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Reversal of female infertility by Chk2 ablation reveals the oocyte DNA damage checkpoint pathway

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Abstract

Genetic errors in meiosis can lead to birth defects and spontaneous abortions. Checkpoint mechanisms of hitherto unknown nature eliminate oocytes with unrepaired DNA damage, causing recombination-defective mutant mice to be sterile. Here, we report that checkpoint kinase 2 (*Chk2; Chek2*), is essential for culling oocytes bearing unrepaired meiotic or induced DNA double-strand breaks (DSBs). Female infertility caused by a meiotic recombination mutation or irradiation was reversed by mutation of *Chk2*. Both meiotically-programmed and induced DSBs trigger CHK2-dependent activation of TRP53 (p53) and TRP63 (p63), effecting oocyte elimination. These data establish CHK2 as essential for DNA damage surveillance in female meiosis, and indicate that the oocyte DSB damage response primarily involves a pathway hierarchy in which ATR signals to CHK2, which then activates p53 and p63.

Fertility, health of offspring, and species success depends on production of gametes with intact genomes. Particularly crucial is the proper synapsis and segregation of homologous chromosomes at the first meiotic division, processes requiring homologous recombination (HR), a high-fidelity DSB repair process. Meiocytes initiate HR by producing proteins (namely SPO11) that create DSBs. In mice, ~10% of the >200 induced DSBs are repaired as crossovers (COs), and the rest by non-crossover (NCO) recombination (1).

Aberrant homolog synapsis or DSB repair trigger checkpoints that eliminate defective meiocytes (2–4). Either defect causes apoptotic elimination of mouse spermatocytes at mid-pachynema of meiotic prophase I (5, 6). In contrast, oocytes defective for both DSB repair and synapsis occurs earlier (within a few days postpartum) than those defective for synapsis alone (~2 months postpartum), suggesting that mammalian oocytes have distinct DNA damage and synapsis checkpoints (2, 7) (Fig. S1). Mutations preventing DSB formation (*Spo11*, *Mei1*) are epistatic to those affecting DSB repair (2). The DNA damage checkpoint acts around the time oocytes enter meiotic arrest (dictyate, or resting stage) and presumably persists, since resting primordial follicles are highly sensitive to ionizing radiation (IR) (8).

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Supplementary Materials:
Materials and Methods
Figures S1–S8
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We focused on CHK2 kinase as a candidate component of the meiotic DNA damage checkpoint. It is a downstream effector of the ATM kinase that responds primarily to DSBs, and can also be activated by the ATR kinase that responds primarily to ssDNA (9, 10). Unlike *Atm* and *Atr, Chk2* is dispensable for fertility and viability. To determine if *Chk2* is required for the meiotic DNA damage checkpoint, we bred mice doubly deficient for *Chk2* and *Dmc1*, a RecA homolog required for interhomolog (IH) repair of meiotic DSBs. (11). *Dmc1* deficiency also prevents synapsis, which is HR-dependent. Whereas 3 weeks postnatal WT or $Chk2^{-/-}$ ovaries contain primordial through antral follicles (Figs.1A, B; S2), $Dmc1^{-/-}$ ovaries are devoid of follicles (Fig. 1D). Deletion of *Chk2* enabled survival of developing oocytes in DMC1-deficient 3-weeks old ovaries (Figs.1E, F). Primordial follicles were absent, however, leading to a nearly complete oocyte depletion by 2 months postpartum (Figs. S2, S3). This pattern of oocyte loss resembles that of *Spo11* or $Spo11^{-/-}$ $Dmc1^{-/-}$ mice (Fig. S1; (*2*)), suggesting that *Chk2* ablation compromises the DSB repair but not synapsis checkpoint.

