DNA damage responses in mammalian oocytes.

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Abstract

DNA damage acquired during meiosis can lead to infertility and miscarriage. Hence it should be important for an oocyte to be able to detect and respond to such events in order to make a healthy egg. Here the strategies taken by oocytes during their stages of growth to respond to DNA damaging events are reviewed. In particular, recent evidence of a novel pathway in fully grown oocyte that helps prevent the formation of mature eggs with DNA damage. It has been found that fully grown germinal vesicle stage oocytes that have been DNA damaged do not arrest at this point in meiosis, but instead undergo meiotic resumption and stall during the first meiotic division. The Spindle Assembly Checkpoint, which is a well-known mitotic pathway employed by somatic cells to monitor chromosome attachment to spindle microtubules, appears to be utilised by oocytes also to respond to DNA damage. As such, maturing oocytes arrest at metaphase I, due to an active Spindle Assembly Checkpoint. This is surprising given this checkpoint has been previously studied in oocytes and thought to be weak and ineffectual because of its poor ability to be activated in response to microtubule attachment errors. Therefore the involvement of the Spindle Assembly Checkpoint in DNA damage responses of mature oocytes during meiosis I, uncovers a novel second function for this ubiquitous cellular checkpoint.

Introduction

An effective response to DNA damage is crucial for all cells including oocytes (Sancar, et al. 2004). Extensive damage occurring throughout meiosis can have severe consequences if an appropriate response is not taken, and can result in infertility or defective embryo development (Adriaens, et al. 2009, Kirk and Lyon 1982, Meirow, et al. 2001). Mammalian oocytes remain arrested at the dictyate stage of meiosis for an extended period; up to several decades in some species including humans (Chiang, et al. 2012, Holt, et al. 2013, Jones, et al. 2013, Mehlmann 2005). Such a lengthy arrest provides an opportunity for the accumulation of DNA damage.

Insults to DNA can also result from exogenous factors throughout a woman's reproductive life, in particular during the treatment for cancer (Roness, et al. 2014). With a large number of effective anti-neoplastic treatments now available, survival rate among cancer patients has increased (Aziz and Rowland 2003, Dillman and McClure 2014). Therefore, an emerging problem is the long-term effects of such life-saving treatments, including the loss of fertility in both sexes. In women, cancer therapy often results in premature ovarian failure (POF) because the lifetime supply of oocytes in the ovary is killed off by aggressive cancer treatment (Maltaris, et al. 2007). The most effective and established method used to preserve fertility in some women is the cryopreservation of embryos and oocytes (ASRM 2013, Maltaris, et al. 2007, Roness, et al. 2014, Skaznik-Wikiel, et al. 2015). Unfortunately these methods cannot be applied to all. One limitation of this technique is that a partner, or willingness to use a donor, is required to provide sperm. Hormonal suppression of ovaries during cancer treatment is another option for women, however the use of such drugs have potential associated risks such as interference with the cancer treatment or survival of eggs with DNA damage (Roness, et al. 2014).

However, the major limitation of cryopreservation methods is that they can only be used in post-pubertal women. Therefore there are currently no established options for young pre-pubertal girls (Skaznik-Wikiel, et al. 2015). Experimental options include ovarian tissue cryopreservation, but this has a variety of risks associated with it (Maltaris, et al. 2007, Skaznik-Wikiel, et al. 2015).

In this review we will focus on the various strategies that oocytes elicit, in the adult, upon damage to their DNA. This includes the apoptosis of primordial follicles, evasion of the G2/M checkpoint, and a metaphase arrest induced by DNA damage. Programmed double strand breaks (DSBs) occur in fetal life during meiotic recombination and pose a potential threat to oocytes if left unrepaired. However, only responses to exogenous sources of DNA damage will be discussed here.

Primordial follicle apoptosis after DNA damage

At birth the reserve of oocytes has been established and is held within primordial follicles arrested at prophase of meiosis I (Pepling 2006, Pepling and Spradling 2001). These follicles are important as they will provide the oocytes for future post-pubertal ovulations throughout reproductive life, and therefore the effect of DNA damage on primordial follicles is of much significance for fertility. There are several types of DNA damage, including crosslinks and base alterations, which are reviewed elsewhere (Sancar, et al. 2004), but one of the more dangerous types of damage is DSBs. This is due to the fact that a variety of chromosomal aberrations can be induced, including chromosomal translocations and rearrangements, if DNA DSB repair is aberrant (Ferguson and Alt 2001, Iarovaia, et al. 2014, Richardson and Jasin 2000). A cell can respond in many ways to DNA damage, including but not limited to, inducing an arrest in the cell cycle, or initiation of apoptosis if the damage is severe (Roos and Kaina 2006, 2013, Sancar, et al. 2004). Indeed, it is well documented that primordial

follicle stage oocytes with damaged DNA readily undergo apoptosis (Kerr, et al. 2012a, Livera, et al. 2008, Roness, et al. 2014, Suh, et al. 2006)(Figure 1). Likewise somatic cells will undergo apoptosis if a G1/S arrest is sustained and contain extensive DNA damage that cannot be repaired (Nowsheen and Yang 2012, Roos and Kaina 2013).

