

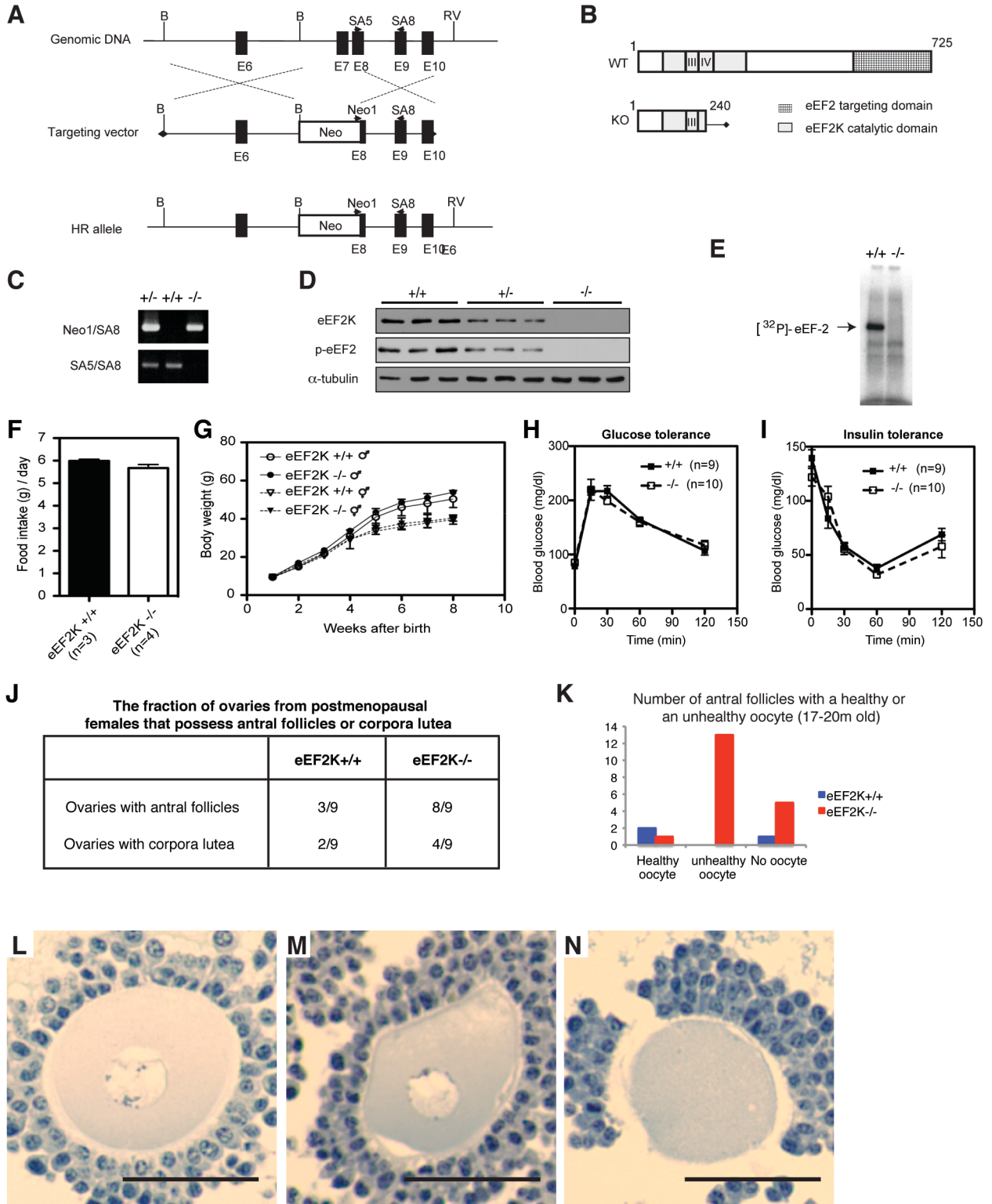
**E**

**The relative intensity of p-eEF2 immunostaining in mouse ovarian cells**

Follicular stages	Oocytes	Granulosa cells	Theca interna cells
Primordial and primary follicles	+	+	n/a
Small preantral follicles	++	+++	n/a
Large preantral follicles	++	+++	+
Small antral follicles	++	+++	+
Large antral follicles	+	+++	+
Corpora lutea	n/a	+	n/a