To test this, we exploited an allele of *Trip13* ($Trip13^{Gt}$) that causes male and female meiotic failure. $Trip13^{Gt/Gt}$ chromosomes undergo synapsis and CO formation, but fail to complete NCO DSB repair (12), causing elimination of the entire primordial follicle pool and nearly all developing oocytes by three weeks postpartum (Fig. 1G), coinciding with the oocyte DNA damage checkpoint (Fig. S1) (12, 13). $Chk2^{-/-}$ $Trip13^{Gt/Gt}$ ovaries had a large oocyte pool at 3 weeks postpartum (Figs. 1H, I; S2), and they retained high numbers of all follicle types after two months (Fig. S3), indicating that the rescue of surviving oocytes from checkpoint elimination was permanent or nearly so (see below). The rescue was not attributable to activation of an alternative DSB repair pathway during pachynema, a consideration since the *Chk2* yeast ortholog *MEK1* influences pathway choice (14); all dictyate $Chk2^{-/-}$ $Trip13^{Gt/Gt}$ oocytes (n=54), like $Trip13^{Gt/Gt}$ oocytes, exhibited abundant γH2AX staining, indicative of persistent unrepaired DSBs (*vs.* 7% of $Chk2^{-/-}$ dictyate oocytes; n=45) (Fig. 2A, B).

Despite bearing DSBs into late meiotic Prophase I, the rescued oocytes proved to be functional. All tested $Chk2^{-/-}$ $Trip13^{Gt/Gt}$ females produced multiple litters (Fig. 2C). Litter sizes were smaller than controls (Fig. 2D), attributable to fewer ovulated oocytes and implanted embryos (Fig. S4). $Chk2^{-/-}$ $Trip13^{Gt/Gt}$ females sustained fertility for many months, yielding 4–7 litters each (Fig. 2C) and over 160 pups collectively. Progeny showed no visible abnormalities up to 1 year of age (n=28). The results suggested that all or most DSBs persisting into late meiosis were eventually repaired. Indeed, there was no evidence of persistent DNA damage (as indicated by γH2AX) in 2 month old primordial, growing, or germinal vesicle (GV) stage preovulatory $Chk2^{-/-}$ $Trip13^{Gt/Gt}$ oocytes (Fig. S5). Thus, repair of DSBs occurred after birth by unknown mechanisms.

Canonically, CHK2 signals to p53 in mitotic cells. In *Drosophila melanogaster*, CHK2-dependent p53 activation occurs in response to SPO11-induced breaks (3). We therefore tested whether p53 deficiency could rescue $Trip13^{Gt/Gt}$ oocytes. Three weeks old $p53^{-/-}$ $Trip13^{Gt/Gt}$ ovaries had significantly more oocytes than $Trip13^{Gt/Gt}$ single mutants (Figs.3B, C; S2), however, they contained far fewer primordial follicles than $Chk2^{-/-}$ $Trip13^{Gt/Gt}$ ovaries at 3 weeks postpartum, and almost no oocytes remained after 2 months (Fig. S3).

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Therefore, CHK2-mediated elimination of *Trip13*$^{Gt/Gt}$ oocytes does not occur exclusively *via* signaling to p53, indicating the existence of another downstream effector(s) that acts perinatally in primordial follicles.

One candidate is *p63*, a *p53* paralog. A predominant isoform called TAp63 appears perinatally in late pachytene and diplotene oocytes, approximately coinciding with DNA damage checkpoint activation. Since TAp63 was implicated in the elimination of dictyate oocytes subjected postnatally to DSB-causing IR (15, 16), and it contains a CHK2 consensus substrate motif LxRxxS (17), we speculated that CHK2 might activate TAp63 in response to DSBs. Indeed, whereas IR induces phosphorylation in WT ovaries (15, 16), TAp63 remained unphosphorylated in CHK2-deficient ovaries (Fig. 3D). Moreover, mutating serine to alanine in the CHK2 phosphorylation motif in p63 also prevented IR-induced TAp63 phosphorylation in cultured cells (Fig. 3E). We next tested if CHK2 is required for the elimination of DSB-bearing dictyate oocytes, presumably *via* TAp63 activation. Whereas the entire primordial follicle pool was eradicated one week after IR-treatment of WT ovaries, CHK2 deficiency prevented oocyte elimination despite the presence of p63 protein (Fig. 3F). Furthermore, irradiated *Chk2*$^{-/-}$ females remained fertile with an average litter size (6.3± 1.8, n=7) similar to unirradiated controls (6±2.3, n=3). If this rescue of fertility was due entirely to abolition of TAp63 activation, then deletion of TAp63 should also restore fertility to irradiated females. Previous studies (15, 16) found that *p63*$^{-/-}$ and *TAp63*$^{-/-}$ oocytes survived 5 days after 0.45–5Gy of IR, but longer term survival was not evaluated. We found that 0.45Gy IR completely eradicated primordial oocytes after 7 days in females homozygous for a viable, TA domain-specific deletion allele of *p63* (*TAp63*$^{-}$)(18, 19), identical to WT (Fig. 4A, B).

