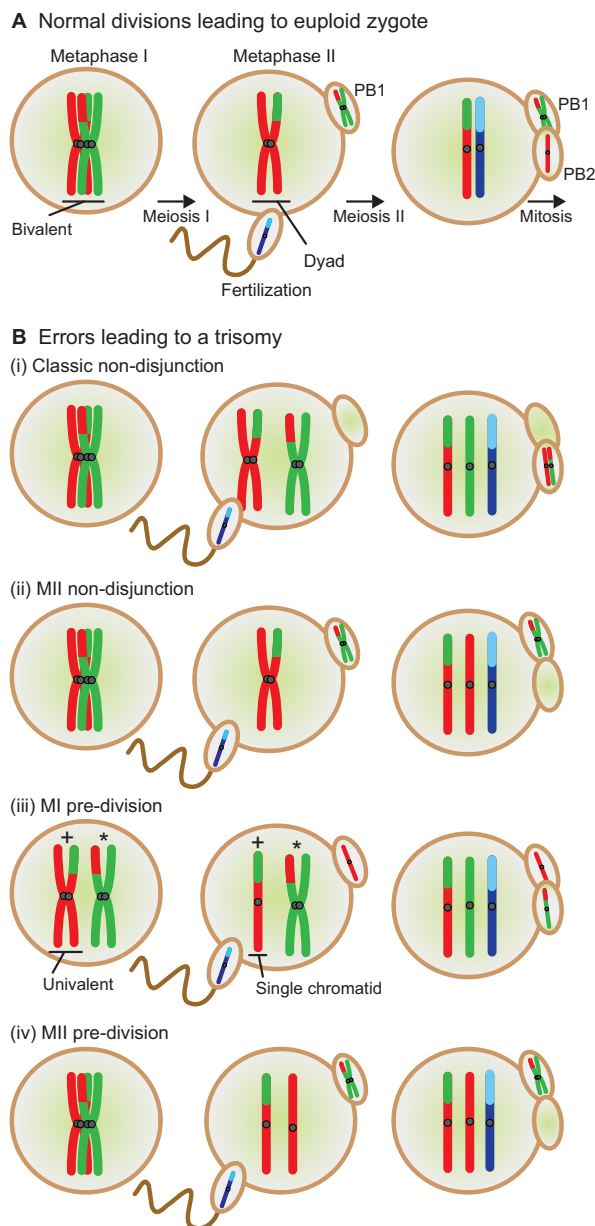at meiosis II (MII non-disjunction, ii) can also result in an extra chromatid in the zygote. Loss of cohesion between the two homologous chromosomes of the bivalent before anaphase I generates two 'univalents' in the oocyte (MI pre-division, iii). These can either segregate intact (*) or can be prematurely divided into chromatids (+) during meiosis I. Random segregation of the chromatid (+) in meiosis II may result in a trisomic zygote. Finally, loss of cohesion within a dyad in meiosis II (MII pre-division, iv) leaves two chromatids that segregate randomly at anaphase II, potentially leading to trisomy (shown here), to euploidy or to monosomy. PB, polar body.

lead to independent segregation of individual chromatids, thus allowing the embryo to inherit the wrong number of chromosomes.

Using classical cytogenetic approaches to screen met II human eggs, the presence of individual chromatids in such eggs has pointed towards a susceptibility to undergo pre-division (Angell, 1991; Angell, 1997). Furthermore, more modern techniques, such as spectral karyotyping and comparative genome hybridization (CGH), appear to back up this claim (Fragouli et al., 2011; Kuliev et al., 2011). However, it is important to appreciate that many of these studies use oocytes from individuals attending IVF clinics, who may not be representative of the general population. Additionally, the oocytes used are often ones that 'failed to fertilize' and so, again, may be far from representative. More subtly, all of the techniques employed on human oocytes are non-dynamic so they cannot capture the events as they happen in real-time. In addition, when counting chromosomes in met II eggs, latent errors that may not have affected chromosome number, such as the breakdown of a dyad into two chromatids, cannot be detected.

In summary, therefore, despite caveats regarding how representative they are, large-scale human egg studies appear to show a complex mix of segregation errors, the origins of which can be in MI or MII. Indeed, it appears that timing of the segregation error may be influenced by which chromosome is being discussed (see Box 4). However, the most clinically relevant aneuploidies in humans associated with spontaneous abortions appear to have their origins in maternal MI (Hassold and Hunt, 2009; Nagaoka et al., 2012). Therefore, we focus here on MI, rather than on MII.