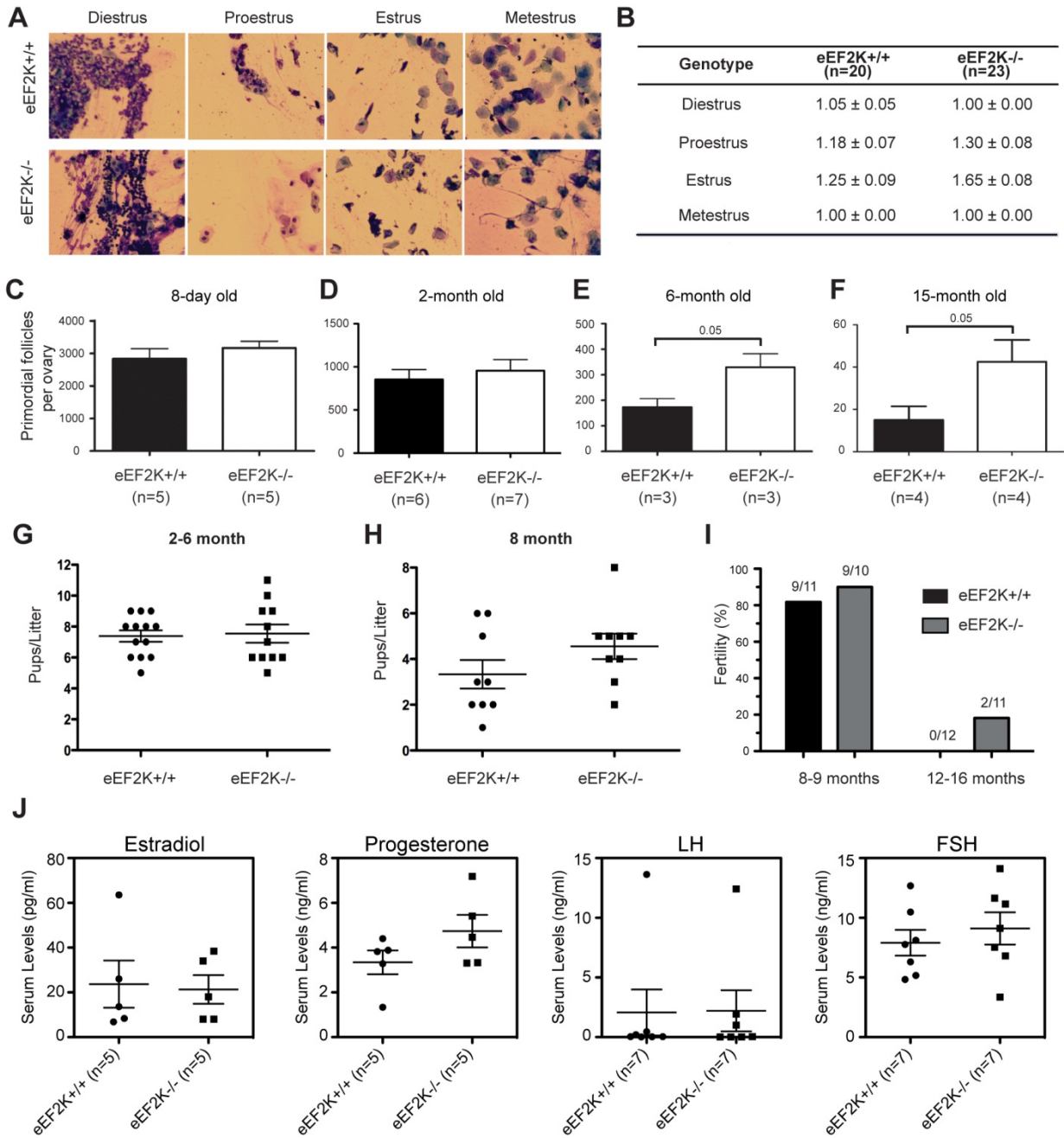
**Figure S1. Phosphorylation of p-eEF2 in ovaries, related to Figure 1. (A-D)** Immunostaining of p-eEF2 in 6-month old wild-type ovaries. **(A)** p-eEF2 was localized in different staged follicles (F) and corpora lutea (CL). The black arrowhead indicates a primordial follicle that transitioned to a primary follicle. The black arrows indicate oocytes. Scale bars represent 50  $\mu\text{m}$ . **(B)** p-eEF2 staining was intense in granulosa cells near oocytes. Interfollicular stromal cells (IFC) were negative for p-eEF2 staining. Scale bars represent 20  $\mu\text{m}$ . **(C,D)** Staining of p-eEF2 in atretic follicles. The black star indicates the degenerated oocyte in a late stage atretic follicle. The green arrows indicate granulosa cells with pyknotic nuclei that have intense p-eEF2 staining. Scale bars represent 25  $\mu\text{m}$ . **(E)** The intensity of p-eEF2 immunostaining in mouse ovarian cells at different stages of follicle development. (+) relative-weak staining. (++) relative-strong staining. (+++) intense staining. (N/A) staining not analyzed.