These results suggested IR-induced DSBs (and perhaps meiotic DSBs) stimulate CHK2 signaling to a protein(s) in addition to TAp63. Suspecting p53, we found that whereas irradiated *p53*$^{-/-}$ ovaries were essentially devoid of oocytes (Fig. 4C) (15, 16), *p53*$^{-/-}$ *TAp63*$^{-/-}$ oocytes (including those in primordial follicles) were rescued (Fig. 4D) to a degree similar to *Chk2* mutants (Fig. 3F). Irradiated *p53*$^{+/-}$ *TAp63*$^{-/-}$ (Fig. 4E) but not *p53*$^{-/-}$ *TAp63*$^{+/-}$ oocytes were partially rescued, indicating that CHK2 signals to both p53 and p63, and that they act in a partially redundant fashion to eliminate DSB-bearing resting oocytes. The marked effects of p53 haploinsufficiency, and the possible inconsistencies with earlier reports showing that deletion of p63 alone could rescue primordial follicles from IR over the short term, indicate that checkpoint responses may be sensitive to quantitative variation.

Since *Chk2* but not *p53* deficiency reversed *Trip13*$^{Gt/Gt}$ female infertility, an outcome similar to the results with postnatal ovary irradiation, we hypothesized that the same DNA damage checkpoint was operative in both pachytene/diplotene and dictyate oocytes. To test this, we first examined patterns of p53 and TAp63 activation in different genotypes of ovaries, with or without IR exposure. As expected for WT, TAp63 phosphorylation and p53 stabilization/expression occurred only after exposure to IR (Fig. 4F). Importantly, we observed p53 protein in unirradiated *Trip13*$^{Gt/Gt}$ neonatal ovaries but not WT (Fig. 4F), implying a role for p53 in the elimination of mutant oocytes with unrepaired meiotic DSBs (and consistent with partial rescue of *Trip13*$^{Gt/Gt}$ *p53*$^{-/-}$ oocytes; Fig. 3C). Stabilization of

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p53 in response to unrepaired meiotic DSBs is CHK2-dependent, since we did not detect p53 in *Chk2*$^{-/-}$ *Trip13*$^{Gt/Gt}$ ovaries (Fig. 4F). TAp63 was absent from neonatal *Trip13*$^{Gt/Gt}$ ovaries bearing residual oocytes (Fig. 4F). Normally, *TAp63* mRNA appears in late meiotic prophase I when meiotic DSBs have been repaired, and is robustly activated in resting oocytes in response to exogenous DNA damage (15, 16). Nevertheless, the absence of TAp63 in *Trip13*$^{Gt/Gt}$ oocytes predicts that it is not responsible for their death. Indeed, no oocyte rescue was observed in wean age *TAp63*$^{-/-}$ *Trip13*$^{Gt/Gt}$ ovaries (Fig. 4I). A potential explanation for TAp63 repression in *Trip13*$^{Gt/Gt}$ oocytes was suggested by our observation (Fig. 4F) that unphosphorylated TAp63 was present in *Chk2*$^{-/-}$ *Trip13*$^{Gt/Gt}$ ovaries lacking detectable p53. These results suggest a regulatory relationship between p53 and TAp63 in the meiotic DNA damage response.

The mutual exclusivity of TAp63 and p53 in *Trip13*$^{Gt/Gt}$ oocytes gives insight into the failure of either single mutant to rescue fertility. We hypothesized that unrepaired DSBs that persist into late pachynema trigger CHK2-dependent p53 activation and oocyte elimination independent of TAp63, but that in the absence of p53, *TAp63* can be expressed and activated by CHK2 to drive oocyte elimination. This predicts that removal of both proteins would abolish the CHK2-dependent checkpoint. Indeed, we found that *p53* heterozygosity could rescue *TAp63*$^{-/-}$ *Trip13*$^{Gt/Gt}$ oocytes (Fig. 4J). Importantly, this rescue included primordial follicles (Fig. 4J, inset; note: nullizygosity for all three genes is embryonically semilethal). These and previous results with single mutants indicate that the DNA damage checkpoint pathway that monitors repair of SPO11-induced DSBs involves CHK2 signaling to both p53 and TAp63, and that this pathway also operates in postnatal resting oocytes (Fig. S6).