An overview of the timing and control of meiosis in oocytes

Given the limitation of studying human oocytes, investigators soon appreciated that for a detailed understanding of meiosis, the mouse, the reproductive biology of which is fairly similar to that of humans, presents an attractive model system. Importantly, in spite of their far shorter reproductive lifespan, mice nonetheless show a similar age-related aneuploidy trend to that seen in humans (Fig. 1). The first meiotic division in murine oocytes, measured *in vitro* from the time of meiotic resumption (marked by dissolution of the nuclear envelope) to first polar body extrusion, typically has a duration that is dependent on strain but is generally between 8 and 12 hours. In humans, the duration of this division is 24-36 hours. Below, we provide an overview of the control and timing of some major events in MI that are of relevance to aneuploidy.

The roles of Cdk1 and separase in meiosis

In MI, and in fact in MII and all mitotic divisions, entry and exit are controlled by the activity of the kinase Cdk1 (cyclin-dependent kinase 1), and separation of the chromosomes are controlled by the thiol protease separase. By modulating the activities of this kinase and this protease, the oocyte successfully navigates its way into and out of each meiotic division. Cdk1 is the primary driver of entry into the meiotic (and mitotic) division by virtue of its ability to phosphorylate a wide range of substrates that are needed to dissolve the nuclear envelope (except in MII, where there is no nuclear envelope), condense chromosomes and establish a spindle. Entry into the meiotic divisions is triggered by a rise in Cdk1 activity and, reciprocally, its activity needs to fall in order to complete each division. Crucial to Cdk1 activity is its ability to bind a cyclin, and the most relevant cyclins in oocytes appears to be the B-type cyclins. Despite the requirement for a rise in Cdk1 activity in order to build the spindle microtubule structure, the separation of the

Box 4. Why should the properties of a chromosome have any influence on when its mis-segregation happens?

In principle, there are a number of considerations that could influence the timing of the mis-segregation event; three are given here.

Size

In humans, chromosome 1 is the largest (corresponding to 8% of nuclear DNA) and chromosome 21 the smallest (~1-2% of nuclear DNA). If the bivalent for chromosome 21 were loaded with the same density of cohesin, it would have only one-quarter to one-eighth the total content of cohesin molecules as the bivalent of chromosome 1. A lower cohesin content could potentially influence how successful the bivalent is in maintaining its integrity upon pulling forces from spindle microtubules during MI, or with respect to maternal aging, the small bivalent may be vulnerable to age-associated cohesin loss.

Crossover

This is the location and number of the homologous recombination events. A distal crossover point located at the telomere may generate a bivalent that is readily pulled apart by microtubules in MI and contains only a small amount of distal cohesin that is susceptible to age-associated loss.

Centromere location on which the kinetochore is built

A centromere located centrally along the chromosome (metacentric) or nearly central (submetacentric) would produce arms of equal or similar length. A centromere that is located closer to the telomeres than to the centre (acrocentric), or one that is at the telomere ends (telocentric) produces arms of very unequal length. No human chromosomes are telocentric: six are acrocentric (13, 14, 15, 21, 22 and Y), as are all mouse chromosomes; the rest are metacentric or submetracentric. An acrocentric chromosome with a single crossover event on the small (p) arm may be very vulnerable to losing its integrity as a bivalent, owing to the very small amount of cohesin associated with the distal region of the p arm.

chromosomes themselves is dependent on dissolution of the cohesin (see Glossary, Box 1) ties holding them together, which is achieved by the regulated activation of separase (Herbert et al., 2003; Terret et al., 2003). At anaphase onset, chromosomes are thus pulled towards the spindle poles by k-fibres (see Glossary, Box 1) that are attached to them through their kinetochores.

The kinase Cdk1, through binding cyclin B1 and being activated by the phosphatase Cdc25, is responsible for meiotic resumption after the prophase I arrest (Solc et al., 2008; Zhang et al., 2008; Oh et al., 2010; Holt et al., 2011). Recent evidence also suggests that cyclin B2 plays a role in meiotic entry (Gui and Homer, 2013). Cdk1 activity during exit from MI and at fertilization in MII, needs to decrease; this is triggered by both cyclin degradation and increased inhibitory phosphorylation of Cdk1 (Nixon et al., 2002; Herbert et al., 2003; Hyslop et al., 2004; Oh et al., 2011; Oh et al., 2013).