# Chu Figure S2



**Figure S2. Knockout of eEF2K leads to complete loss of eEF2 phosphorylation in mice, and the accumulation of unhealthy oocytes in eEF2K<sup>-/-</sup> aged mice, related to Figures 2 and 3.**

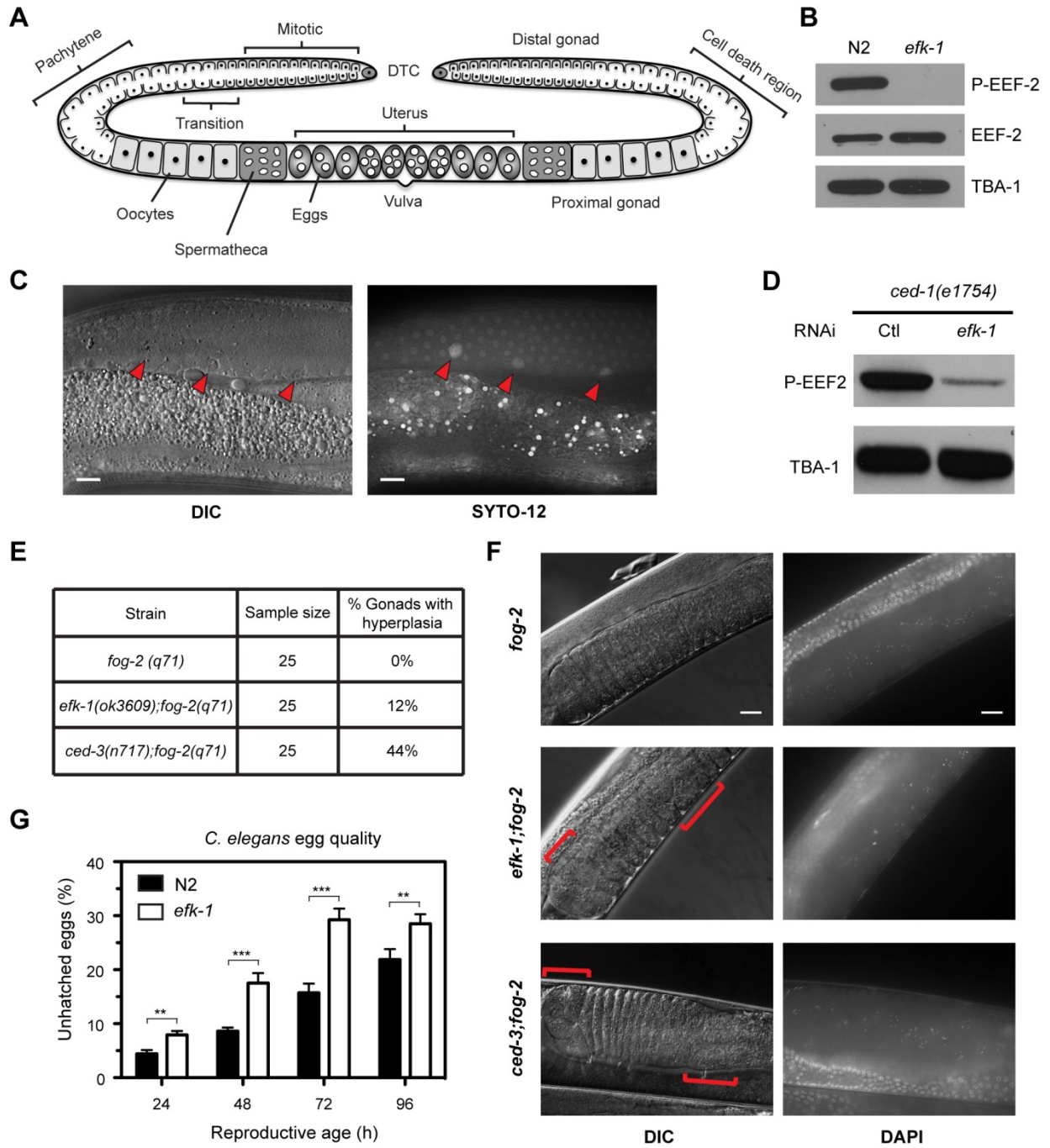
**(A,B)** Schematic representation of eEF2K knockout constructs. eEF2K<sup>-/-</sup> mice were generated using a targeting vector to delete exons 7 and 8 of the murine eEF2K gene through the addition of a neomycin selection cassette. **(C)** eEF2K<sup>-/-</sup> mice were identified by PCR. **(D)** Western blot of eEF2K and p-eEF2 from eEF2K wild-type, heterozygous, and knockout mouse tissues. **(E)** Abolition of eEF2 phosphorylation in eEF2K<sup>-/-</sup> mouse tissues was confirmed by *in vitro* kinase assay in the presence of [ $\gamma$ -<sup>32</sup>P]-ATP. **(F)** Daily food intake for eEF2K<sup>+/+</sup> and eEF2K<sup>-/-</sup> mice. **(G)** Weekly body weight increase after birth for eEF2K<sup>+/+</sup> and eEF2K<sup>-/-</sup> mice. **(H)** Glucose tolerance tests. Wild-type mice (male, n=9) and eEF2K<sup>-/-</sup> mice (male, n=10) were fasted overnight before an IP injection of 2 g/kg glucose; blood glucose concentration was measured at indicated time points. **(I)** Insulin tolerance tests. Wild-type mice (male, n=9) and eEF2K<sup>-/-</sup> mice (male, n=10) were fasted for 5h before IP injection of 0.75 U/kg insulin and blood glucose concentration was measured at indicated time points. **(F-I)** Data are represented as mean +/- S.E.M. **(J)** The fraction of ovaries from postmenopausal female mice that possess antral follicles or corpora lutea. Results were based on the histology of serial ovarian sections, obtained from 17~21 month old females (9 wild type mice; 9 eEF2K-deficient mice). **(K)** Ovaries of 17-20 month old mice were serial sectioned and the number of large antral follicles with a healthy or unhealthy oocyte was counted. Irregular shaped oocytes or those with no nuclear staining were identified as unhealthy oocytes. eEF2K<sup>-/-</sup>, n= 19; eEF2K<sup>+/+</sup>, n=3. **(L-N)** Iron hematoxylin/aniline blue staining of ovary from 17-20 month old mice. Scale bars represent 50  $\mu$ m. **(L)** Healthy oocyte with an oval shape and clear nuclear staining. **(M)** Unhealthy oocyte with an irregular shape and nuclear staining. **(N)** Unhealthy oocyte with no nuclear staining.