A remaining question concerns the upstream activator(s) of CHK2. Canonically, ATM phosphorylates CHK2 in response to DSBs, whereas ATR responds to single stranded DNA by activating CHK1 (20, 21). However, ATR and ATM have other activities in mouse meiosis. ATM negatively regulates SPO11, causing *Atm*$^{-/-}$ oocytes to sustain extensive DSBs and triggering elimination by the meiotic DNA damage checkpoint (Fig. S1) (2), (22). Therefore, CHK2 is likely activated by a different kinase. Indeed, *Chk2* deficiency rescued *Atm*$^{-/-}$ oocyte depletion (Fig. S7) to a degree similar to the rescue of DMC1-deficient ovaries. The facts that: a) CHK2 can trigger apoptosis in the absence of ATM in somatic cells (9), b) CHK2 can be activated in an ATR-dependent manner (10) and c) ATR localizes to sites of meiotic DSBs in mice (23), prompt us to propose that the DNA damage checkpoint pathway in mouse oocytes involves signaling of ATR to CHK2, which in turn signals to p53 and TAp63 (Fig. S6). Intriguingly, spermatocytes may have a distinct DNA damage response pathway; we did not observe histological evidence for rescue of DSB repair-defective/synapsis-proficient spermatocytes by deletion of *Chk2* or *p53* (Fig. S8).

Our results are of biomedical interest with respect to the primordial follicle pool depletion and premature ovarian failure that can occur following cancer radiotherapy or chemotherapy. CHK2 is an attractive target since chemical inhibitors are available, and *Chk2* insufficiency is of minor phenotypic consequence in mice (24).

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Supplementary Material

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Acknowledgments

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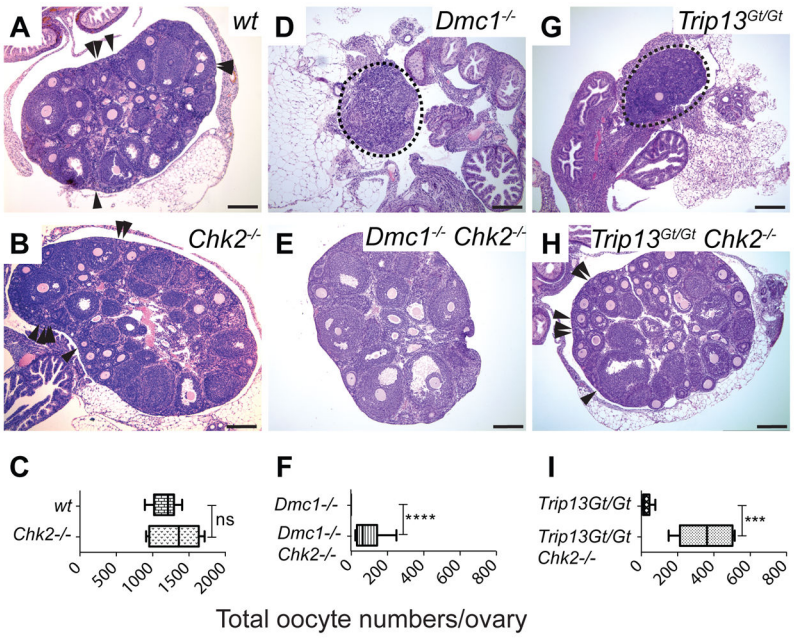

Fig. 1. Evidence of a specific DNA damage checkpoint in mouse oocytes

(**A, B, D, E, G, H)** Histology of 3 weeks postpartum ovaries. Follicle-devoid ovaries are denoted by dotted outline. Arrowheads (A, B, H) indicate primordial follicles. **(C, F, I)** Oocyte quantification in mutants. Bar = 200μm.