Separase is responsible for cleaving a specific subunit, the kleisin component, of cohesin (Nasmyth, 2011). Cohesin is thought to form a ring-like structure around newly replicated chromosomes in S phase, and so prevent their separation until anaphase (Nasmyth and Haering, 2009). Separase is kept inactive by at least two inhibitory mechanisms. The first involves negative phosphorylation by Cdk1 and the second involves binding to a chaperone protein called securin (initially called PTTG in human cells) (Shindo et al., 2012). During exit from both MI and at fertilization in MII, separase needs to be activated (Kudo et al., 2006; Kudo et al., 2009). This is triggered directly by a loss of securin and indirectly

via a loss of cyclin B1, both of which are effected by the anaphase-promoting complex/cyclosome (APC), as discussed below.

Anaphase-promoting complex-mediated control of meiosis

In oocytes, as in all cells, degradation of both cyclin B1 and securin is brought about by the APC, an E3 ubiquitin ligase that polyubiquitylates substrates, marking them for immediate proteolysis by the 26S proteasome (Manchado et al., 2010; Jones, 2011). APC substrates such as cyclin B1 and securin bind to Cdc20 (cell division cycle 20), which is a co-activator of the APC, by virtue of discrete motifs known as 'degrons' in their primary sequence. Therefore, APC^{Cdc20} activation leads to loss of cyclin B1 and securin, and consequently to a drop in Cdk1 activity and to the activation of separase, respectively.

The essential role of the spindle assembly checkpoint

The SAC is a signalling system that inhibits APC^{Cdc20}. Its constituent proteins were first discovered in yeast but, functionally, it has been best characterized in somatic cell lines. It determines when the chromosomes in a cell are ready to be divided, and only once this state is achieved does it relinquish its inhibition of the APC. In other words, its role is to couple complete and correct chromosome attachment to the spindle with anaphase onset. Therefore, there has been much interest over recent years in understanding how the APC is controlled by the SAC in meiosis, in the anticipation that there may be differences that could account for the high rates of maternal meiotic aneuploidy.

First, we must examine somatic cells in which the most is known. It is required that each chromosome be bi-orientated, i.e. its two sister kinetochores attached by microtubules to opposing spindle poles in order to switch off the SAC. There is also evidence that tension must be generated between the two kinetochores, although whether this contributes directly or indirectly to satisfying the SAC is uncertain (Khodjakov and Pines, 2010; Musacchio, 2011). Once the criteria of bi-orientation are met (for every chromosome), the SAC is switched off, the APC is promptly activated and anaphase follows shortly afterwards (Fig. 5). Its effectiveness has been demonstrated in what is now regarded as a classic experiment using laser ablation to eradicate the last remaining unattached kinetochore in a metaphase cell (Rieder et al., 1995). Anaphase follows shortly afterwards, indicating that the single, now ablated, kinetochore is sufficient to generate a SAC 'wait anaphase' signal and so prevent anaphase by inhibiting the APC.