**Figure S3. Knockout of eEF2K delays estrus cycle progression and preserves primordial follicles in aged female mice, related to Figure 3.** (A) Representative images of vaginal smears from female mice under different phases of the estrus cycle. (B) Duration of each phase of estrus cycle in days. Significant prolongation in estrus phase (Mann-Whitney *U*-test, two-tails,  $P=0.002$ ) is shown. (C-F) Primordial follicle counts (C) 8-day old, (D) 2-month-old, (E) 6-month-old, and (F) 15-month-old eEF2K wild-type and knockout mice. Statistical significance (Mann-Whitney *U*-test, one-tail) is shown. (G,H) Pups number per litter for 2-6 month old and 8-month-old female mice. (B-H) Data are represented as mean  $\pm$  S.E.M. (I) Female fertility as the percentage of females who are able to be pregnant at the indicated ages. Female mice were mated with young (2-6 month old) wild-type males. (J) Hormone levels of estradiol, progesterone, LH and FSH were measured. Blood was collected from 3-month old females in estrus phase.

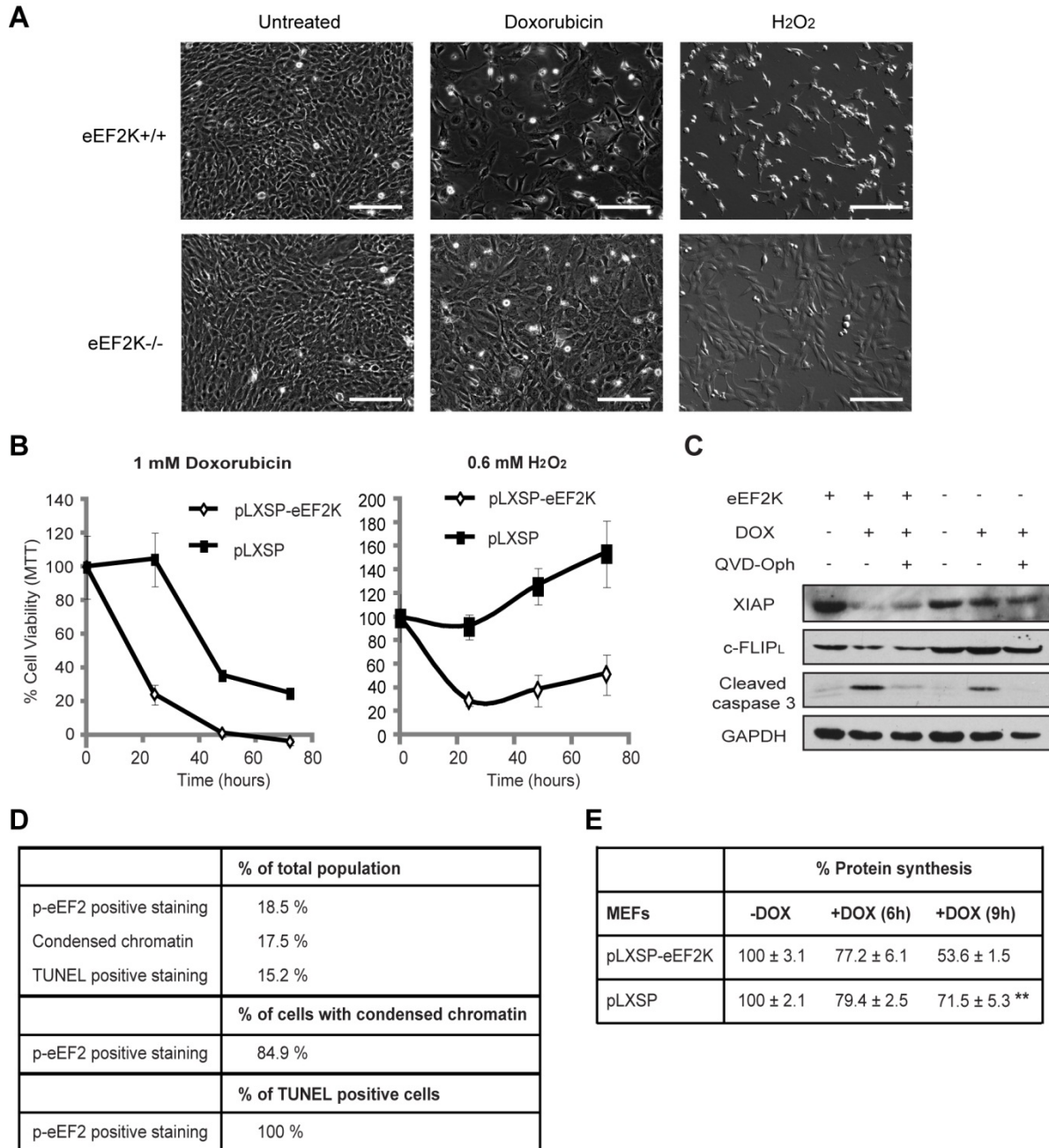


# Chu Figure S4



**Figure S4. Elongation factor 2 kinase functions to promote germ cell death and maintain germ cell quality in *C. elegans*, related to Figure 4.