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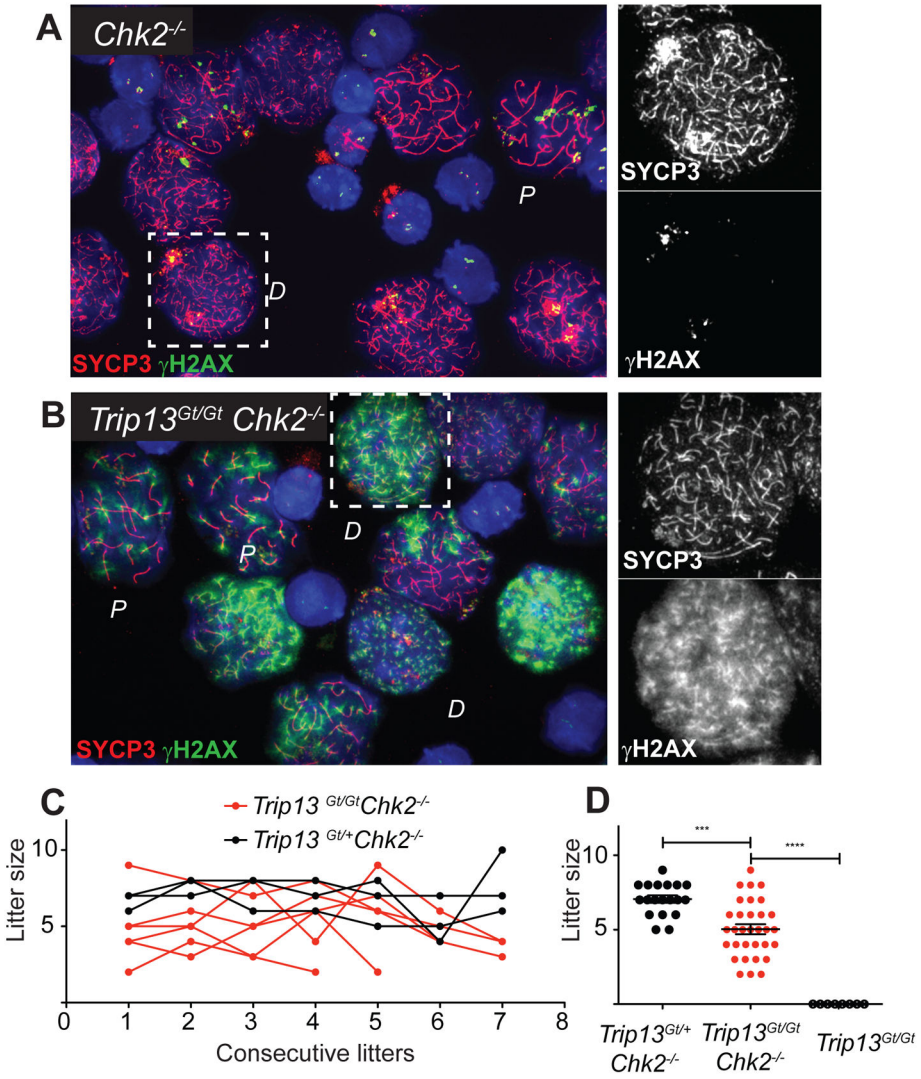

Fig. 2. DSBs in *Trip13*$^{Gt/Gt}$ *Chk2*$^{-/-}$ newborn oocytes are eventually repaired and yield offspring

(A) Co-immunolabeling of neonatal oocytes. **(B)** *Trip13*$^{Gt/Gt}$ *Chk2*$^{-/-}$ oocytes progress to dictyate ("D") with DSBs. P = pachytene. Boxed nuclei magnified (inset). **(C)** Female reproductive longevity and **(E)** fecundity.