The nature of the inhibitory signal has been keenly studied and is universally present in eukaryotic cells. It is comprises at least six core members: Mad1 (Mad1l1; MAD1 mitotic arrest deficient 1), Mad2 (Mad2l1; MAD2 mitotic arrest deficient-like 1), Bub1 (budding uninhibited by benzimidazoles 1 homolog), Bub3 (budding uninhibited by benzimidazoles 3 homolog), BubR1 (Bub1b; budding uninhibited by benzimidazoles 1 homolog β) and Mps1 (monopolar spindle 1, also known as Ttk protein kinase). The signal transduction pathway between the kinetochore and the APC is shown in Fig. 5 and has been reviewed previously (Lara-Gonzalez et al., 2012). The most upstream components of the SAC signal are assembled on kinetochores that are not attached to k-fibres (Fig. 5A). Such a lack of attachment would predominate during the early stages of spindle assembly. The unattached kinetochore acts as a platform to produce a diffusible inhibitory signal comprising the SAC proteins Mad2, Bub3 and BubR1, which additionally and crucially incorporate the APC activator protein Cdc20 (this complex is often referred to as the 'mitotic checkpoint complex'). This inhibits the APC by both sequestering
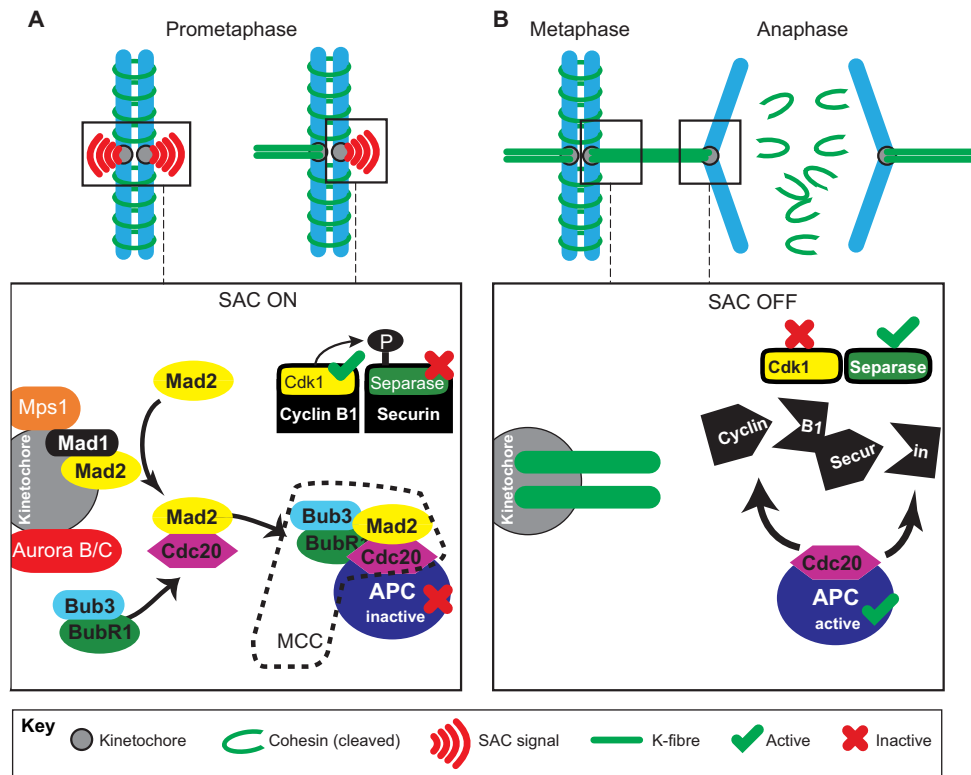
Fig. 5. The spindle assembly checkpoint pathway. (**A**) Chromosomes (blue) held together by cohesin proteins (green rings) progress through mitosis, accumulating microtubule attachments (green) to their kinetochores (grey) as they do. In prometaphase, unattached kinetochores act as a platform for the generation of the inhibitory SAC signal (red, see inset). Aurora kinase B/C, which is increasingly seen as a SAC component, can destabilize weak kinetochore-microtubule interaction, but is physically removed from substrates when tension develops. Mps1 helps recruit Mad2 to the kinetochore, which is converted by a Mad1-Mad2 heterodimer into a Cdc20-binding protein. This combines with BubR1 and Bub3 to form the mitotic checkpoint complex (MCC), and together presents Cdc20 as a pseudo-substrate of the APC. This protects cyclin B1 and securin from degradation and thus protects cohesin from cleavage. (**B**) Later, at metaphase, following attachment of all kinetochores, the propagation of the SAC signal is lost (see inset). The APC binds Cdc20, allowing it to target cyclin B1 and securin. Degradation of cyclin B1 and securin leads to inactivation of Cdk1 and activation of separase, respectively. Active separase then cleaves cohesin proteins, allowing anaphase to occur. Bub3, budding uninhibited by benzimidazoles 3 homolog; BubR1 (Bub1b), budding uninhibited by benzimidazoles 1 homolog β; Cdc20, cell division cycle 20; Cdk1, cyclin-dependent kinase 1; Mad1 (Mad1l1), MAD1 mitotic arrest deficient 1; Mad2 (Mad2l1), MAD2 mitotic arrest deficient-like 1; Mps1, monopolar spindle 1 (also known as Ttk protein kinase). SAC, spindle assembly checkpoint.

its activator Cdc20 and causing degradation of Cdc20 by the APC (Mansfeld et al., 2011; Foster and Morgan, 2012; Uzunova et al., 2012) (Fig. 5A). Once all kinetochores are attached to microtubules and under tension, the SAC is switched off and anaphase onset occurs (Fig. 5B).

How does aneuploidy in mammalian eggs arise?
The SAC is present and functional in oocytes
One reasonable hypothesis to explain their higher segregation error rate is that oocytes lack a SAC altogether, and that the APC is regulated independently of this surveillance mechanism. Indeed, MI in frog oocytes was initially proposed to be regulated independently of the APC (Peter et al., 2001; Taieb et al., 2001). However, the involvement of the APC (Reis et al., 2007; Jin et al., 2010) and the presence of a full complement of SAC proteins (Table 1) in mouse oocytes would appear beyond question. All the SAC proteins investigated in mouse oocytes are present and functional: their loss leads to increased mis-segregation or aneuploidy, as well as a shortening of MI, as would be predicted if APC activation is initially SAC inhibited. Therefore, if the SAC components are all present and functioning why should this mechanism fail to prevent mis-segregation of chromosomes in oocytes?