p53, a transcription factor, is necessary for the maintenance of the G1/S checkpoint in a somatic cell with DNA damage (Basu and Haldar 1998). The initiation of this checkpoint requires activation of the master kinases ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad-3 related (ATR) (Smith, et al. 2010). In response to DSBs, these kinases are known phosphorylate histone 2AX (H2AX) at serine 139 (Bakkenist and Kastan 2003, Burma, et al. 2001). Such post-translational modification at the site of damage provides a platform for other DNA damage response (DDR) proteins to assemble on DNA in the event of damage. ATM/ATR kinases also phosphorylate, and activate, several other DDR signalling proteins (Shiloh and Ziv 2013). p53 phosphorylation by ATM and ATR kinases at serine 15 (Loughery, et al. 2014), and by CHK1/CHK2 kinases at serine 20 aid its activation (Chehab, et al. 1999). Modifications to MDM2, the p53 ubiquitin ligase binding partner, have also been reported to allow the two to dissociate and to stabilise p53 (Cheng, et al. 2009). Post-translational modifications of p53 retain it within the nucleus, allowing it to upregulate p21, a cyclin dependent kinase (CDK) inhibitor, as well as directly blocking the transcription of cell cycle regulators (Figure 2A).

Loss of p53 in mice leads to greater susceptibility to spontaneous and induced tumours, giving it the moniker ‘guardian of the genome’ (Donehower, et al. 1992). Interestingly, results obtained from *p53*$^{-/-}$ oocytes suggested that it is not an essential component for DNA damage induced apoptosis in the female germ line (Suh, et al. 2006), and instead the role is fulfilled by other members of the p53 family such as p63 and p73 (Levrero, et al. 2000). Indeed, in oocytes one important ‘guardian’ appears to be trans-activating p63 (TAp63) (Kerr,

et al. 2012b, Livera, et al. 2008, Suh, et al. 2006) (Figure 2B). Experiments using PCR and studies on knockout mice revealed that the prevalent form of TAp63 in oocytes is TAp63α (Livera, et al. 2008). The expression profile of TAp63 has also been mapped throughout oogenesis and oocyte maturation in mice. Embryonic expression is very limited, however by postnatal day 5 all oocytes express the transcription factor (Kim and Suh 2014, Suh, et al. 2006). The lack of TAp63 expression allows embryonic oocytes to evade apoptosis, whereas oocytes retrieved from the ovaries of 5 day old mice die within a few days of irradiation (Kim and Suh 2014). To highlight the importance of TAp63 in the DNA damage induced apoptosis, *TAp63*$^{-/-}$ mice were irradiated and ovaries were harvested several days later. In such mice, primordial follicles did not undergo apoptosis after exposure to gamma-irradiation, strongly implying TAp63 is essential for the induction of apoptosis (Suh, et al. 2006).

To activate TAp63 after DNA damage induction ATM kinase and CHK2 are required (Bolcun-Filas, et al. 2014, Livera, et al. 2008, Suh, et al. 2006). The requirement for phosphorylation in the activation of TAp63 has been shown using phosphatase treatment, as this prevented its mobility shift seen on immunoblots following ionising-radiation (Livera, et al. 2008, Suh, et al. 2006). This shift is only seen in mice from postnatal day 5 onwards and so is absent in new-born oocytes (Kim and Suh 2014), implying that prior to this the kinases responsible are under inhibitory regulation. The involvement of ATM kinase specifically in activating TAp63 was recently shown by Kim and Suh (2014) where treatment with pharmacological inhibitors, KU55933 or Wortmannin, blocked apoptosis. CHK2 has also been found to be involved in activation of TAp63 (Bolcun-Filas, et al. 2014). In *Chk2*$^{-/-}$ ovaries TAp63 remained un-phosphorylated after ionising radiation exposure, and its absence allowed oocytes to survive despite the presence of DNA damage (Bolcun-Filas, et al. 2014).