** (A) Schematic representation of the hermaphrodite *C. elegans* gonad, showing the two U-shaped gonad arms joined at a central uterus. Shown are the distal tip cell (DTC), the distal gonad, including mitotic region, transition region, and pachytene/meiosis I region, and the proximal gonad, including maturing oocytes, spermatheca, and embryos, as well as the uterus and vulva. (B) Western blot analysis of N2 and *efk-1(ok3609)* for phospho-EEF-2, EEF-2, and TBA-1 ( $\alpha$ -tubulin) in adult hermaphrodites. Phosphorylation of EEF2 is completely abolished in *efk-1(ok3609)* mutants. (C) Germ cell corpses observed by SYTO-12 staining and by Nomarski optics (shown by red arrowhead). Scale bars represent 10  $\mu$ m. (D) Western blot analysis of P-EEF2 and TBA-1 ( $\alpha$ -tubulin) in *ced-1(e1754)* mutants treated with control and *efk-1* RNAi. (E) Percentage of proximal gonads with hyperplasia amongst mature stacked oocytes. (F) DIC and DAPI-stained images for results shown for (E). Hyperplasia in the proximal gonad can be identified by red brackets. Scale bars represent 20  $\mu$ m. (G) Hermaphrodites were subjected to 0.5% hypochlorite treatment at various stages of adult reproductive development in order to quantify the proportion of eggs that failed to hatch. For each time point, populations (>30 per genotype) of ~25-50 hypochlorite-treated eggs were used to calculate the percentage of unhatched eggs. Data are represented as mean +/- S.E.M. where \*\*\*<0.001, \*\*<0.01, and \*<0.05 (Mann-Whitney U-test).