What satisfies the SAC surveillance mechanism in oocytes?
An interesting series of observations that set the scene for examining how the SAC functions in oocytes have been gained from studying the behaviour of univalents in MI. When univalents are generated by blocking homologous chromosome recombination, oocytes do not show a protracted MI arrest, nor do the univalents undergo a strict equational division. Instead, some of the univalents undergo a division whereby they remain intact, i.e. equivalent to a normal reductional division, but lacking the homologue partner (Nagaoka et al., 2011). How is the SAC satisfied when presented with these univalents? The lack of proper alignment suggests that some of these univalents do not form attachments to both spindle poles and come under tension – a condition that would make them line up at the spindle equator. Instead, the observations are consistent with the SAC being satisfied by univalent attachment to k-fibres, regardless of whether such attachment is to only one pole or to both. Thus, a lack of bi-orientation in a proportion of the univalents is not sufficient to induce a SAC arrest. However, despite the division, a delay of a few hours is still observed in the completion of MI. This may be caused by some SAC activity, which is attempting to block MI. In addition, the fate of the univalents depends on the genetic background of the mice (Woods et al., 1999).

DEVELOPMENT

Table 1. Functionality of SAC proteins in mouse oocytes

SAC component	Intervention	Acceleration of meiosis I	Aneuploidy	Reference
Mad1	Antibody injection	Not determined	Not determined (misalignment high)	(Zhang et al., 2005)
Mad2	Knockdown	At 2 hours	30%	(Homer et al., 2005b)
BubR1	Dominant negative	At 3 hours	Not determined	(Tsurumi et al., 2004)
Bub1	Knockout	At 3 hours	100%	(McGuinness et al., 2009)
Bub3	Knockdown	No	80%	(Li et al., 2009)
Mps1	Mutant (N-terminal deletion)	At 2.5 hours	70%	(Hached et al., 2011)
Aurora B/C Kinase	Dominant negative	At 3 hours	30%	(Yang et al., 2010;
	Pharmacological inhibition			Lane et al., 2010)

The acceleration of meiosis I is measured relative to controls; the aneuploidy rate is the absolute level achieved following inhibition of the SAC component (this is usually on background aneuploidy rate of <10%).

Despite the above concerns, there are several supporting studies that all concur with the interpretation that the SAC is not a surveillance mechanism that can act in response to all types of incorrect microtubule attachment. First, univalents generated by other means also fail to stall MI: this is true for the single X chromosomes in XO female oocytes (LeMaire-Adkins et al., 1997) and for univalents that are generated in interspecies crosses of mice (Sebestova et al., 2012). Furthermore, a number of subsequent studies using mice in which recombination has not been altered, all show that bivalent alignment on a metaphase plate is not a prerequisite for initiation of chromosome segregation (Woods et al., 1999; Gui and Homer, 2012; Kolano et al., 2012; Lane et al., 2012; Sebestova et al., 2012). Kolano and colleagues used an oocyte-specific, mutant of nuclear mitotic apparatus protein (NuMA), a protein known to be involved in mitotic spindle assembly. The mutant NuMA lacked its microtubule-binding domain and thus mislocalized, consequently causing changes in spindle assembly kinetics. In this study, the authors observed defects in bivalent congression and a lack of tension development across bivalents. Despite this, Mad2, an important SAC mediator whose inhibitory signal at the kinetochore lies at the heart of the SAC surveillance mechanism, was lost from kinetochores with normal dynamics, leading to completion of MI with normal timing. Gui and Homer used knockdown of CENPE, a plus-end-directed kinesin 7 motor protein, to interfere with the formation of microtubule-kinetochore attachments. In these CENPE-deficient oocytes, loss of Mad2 from kinetochores was delayed but, crucially, when Mad2 loss from kinetochores first occurred it was in the absence of stable k-fibre attachments, demonstrating that initial unstable attachments between microtubules and kinetochores are sufficient for Mad2 removal. Similarly, non-aligned bivalents in wild-type oocytes, which were not under tension and often either lacked or had incorrect attachments, were common during MI, and were unable to retain sufficient Mad2 protein to influence APC activity negatively (Lane et al., 2012).