As well as the upstream components that lead to apoptosis, the downstream signalling of the p53 family is of considerable interest. In somatic cells p53 initiates apoptosis by increasing

the expression pro-apoptotic factors such as NOXA, PUMA and BAX (Basu and Haldar 1998, Roos and Kaina 2006, 2013). These proteins are members of the BCL2 family and act as pro-apoptotic factors by leading to activation of caspase-9, a crucial caspase during intrinsic apoptosis (Elmore 2007). As one may expect, TAp63 is the essential transcription factor for the expression of NOXA and PUMA in oocytes from 5 day old mice (Kerr, et al. 2012b). *Puma-/-, Noxa -/-* and *Puma-/-Noxa-/-* ovaries maintain many primordial follicles after ionising radiation treatment compared to wild-type controls, which rapidly deplete. This suggests that the expression of PUMA and NOXA is what drives primordial follicle apoptosis after DNA damage. Not only are these follicles protected from loss, but the knockout also preserved fertility, indicated by the production of multiple litters without gross abnormalities. The lack of abnormality suggests that these irradiated oocytes, which do not undergo apoptosis, have the ability to repair DNA damage over time (Kerr, et al. 2012b). As well as the preservation of fertility, NOXA and PUMA knockout mice have no increased susceptibility to cancer. For the future, if NOXA and PUMA could be targeted when women undergo cancer treatment it could potentially be used as a way to reduce the prevalence of POF in these women without an increased cancer risk caused by the treatment itself.

GV oocytes possess a weak G2/M checkpoint.

TAp63 expression is dramatically lost when a follicle is recruited for ovulation (Suh, et al. 2006). Therefore, it was unknown what effect DNA damage has on oocytes from larger antral follicles, once fully grown and meiotically competent (Suh, et al. 2006). While primordial follicles constitute the vast majority of the population of oocytes in the ovary, it is interesting to determine how fully grown oocytes behave in response to DNA damage as these are temporally closer to creating an embryo.

Whilst an oocyte is growing it remains arrested in prophase of meiosis I (Mehlmann 2005). As well as this, several factors within an oocyte need to reach a threshold level such as Cdk1, in order for the oocyte to become competent to complete meiosis (deVantery, et al. 1996). The biochemical mechanism of prophase arrest and meiotic resumption has been extensively reviewed elsewhere and so will not be discussed further here (Holt, et al. 2013, Jones, et al. 2013, Mehlmann 2005) (Figure 3A). However, it is noteworthy that there are several similarities in the transition from GV arrest to meiotic resumption and the G2/M transition of a somatic cell. (Solc, et al. 2010). Most notable here is that both processes are triggered by CDK1 (Adhikari and Liu 2014, Adhikari, et al. 2012). Due to this similarity it was assumed that the oocyte would have the ability to initiate a GV arrest when exposed to genotoxic agents because somatic cells arrest at G2 in response to DNA damage. However, the first studies that looked into the effect of DNA DSBs in fully grown GV oocytes in mice revealed that in contrast to mitotic cells, oocytes do not induce a robust G2/M checkpoint after exposure to the drug etoposide (Marangos and Carroll 2012, Marangos, et al. 2015) (Figure 1). Etoposide induces DSBs by inhibiting the release of topoisomerase II from DNA (Nitiss 2009). This creates a protein-DNA complex that has to be cleaved, which forms a DSB capped by remnants of the topoisomerase enzyme. It is only very high concentrations of either etoposide or doxorubicin that delay meiotic entry. Similar findings have since been observed by other groups, again using etoposide (Collins, et al. 2015), and other DNA damaging agents such as neocarzinostatin (NCS) (Mayer, et al. 2016, Yuen, et al. 2012), bleomycin, ionising radiation and UV-B exposure (Collins, et al. 2015). Ionising radiation induces a majority of its DSBs through the generation of reactive oxygen species (ROS) (Desouky, et al. 2015). Chemical agents such as Bleomycin and NCS also induce DSBs in DNA by acting as ionising radiation mimetics (Chen and Stubbe 2004). UV damage can induce several forms of DNA damage including pyrimidine dimers, oxidative damage to

bases, and also DSBs primarily through the formation of ROS but also as a secondary effect of dimer repair (Rastogi, et al. 2010, Sinha and Hader 2002). There is likely to be a lack of a G2 checkpoint in all mammalian species, not just mice, because porcine oocytes do not appear to initiate a checkpoint either (Wang, et al. 2015). Recently it has been suggested that the presence of cumulus cells, may allow for oocytes to remain GV arrested when their DNA is damaged (Sun, et al. 2015). This could potentially provide some protection against the formation a fully mature egg with DNA damage *in vivo.*