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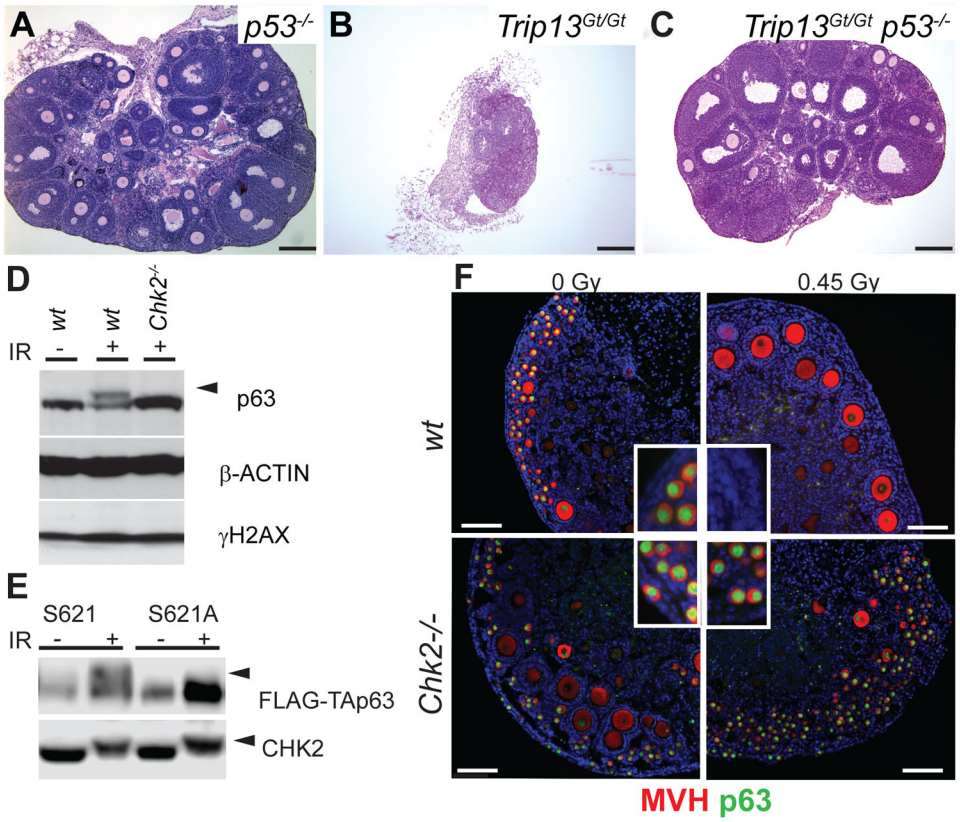

Fig. 3. Genetic and molecular analysis of the oocyte DNA damage checkpoint

(A–C) *Trip13*$^{Gt/Gt}$ oocyte depletion is partially rescued by p53 deficiency. Bar = 200 μm. **(D)** DNA damage-induced TAp63 phosphorylation in newborn ovaries is CHK2-dependent. Neonatal ovaries (4) received 3Gy IR before protein extraction 2 hrs later. Note: increased p63 in *Chk2*$^{-/-}$ is likely due to increased oocytes in this genotype. **(E)** p63 contains a CHK2 phosphorylation site. HeLa cells bearing FLAG-tagged TAp63 with WT (LxRxxS) or mutant (LxRxxA) CHK2 motifs. Shifted CHK2 (arrowhead) is phosphorylated. IR dose=3Gy. **(F)** Depletion of p63-positive primordial follicles by IR is CHK2-dependent. Ovaries were cultured 7 days after irradiation. Bar=100 μm. MVH marks oocytes. Inset: ovary cortical region containing primordial follicles.

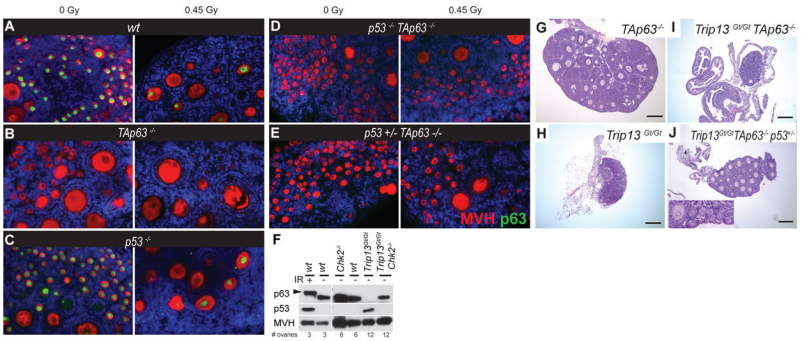

Fig. 4. CHK2 signals to both p63 and p53 in oocytes

(A–E) Depletion of primordial follicles by IR requires p53 and TAp63. Week old ovaries were irradiated, cultured 7 days, then immunostained. p63 and MVH are oocyte-specific. **(F)** Dynamic signaling to p53 and p63 in response to meiotic and induced DSBs. Shown are Western blots of neonatal ovarian protein. The irradiated sample was collected 2 hrs post-IR (3Gy). Arrowhead: phosphorylated p63 (15, 16). *Trip13* mutants are undergoing oocyte elimination (reflected by MVH), hence use of more ovaries. **(G–J)** p53 and *TAp63* are required for complete elimination of DSB repair-defective oocytes. Ovaries are 3 weeks postpartum. Inset (J) shows primordial follicles. Bar=200 μm.

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