Clearly, a compelling important conclusion can be drawn from these observations from several independent groups: that the SAC surveillance mechanism in oocytes does not need all bivalents to be bi-orientated in order to be satisfied. It may be that only the majority of kinetochores needs to be connected to microtubules in order to satisfy the SAC. Alternatively, weak lateral interactions of microtubules with kinetochores are capable of depleting those kinetochores of Mad2, and so satisfying the SAC. Indeed such interactions of microtubules with kinetochores are a common feature during MI in mouse oocytes (Brunet et al., 1999).

Nevertheless, one other study has suggested that a single bivalent can, in some circumstances, be sufficient to inhibit the APC (Hoffmann et al., 2011). However, the study was performed in bisected oocytes that, although containing the full complement of chromosomes, were half the normal volume. It is possible that the decreased oocyte volume is responsible for the increased efficacy of the SAC. Evidence to support the relationship between SAC activity and nuclear volume comes from *Xenopus* oocytes, which are far larger in volume and completely lack a SAC response (Shao et al., 2013). In *Xenopus* cell-free extracts, a SAC response is also absent, but can be induced by increasing nuclear density through addition of sperm nuclei (Minshull et al., 1994).

The slow pace of meiosis I gives time to achieve bi-orientation

The satisfaction of the SAC during MI leads to APC activation, and therefore to cyclin B1 and securin degradation. The clock can therefore be considered to be started and in countdown to anaphase from this time forwards. Erroneous attachments of kinetochores to microtubules, if unrepaired, would therefore lead to mis-segregation and likely cause aneuploidy. It is therefore fortunate that there are several hours between initiation of APC activation, measured as the onset in degradation of its substrates cyclin B1 and securin [revealed both by immunoblotting their levels in MI and by monitoring degradation of exogenously expressed GFP constructs (Herbert et al., 2003; Reis et al., 2007)], and anaphase onset for this to be achieved. In fact, over these hours one can observe in mouse, via live imaging, the repair mechanism in place as bivalents progressively achieve bi-orientation (Kitajima et al., 2011; Lane et al., 2012). It has been calculated that, on average, each bivalent undergoes three rounds of error correction before successful attachment is reached (Kitajima et al., 2011). Several similar events are also observed in chromosomes in mitotic cells before anaphase, but over a much shorter time scale (Magidson et al., 2011).

How are incorrect attachments destabilized and correct attachments promoted? In the case of mitosis, weak outer kinetochore-microtubule interactions allow aurora kinase B, which is located on the centromere (see Glossary, Box 1), to phosphorylate and destabilize the binding of outer kinetochore proteins with such microtubules. Following end-on microtubule attachment to both kinetochores, which bi-orientates the chromosomes, the centromeres become stretched due to k-fibre pulling forces, so distancing Aurora kinase from the outer kinetochore and thus allowing a stabilization of correct attachments (Liu et al., 2009). It is likely that the destabilizing of erroneous weak microtubule interactions and the stabilization of correct, strong, k-fibres is the same in MI as it is in mitosis. However, what is different in MI is the configuration of the kinetochores, because two sister kinetochores act as a single functional unit and so must attach to a single pole (Box 3).

The kinetochore is thus both the platform on which the SAC is launched and the tether through which successful poleward

chromosome movements are made. Because of its central role in division and the fact that it has a unique property in MI, such that sister kinetochores act as a functional unit, a better understanding of kinetochore anatomy and regulation will undoubtedly aid our understanding of meiosis control and aneuploidy.

Maternal age and aneuploidy

Until now, we have focused our attention on meiosis in oocytes without any concern for the influence of maternal aging. Given the increasing incidence of aneuploidy with maternal age (Fig. 1), it is important to study additional factors that may affect the fidelity of chromosome segregation.

It is increasingly accepted that the cohesive ties holding together chromosomes are weakened with maternal age, and this has recently been reviewed elsewhere (Jessberger, 2012; Nagaoka et al., 2012). In brief, cohesin is loaded onto newly replicated chromosomes in oogonia during foetal life. However, oocytes have only a limited capacity to reload this complex once S phase is complete (Revenkova et al., 2010; Tachibana-Konwalski et al., 2010), and consequently there is a loss of the cohesion over time (Chiang et al., 2010; Duncan et al., 2012b).

If all cohesion were lost from chromosomes, the anticipated outcome would be the presence of single chromatids in MI, as each bivalent is resolved into its four constituent single chromatids. However, there is no evidence to suggest that such single chromatids are a common feature of MI; therefore, it may be that there is an excess of cohesin loaded onto chromosomes during S phase, which is sufficient in most cases to maintain the bivalent integrity late into the reproductive lifespan. Indeed, very small amounts of Rec8, a cohesin component, can be observed on bivalents from aged oocytes that still retain their integrity (Chiang et al., 2010).