Nevertheless, the absence of an efficient DNA damage checkpoint in prophase arrested oocytes is thought to be due to a lack of ATM kinase activation (Marangos and Carroll 2012). This contrasts to a somatic cell in which the response to DNA damage at the G2/M checkpoint switches on in this kinase (Bakkenist and Kastan 2003) (Figure 3B). Only very high levels of DNA damage in oocytes were able to activate ATM (Marangos and Carroll 2012, Wang, et al. 2015). In mouse oocytes this culminates in a CHK1-dependent inhibitory phosphorylation of CDC25B, and so maintenance of GV arrest (Marangos and Carroll 2012). The lack of ATM activation in oocytes, compared to somatic cells, is thought to be due to low levels of ATM expression, and possibly the specific chromatin configuration in fully grown oocytes, leading to a failure of the DDR pathway to be fully implemented (Marangos and Carroll 2012).

An oocyte-specific DNA Damage Checkpoint

Once it was established that oocytes do not induce a robust checkpoint if exposed to genotoxins when GV arrested, it was of interest whether or not an alternative mechanism exists at some point later in meiosis to prevent the formation of a fertilisable egg. Having undergone GV breakdown the oocyte then needs to progress through meiosis I, and arrest at metaphase of meiosis II, where it remains until fertilisation (Jones and Lane 2013). However,

fully grown GV oocytes exposed to genotoxic agents such as NCS (Yuen, et al. 2012), etoposide (Collins, et al. 2015, Marangos, et al. 2015), UV-B and ionising radiation (Collins, et al. 2015) do not reach metaphase II, and instead arrest in meiosis I (Figure 1). Interestingly treatment with mitomycin C (Yuen, et al. 2012), to induce interstrand crosslinks, or treatment with very low doses (ng/ml) of NCS (Mayer, et al. 2016), does not appear to prevent polar body extrusion. The lack of response to interstrand crosslinks could allow this type of damage to be present in the mature oocyte. If left unrepaired such genetic insults could lead to severe perturbations during embryonic development if fertilised.

The block in meiosis I seen after most forms DNA damage occurs prior to the metaphase to anaphase transition (Collins, et al. 2015, Marangos, et al. 2015). This transition is one of the major events in oocyte maturation, with bivalents reductionally segregating into sister chromatids. The bivalent structure is maintained by cohesin. To allow the physical separation of bivalents requires the cleavage of cohesin, and is achieved by the protease separase (Terret, et al. 2003). Separase is kept inactive until anaphase-onset by CDK1-dependent phosphorylation and a chaperone binding protein securin (Terret, et al. 2003) . Therefore, in order to achieve anaphase, securin loss is essential, as well as a decrease in CDK1 activity which is caused by the loss cyclin B1 (Herbert, et al. 2003). Both cyclin B1 and securin loss is brought about by ubiquitylation from the Anaphase Promoting Complex/Cyclosome (APC) (Homer 2013). DNA damage in oocytes appears to prevent APC activation (Collins, et al. 2015).

A well characterised M-phase arrest brought about by APC inhibition is observed in somatic cells at a time when chromosomes are not fully attached to microtubules and under tension from the mitotic spindle. The surveillance system, that keeps the APC inactive is the Spindle Assembly Checkpoint (SAC); and prevents mis-segregation of chromosomes by coupling anaphase with correct chromosome alignment (Khodjakov and Pines 2010). Many of its

components were first discovered in yeast, but have since been identified in mammalian model systems and oocytes including MAD1, MAD2, BUBR1 and MPS1. However, the female meiotic SAC is thought to be less effective at preventing mis-segregation events (Gui and Homer 2012, Kitajima, et al. 2011, Kolano, et al. 2012, Lane, et al. 2012, Nagaoka, et al. 2011, Sebestova, et al. 2012); and such ineffectiveness has been associated with the higher rates of bivalent mis-segregation in oocytes leading to aneuploidy (Jones and Lane 2013, Nagaoka, et al. 2011).