**Figure S5. Knockout of eEF2K suppresses apoptosis induced by oxidative stress in MEFs, related to Figure 5. (A)** Phase-contrast images of cells. MEFs were treated for 24 hours with 1.6  $\mu$ M doxorubicin or 400  $\mu$ M hydrogen peroxide. Scale bars represent 50  $\mu$ m. **(B)** MTT assay of eEF2K<sup>-/-</sup> MEFs expressing pLXSP empty vectors or eEF2K cDNA in pLXSP vectors treated with hydrogen peroxide or doxorubicin for different time periods. Data are represented as mean  $\pm$  S.E.M. **(C)** MEFs with or without eEF2K were treated with 1 $\mu$ M doxorubicin for 24h. Caspase activity was inactivated by adding the broad spectrum caspase inhibitor, QVD-OPh (20  $\mu$ M), one hour prior to the doxorubicin treatment. Western blot shows that the protein levels of XIAP and c-FLIP<sub>L</sub> were not restored in QVD-OPh pre-treated cells. **(D)** TUNEL-positive cells contain intense phosphorylation of eEF2. HeLa cells were treated with 1.6  $\mu$ M doxorubicin for 12h and stained for p-eEF2 and TUNEL staining. Condensed chromatin was visualized by DAPI staining. At least 300 cells were counted in each group. All cells with positive staining of TUNEL are also positive stained by p-eEF2. **(E)** Protein synthesis rate in eEF2K<sup>-/-</sup> MEFs infected with retrovirus containing eEF2K cDNA or empty vector pLXSP. Protein synthesis was measured during 3h periods following 6h or after 9h incubation with doxorubicin. The rate of protein synthesis was determined by estimating the <sup>14</sup>C/<sup>3</sup>H ratios. Values are means  $\pm$  SD, \*\* represents a significant difference where  $P=0.005$  (two-tailed  $t$ -test).

Chu Figure S6

Mus musculus 1 -----MADEDIITCLEGVDDGR-----CSR-----AGHNADSDTSDSDDDGYFICHTDDHMSNQNVSSKQSVYSNLTKTEC-G----STGPSASSFFH  
Danio rerio 1 -----MAEEDIMSMEEVGSARDGSRNRCKVNRVCQNSASDSDDEDEEDDLRHCPTDDIISPTKGF--CEFPKNLAHQN-L----SNSLPKNSFTY  
Strongylocentrotus purpuratus 1 -----MAEDDQIEITDLDDHQ-----VNGYNGNHNHSSDEDEMLLEPLIDFLKEPTT-H-----STP-INKN-I----NKKTKRSPYCA  
Crassostrea gigas 1 MSHSISNDMDFGDIIVLFFHHSDDDEEQE-D-----LTESMDASDERESEHVEEIKKAKAMTLRQRRLSKS-----YAP-LA-N-L----KLVKPGQSSA  
Ixodes scapularis 1 -----MTDITNESD-NSPNPSGLA-SARTFSLNASKMVRITDDYDFEVEFLEQNDVVIEKPRMDLHVRLK  
Caenorhabditis elegans 1 -----MS-----QVDCDTMI-----QNDNDLNSQKKNNSVSKLILKARSLSIVDSIK-----ESYPED-KSDQH-N---PIHISKSPFNS  
Hydra magnipapillata 1 -----MS-----QVDCDTMI-----QNDNDLNSQKKNNSVSKLILKARSLSIVDSIK-----ESYPED-KSDQH-N---PIHISKSPFNS  
Amphimedon queenslandica 1 -----MS-----QVDCDTMI-----QNDNDLNSQKKNNSVSKLILKARSLSIVDSIK-----ESYPED-KSDQH-N---PIHISKSPFNS