Less extreme loss of cohesion may allow the bivalents to separate into two univalents during MI (MI pre-division, Fig. 4B, part iii) as can occur even in young mice lacking one member of the cohesin complex (SMC1β) (Hodges et al., 2005). Separation of the bivalent into univalents would be predicted in situations where a single recombination event known as crossover, which involves tethering the homologous chromosomes of the bivalent together, is located close to the telomere, and/or in shorter chromosomes, where there is less arm cohesin distal to the crossover to maintain bivalent integrity. Indeed in the SMC1B knockout, MI pre-division is observed more frequently in shorter chromosomes (Hodges et al., 2005). However, this pre-division was rarely observed in either young or aged wild-type MI oocytes (Hodges et al., 2005; Lister et al., 2010). The fate of univalents generated from a bivalent that has lost its integrity may be to bi-orientate and so divide equationally in MI, as occurs when univalents are generated by loss of Sycp3, a component of the synaptonemal component (Kouznetsova et al., 2007). Alternatively, they may divide intact during MI, forming interactions with microtubules that satisfy the SAC, without bi-orientation, as some univalents from the XO mouse have been reported to do (LeMaire-Adkins et al., 1997).

In a normal physiological context, it seems likely that even with age the losses in cohesion are insufficient to perturb the integrity of the bivalent. However, they may nonetheless be effective at promoting incorrect microtubule-kinetochore attachment and chromosome segregation during the meiotic divisions. In support of this, aneuploidy is found in MII eggs at a far higher frequency than univalents are found in MI oocytes (i.e. aneuploidy is still being generated in large numbers of oocytes that maintain the integrity of their bivalents). This can be seen dramatically in mice that are heterozygous for mutations in meiosis-specific members of the

cohesin ring (Murdoch et al., 2013). Here, in MI oocytes the presence of univalents is very low in both wild-type and heterozygous oocytes, but the rate of PSSC observed in MII eggs increases up to sixfold.

Cohesin loss can be conveniently measured by an increase in the distance between the sister kinetochores (inter-kinetochore distance), which may be immunolabelled, in both mouse (Chiang et al., 2010; Lister et al., 2010) and human oocytes (Duncan et al., 2012b). Importantly, an increased distance correlates with those mouse oocytes that are aneuploid in maternal aging studies (Merriman et al., 2012); even in young mice where aneuploidy rates are much lower, those oocytes that are aneuploid tend to have higher distances (Merriman et al., 2013). It is feasible that small changes in the proximity of the two sister kinetochores that constitute a functional unit within each homologue of a bivalent have a dramatic consequence on the efficiency of that pair to establish an attachment to just one pole. At the molecular level, it is currently unclear how the sister kinetochore pair becomes a functional unit in mammalian oocytes during MI. However, if this functionality is lost, such that the sister kinetochores stop acting as a pair and instead begin to act independently, the likely outcome will be a promotion of incorrect attachment to microtubules that would result in incorrect segregation at anaphase (see Box 3).

Controlling meiotic fidelity in MII

Finally, we take the opportunity to summarize the controls operating in the second meiotic division MII. A focus on this division is given merit by the fact that recent very extensive CGH analysis of human oocytes suggest that proportionally more MII errors are observed with advanced maternal age than MI errors (Fragouli et al., 2011), although it remains important to bear in mind that CGH counting methods may under-represent MI errors because they can only measure the error when it becomes detectable rather than when it occurs.

Cdk1 activity is high in met II-arrested mouse eggs and, prior to fertilization, is maintained in this state by the Cdk1-activating phosphatase Cdc25 (Oh et al., 2013), as well as by low cyclin B1 degradation caused by the presence of the APC inhibitor Emi2 (endogenous meiotic inhibitor 2; early mitotic inhibitor 2, also known as Erp1; F-box protein 43) (Madgwick et al., 2006; Shoji et al., 2006). Fertilization causes Emi2/Erp1 degradation, resulting in an acceleration of cyclin B1 degradation (Nixon et al., 2002); lowered Cdk1 activity and meiotic exit also requires Wee1B (Wee2) activity, which is an inhibitory kinase of Cdk1 (Oh et al., 2011). During the time of met II arrest, inhibition of separase is bought about by binding its chaperone securin, and the rise in APC activity at fertilization therefore frees separase by causing the degradation of securin (Nabti et al., 2008).