Despite the previous labelling of the oocyte SAC as being weak or ineffectual in responding to microtubule attachment errors it does appear to be by contrast remarkably effective at preventing anaphase after treatment with genotoxic agents (Figure 4). Several SAC components including MAD2, BUBR1 and MPS1 have all been shown to be heavily involved in this arrest (Collins, et al. 2015, Marangos, et al. 2015). Activation of the SAC after DNA damage does not appear to occur at the sites of DNA damage, instead evidence suggests that DNA damage is sensed at the kinetochore, where these proteins usually accumulate during canonical SAC signalling (Collins, et al. 2015, Marangos, et al. 2015). DNA damage caused by DSBs would have the potential to fragment DNA. As such bivalent fragments could contain only a single pair of sister kinetochores that may have the capacity only to mono-orientate, and so activate the SAC due to lack of tension development. However, such bivalent fragments do not appear to be the cause of arrest as they are not present consistently or in sufficient number in DNA damage arrested oocyte (Collins, et al. 2015). Also oocytes with biorientation errors induced by the spindle poison, nocodazole, still undergo anaphase without delay (Collins, et al. 2015) (Figure 4).

The presence of such a checkpoint raises questions about the signalling cascade that takes place in oocytes upstream of SAC activation. Of particular interest is whether a link between the DNA damage checkpoint and the SAC exists in oocytes. There are already links

uncovered between the two cellular checkpoints in somatic cells. For instance, BUBR1 and BUB1 have been shown to be required for the DNA damage response in *Drosophila* embryos and HeLa cells respectively (Royou, et al. 2005, Yang, et al. 2012). Also, the DDR protein CHK1 appears to be involved in SAC function in avian (Zachos, et al. 2007) and mammalian cell lines (Peddibhotla, et al. 2009). Several SAC components, including MPS1 and MAD2, have been shown to be crucial for the DNA damage induced metaphase arrest in oocytes (Collins, et al. 2015, Marangos, et al. 2015). Also, the MOS/MAP kinase (MAPK) pathway, known to have a role in activating the meiotic SAC (Nabti, et al. 2014), appears to be integral for activating the DNA damage checkpoint in oocytes (Marangos, et al. 2015).

Although ATM is involved in DNA damage induced apoptosis in primordial follicles (Kim and Suh 2014) it appears not to contribute to SAC activation after DNA damage induction in fully grown GV oocytes, as pharmacological inhibition of the kinase does not rescue polar body extrusion in damaged oocytes (Marangos, et al. 2015). This may not be too surprising given the reported lack of ATM activation in GV-stage oocytes following DNA damage (Marangos and Carroll 2012). This contrasts with somatic cells where ATM has been implicated in SAC activation after nocodazole treatment (Eliezer, et al. 2014). Another DDR protein, MDC1, has also been suggested to be able to directly interact with the APC, an interaction that is heightened after DNA damage (Coster, et al. 2007).

An alternative candidate for activating the oocyte DNA damage checkpoint would be ATR. This kinase is known to be involved in H2AX phosphorylation after UV exposure in somatic cells (Hanasoge and Ljungman 2007) and is activated by single stranded DNA generated during the repair of DNA damage (Zou and Elledge 2003). It also functions in establishing a G2/M checkpoint in mammalian cell lines independent of ATM kinase activity (Xue, et al. 2015). Another DDR protein that could be involved in DNA damage induced SAC activation

is CHK1. Manipulating the levels of this protein has highlighted its involvement in maintaining prophase arrest and in potentially activating the SAC (Chen, et al. 2012).

Regardless of the mechanism activating the checkpoint it is clear that for most DNA damaging agents tested, a robust arrest in meiosis I is initiated and maintained (Collins, et al. 2015). Future work is likely to focus on the upstream signalling prior to SAC activation and whether any other traditional DDR proteins are involved in the oocyte checkpoint.

Conclusions

The oocyte studies presented here have begun to uncover the strategies employed to prevent the formation of a mature egg with DNA damage. Although it is clear that TAp63 induced apoptosis is responsible for the loss of damaged oocytes from primordial follicles, this pathway is lost once a follicle is recruited for ovulation. Furthermore fully grown oocytes from mature follicles, ready for ovulation do not undergo apoptosis and have a very poor 'G2/M' checkpoint when DSBs are induced. Instead oocytes with DNA damage go on to arrest in meiosis I through the actions of the SAC, which can now be viewed as a major checkpoint in the preventing the creation of embryos with DNA damage. Future studies need to address the full set of players in this pathway, particular the involvement of traditional DNA damage response proteins described in somatic cells.