Mus musculus 81 MEAKKHAIEKAK-HNPPWAEFHLIEDI-ATSHATRHRRYNAVTEGMLKDEVLKHNASOPFGGAMRECFRKKLSNPLHAQQMGASNYVAKRYI-EPVDRSVYFEDVWOL  
Danio rerio 89 KATWKHAIEKAK-ANPPWAEFHLIEM-ETERCIRVRYSAITGEMAKDELFRVSSQTFGGAMRECFRKKLSNPSHSHNMSASNYVAKRYM-EIVDRSVYFEDVWOL  
Strongylocentrotus purpuratus 72 KLKWKWAFQOVR-KNDDPWAEFHLIEDI-HIDTAIRHRRYNAVTEGMLKDEVLKHNASOPFGGAMRECFRKKLSNPSHSHNMSASNYVAKRYM-EIVDRSVYFEDVWOL  
Crassostrea gigas 87 QNEWIMAKKAK-DESOPWAEFHLIEDI-KHDFCIRHRRYNPHKKTWKDEVOVRKMGSESPHGGAMRECFRKKLSNPSHSHNMSASNYVAKRYI-EIVDRSVYFEDVWOL  
Ixodes scapularis 4 AQRWQARRRAL-HNEDPWAEFHLIERI-QEVVRVHRRYNATREWEDDVALVKMEAOQPPFGGAMRECFRKKLSNPSHSHNMSASNYVAKRYI-EIVDRSVYFEDVWOL  
Caenorhabditis elegans 67 METRKAARRARTNYIDPWDENTHEY-IVORARRRYSAITRQWEDDVALVKMEAOQPPFGGAMRECFRKKLSNPSHSHNMSASNYVAKRYI-EIVDRSVYFEDVWOL  
Hydra magnipapillata 67 HGNWIDAKKAK-KVQDPWAEFHLIEM-ETERCIRVRYSAITGEMAKDELFRVSSQTFGGAMRECFRKKLSNPSHSHNMSASNYVAKRYM-EIVDRSVYFEDVWOL  
Amphimedon queenslandica 7 KHEWEAKKAK-SLEDPWAEFHLIEDI-DEKVAIRHRRYNPHKKTWKDEVOVRKMGSESPHGGAMRECFRKKLSNPSHSHNMSASNYVAKRYI-EIVDRSVYFEDVWOL

Mus musculus 188 MEAKLNGEDYRHRHPPKQVDIQQNCIIEKLDK-HGQPLHLEHFELEGYIKYNSNSGFLSD-----DNIRLTPQAFSHFFERSGHOLIIVDIOGVGDLYTDPQIHTLHMD  
Danio rerio 196 MEAKLNGEENRHRPPKQVDIQQNCIIEKLDK-HGQPLHLEHFELEGYIKYNSNSGFLSD-----DNIRLTPQAFSHFFERSGHOLIIVDIOGVGDLYTDPQIHTLHMD  
Strongylocentrotus purpuratus 179 MDAKLNGEENRHRPPKQVDIQQNCIIEKLDK-HGQPLHLEHFELEGYIKYNSNSGFLSD-----DNIRLTPQAFSHFFERSGHOLIIVDIOGVGDLYTDPQIHTLHMD  
Crassostrea gigas 194 MDAKLNGEENRHRPPKQVDIQQNCIIEKLDK-HGQPLHLEHFELEGYIKYNSNSGFLSD-----DNIRLTPQAFSHFFERSGHOLIIVDIOGVGDLYTDPQIHTLHMD  
Ixodes scapularis 111 MDAKLNGEENRHRPPKQVDIQQNCIIEKLDK-HGQPLHLEHFELEGYIKYNSNSGFLSD-----DNIRLTPQAFSHFFERSGHOLIIVDIOGVGDLYTDPQIHTLHMD  
Caenorhabditis elegans 173 MDAKLNGEENRHRPPKQVDIQQNCIIEKLDK-HGQPLHLEHFELEGYIKYNSNSGFLSD-----DNIRLTPQAFSHFFERSGHOLIIVDIOGVGDLYTDPQIHTLHMD  
Hydra magnipapillata 176 MDAKLNGEENRHRPPKQVDIQQNCIIEKLDK-HGQPLHLEHFELEGYIKYNSNSGFLSD-----DNIRLTPQAFSHFFERSGHOLIIVDIOGVGDLYTDPQIHTLHMD  
Amphimedon queenslandica 114 MDAKLNGEENRHRPPKQVDIQQNCIIEKLDK-HGQPLHLEHFELEGYIKYNSNSGFLSD-----DNIRLTPQAFSHFFERSGHOLIIVDIOGVGDLYTDPQIHTLHMD