The dyads produced at the end of MI now behave in their division as sister chromatids would in mitosis. The most prominent feature of this is that the sister kinetochore pair of a dyad no longer behaves as a functional unit, but instead the two kinetochores establish independent attachment to opposite spindle poles in order to segregate equationally in MII (i.e. exactly the same configuration as for sister chromatids in mitosis in Fig. 5).

At this point it should be obvious that some cohesin needs to remain on the dyad following completion of MI in order to prevent it from being pulled apart when k-fibre attachment occurs. In mouse oocytes it has been elegantly shown that it is cohesin, located near the centromere (centromeric cohesin), that performs this task (Tachibana-Konwalski et al., 2010), and it can do this because during MI it has been protected from separase-mediated

loss by the actions of Sgo2 (Lee et al., 2008; Llano et al., 2008). Sgo2 localizes to centromeres, and it is thought that its association with the protein phosphatase 2A (PP2A) prevents local phosphorylation of the Rec8 component of cohesin (Xu et al., 2009), an event that is thought to sensitize Rec8 to the actions of separase. One of the most recent exciting developments in mouse oocytes is the discovery of how Rec8 is deprotected in MII, through centromeric recruitment of a PP2A inhibitor (Chambon et al., 2013). What is intriguing is that the recruitment of the inhibitor of PP2A, known as IPP2A (also known as SET, PHAP-II or TAF-1b), to the centromere is not dependent on the presence of a dyad, because the same recruitment is induced even when bivalent integrity is maintained at MI completion, such that a MII spindle is assembled with bivalents. This means that the timing of IPP2A association with the centromere has to be well regulated to ensure that it does not happen until the first meiotic division is fully completed. Future studies are needed in order to understand how IPP2A transitions to the centromere, and in this regard it is interesting to note that normal sister chromatid segregation in MII is wholly dependent on Cdk1 and cyclin A2 activity, but not when exit is induced by a PP2A inhibitor (Touati et al., 2012). It may be that Cdk1 and cyclin A2 activity promotes IPP2A translocation to the centromere in order to permit separase-mediated cleavage of Rec8.

Conclusions and perspectives

What the SAC can sense and respond to in oocytes has become clearer over recent years. It has long been appreciated that oocytes can respond to the disruption of microtubule-kinetochore interactions, by spindle poisons, with a metaphase arrest that is SAC dependent (Wassmann et al., 2003; Homer et al., 2005a). However, it is only more recently that the question of what the SAC is capable of monitoring has been investigated. These studies all show that MI SAC satisfaction in oocytes, which through APC activation begins the countdown to chromosome segregation, is not dependent on correct stable interactions of bivalent kinetochores with microtubules. With this backdrop, it is much easier to appreciate the cause of maternal age-related aneuploidy where those origins are in MI, given that the aging process would help promote incorrect attachments through weakened cohesion.

It remains possible that there is something fundamental about bivalent architecture that prevents a more robust SAC surveillance mechanism. As such, the incidence of aneuploidy is affected by other factors such as how common incorrect attachments are and how well they are detected and corrected by other means. At a molecular level, the inability of the SAC to detect certain attachment errors could be a consequence of how the SAC components interact with the sister kinetochore pair of the bivalent. To move the field forward, we suggest that the following are key questions that need to be addressed in mammalian oocytes. What makes up the kinetochore in MI and MII? How does the sister kinetochore act as a functional unit in MI, and how does deprotection of centromeric cohesin work in MII? How do SAC proteins interact with kinetochores? Answers to these questions will come from more detailed imaging of the MI and MII events coupled with improvements in proteomic approaches that allow sequencing from small samples (which is key when working on small numbers of oocytes, and possibly immunopurifying their kinetochores). With respect to age, is there a specific loss of chromosome-associated proteins (e.g. as shown for Sgo2) or are there wholesale losses in all proteins? If the latter is true, we would expect the consequences of aging to be variable, depending on which proteins had been particularly affected in individual oocytes. Finally,

it is relevant to ask whether a detailed understanding of how segregation errors arise will impact on human fertility treatment. Given that we now understand some of the molecular components that regulate segregation of chromosomes in oocytes, we have the potential to modify their regulation and assess the consequence. The ability to increase the fidelity of chromosome segregation by drug additions *in vitro* is therefore not so far-fetched. But maybe the greatest societal benefit will come from ameliorating the effects of aging on oocytes; here, the goal is still one of cataloguing the effects of maternal aging in tractable systems outside of humans.

Competing interests statement
The authors declare no competing financial interests.

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