Declaration of Interests

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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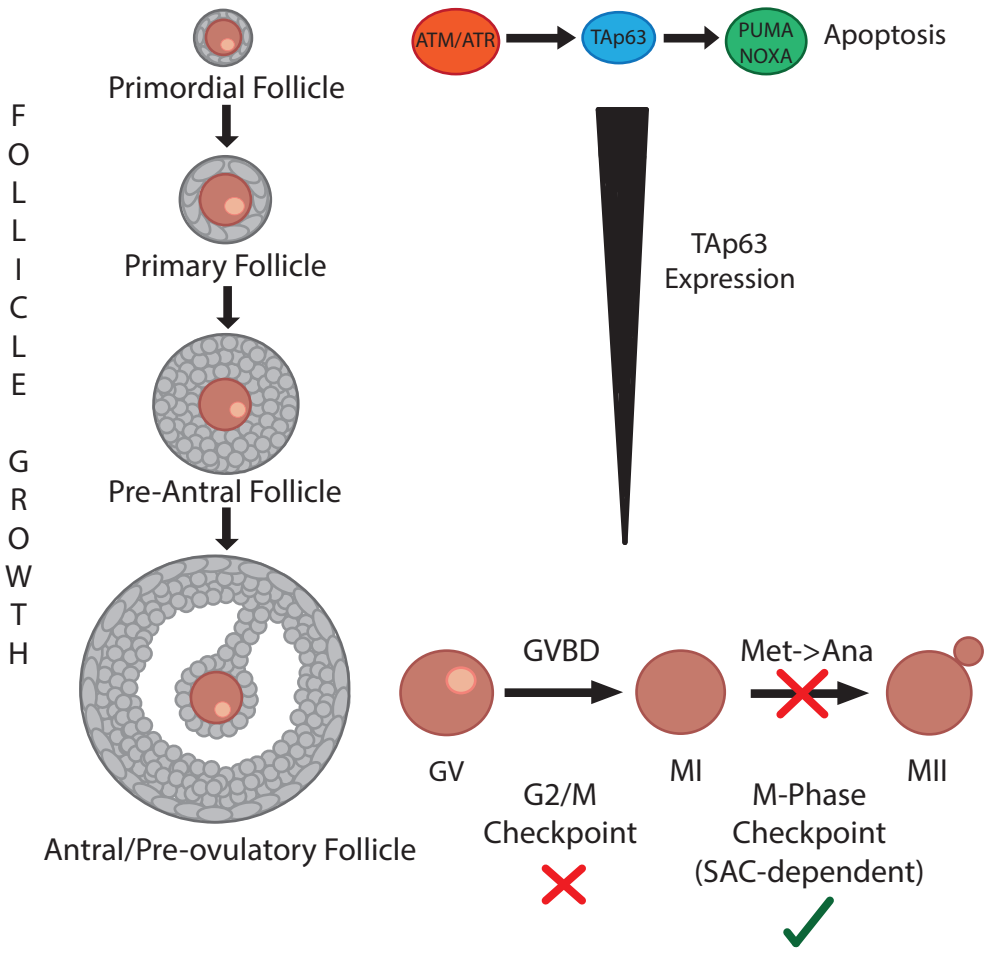
FOLLICLE GROWTH
Primordial Follicle
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ATM/ATR
TAp63
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NOXA
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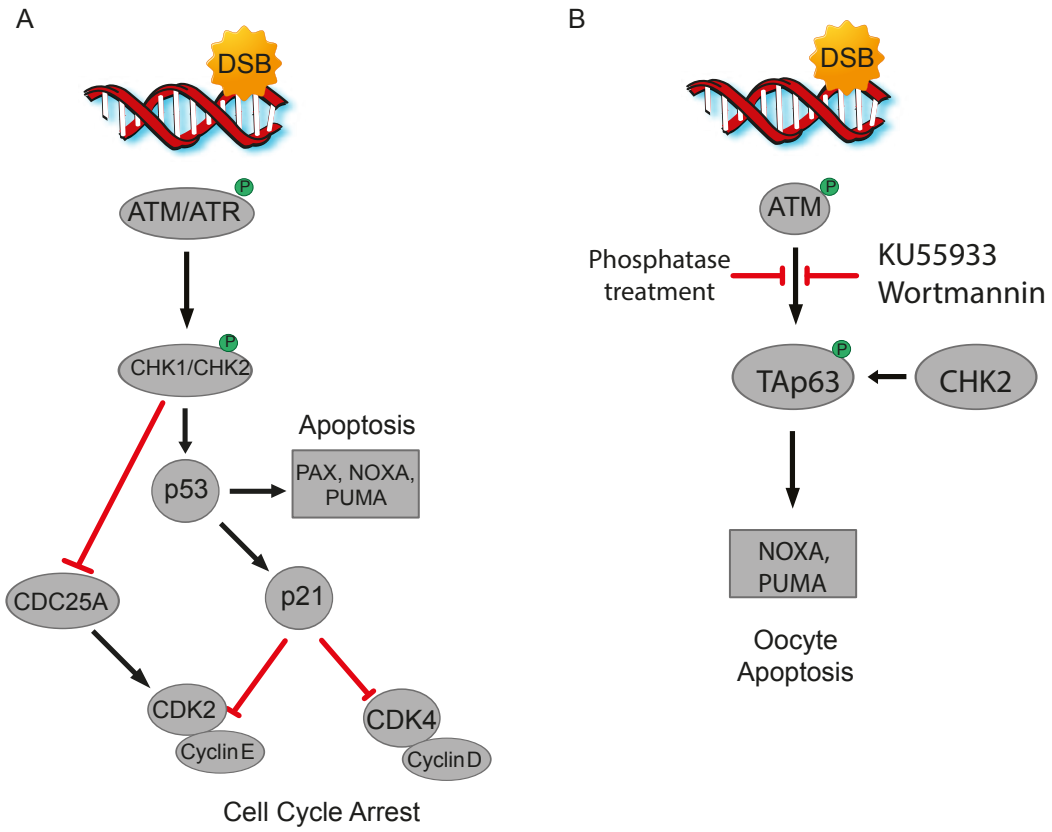
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CDK4
Cyclin D
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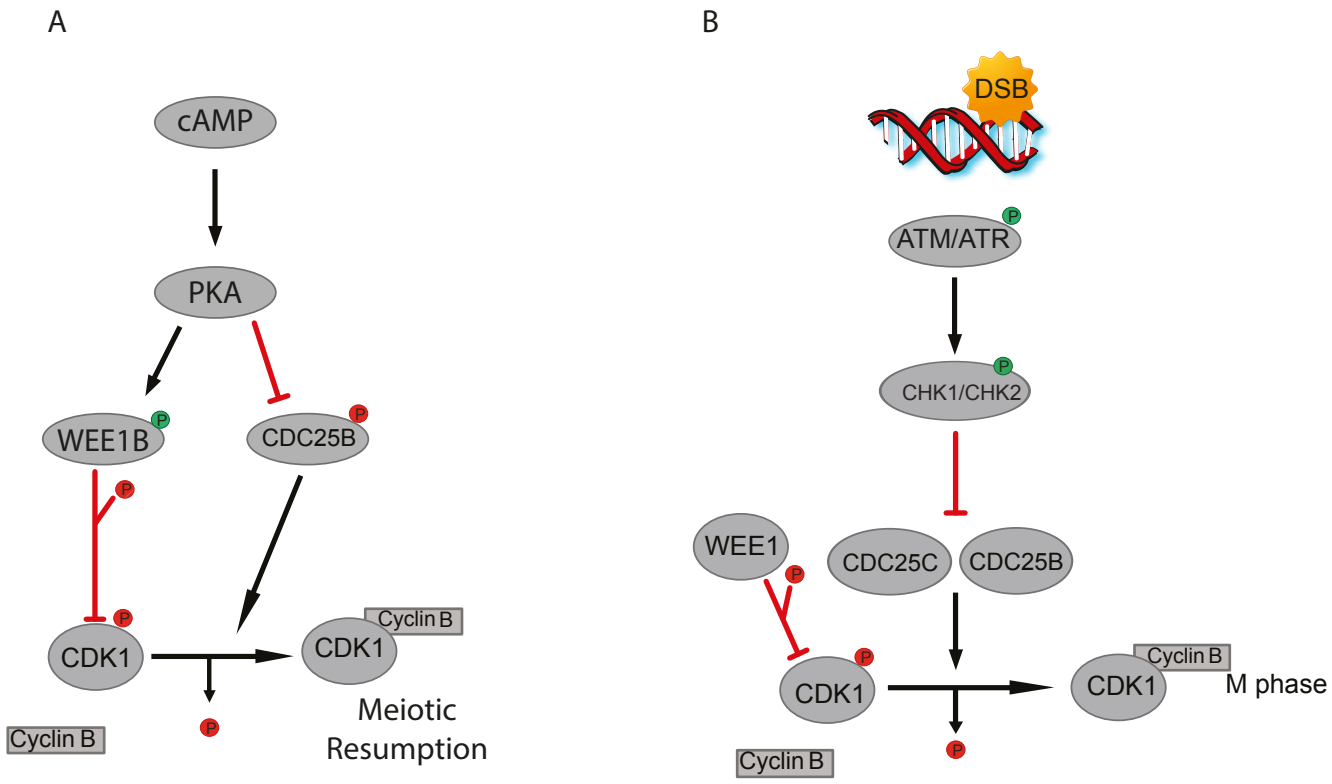
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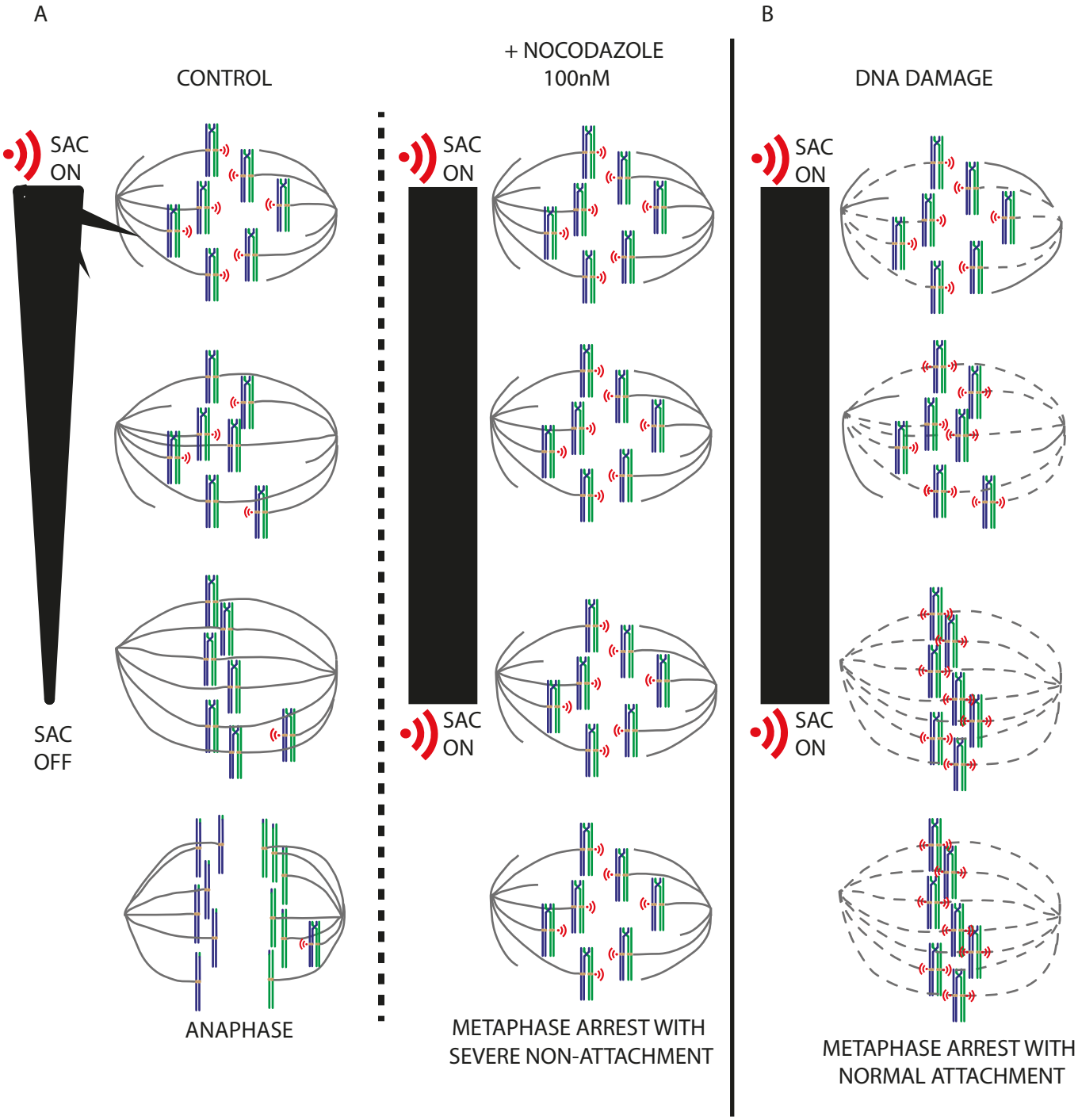
A
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CONTROL
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DNA DAMAGE
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ON
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OFF
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ANAPHASE
METAPHASE ARREST WITH
SEVERE NON-ATTACHMENT
METAPHASE ARREST WITH
NORMAL ATTACHMENT