Mus musculus 294 FGDGNLGVRGMAFFVSHACNRIQOSMGLVPLDPSRPODAVNOSTRLOSAKVLLRGTEEEKGSPRITLSSSRPPLL-----RL-SEN-S--GDENM-----  
Danio rerio 302 FGDGNLGVRGMAFFVSHACNRIQOSMGLVPLDPSRPODAVNOSTRLOSAKVLLRGTEEEKGSPRITLSSSRPPLL-----RL-SEN-S--GDENM-----  
Strongylocentrotus purpuratus 285 FGDGNLGVRGMAFFVSHACNRIQOSMGLVPLDPSRPODAVNOSTRLOSAKVLLRGTEEEKGSPRITLSSSRPPLL-----RL-SEN-S--GDENM-----  
Crassostrea gigas 290 FGDGNLGVRGMAFFVSHACNRIQOSMGLVPLDPSRPODAVNOSTRLOSAKVLLRGTEEEKGSPRITLSSSRPPLL-----RL-SEN-S--GDENM-----  
Ixodes scapularis 217 FGDGNLGVRGMAFFVSHACNRIQOSMGLVPLDPSRPODAVNOSTRLOSAKVLLRGTEEEKGSPRITLSSSRPPLL-----RL-SEN-S--GDENM-----  
Caenorhabditis elegans 278 FGDGNLGVRGMAFFVSHACNRIQOSMGLVPLDPSRPODAVNOSTRLOSAKVLLRGTEEEKGSPRITLSSSRPPLL-----RL-SEN-S--GDENM-----  
Hydra magnipapillata 285 FGDGNLGVRGMAFFVSHACNRIQOSMGLVPLDPSRPODAVNOSTRLOSAKVLLRGTEEEKGSPRITLSSSRPPLL-----RL-SEN-S--GDENM-----  
Amphimedon queenslandica 220 FGDGNLGVRGMAFFVSHACNRIQOSMGLVPLDPSRPODAVNOSTRLOSAKVLLRGTEEEKGSPRITLSSSRPPLL-----RL-SEN-S--GDENM-----

Mus musculus 385 ---SDVTFDSLSPSPS-----SATPHSQKLDHLHVPVFGDLNDMGRDHRDMDNRDSENSGDSGYPSEKNSDLDDY--EPREHGHSNGNR-----HEDEDSL  
Danio rerio 394 ---S-DTDSHPSPSL-----SMGV-SVGRSPMGWSFMNEMNE---THRSQTEQQDSENGGDSGYPSEKNSDLDDY--EPREHGHSNGNR-----HEDEDSL  
Strongylocentrotus purpuratus 391 ---RRISFQDLPSSPD-----DVAM-----SMSP--PSVLNQFORRTA-----HRLDSESE  
Crassostrea gigas 399 ---DPSMDD-----EPM-----SVUSP--SPSGVPY--GVR--RSPSDT  
Ixodes scapularis 305 ---RVMTDLSPLPFTLGESVDSVGLERMCRRKNSQFWRLLNGAQRQ--LDKRVVTRFRFSGELTRIGYGSQTAKRLPOP--EQRQRGQORRRGR--PDPQDIT  
Caenorhabditis elegans 381 HNEDCVCFPIVVDLCEPCSEDEED-----EE---EDYPRSEKSGNSQKRRSRMSISTR--SSGVEASAPRKKCFVDLNSLRQRHDSR--SSVYHIS  
Hydra magnipapillata 359 ---KSISSVQSNR-----EFSEU--PSPRVVDDGNDM-----YDQHLHIS  
Amphimedon queenslandica 311 ---SSVITEN-----KHISDDVFNK-----

Mus musculus 475 --GSSG--RVCVSTNLIINPSR-----LHLRPS-AVALEVQL-----N-ALDGRKIKSVLGLKVIHNAVRYHAGRPECED--E-----EWD  
Danio rerio 457 --EHHG--QLTEERKSFYHSSR-----SHHRPS-CVAVEVQL-----N-ALLLEKKIKSVLGLKVIHNAVRYHAGRPECED--E-----PMD  
Strongylocentrotus purpuratus 434 --G-ETLSHDEKRAKFI-----ERNVHRPS-CVNEVQLRK-----TMVVTSGGSLGQIHLDMAYHAGRAPDDNS-----RN  
Crassostrea gigas 433 --QSSMZKEEERAKFRM-----AGSQNARPS-CVALEKDFRK-----LM-NHKIGDSLGLKVIHNAVRYHAGRPECED--E-----KLDI  
Ixodes scapularis 401 --NNAN--PRCNRVPPFQEHDRAEFQRLVAQRAPS-CVLEVQLRQ--L-GQOPATQSRSLGQIHLDMAYHAGRAPDDNS-----DMD  
Caenorhabditis elegans 471 MNSRQTRDTEKDFPKVIRKQS-----VPANISGLOQMAANLENDEVPVVTGHPQSLGQIHLDMAYHAGRAPDDNS-----DMD  
Hydra magnipapillata 398 ---SSAA--PME--TVTRM-----QENVKRIIS-SVALEMEGD-----TIVESYSLEQKNVNLGQVHVMATYHSGRTESEP-----D  
Amphimedon queenslandica 333 --GSEA--PME--TVTRM-----QENVKRIIS-SVALEMEGD-----TIVESYSLEQKNVNLGQVHVMATYHSGRTESEP-----D

Mus musculus 547 RSEATPHHNDLQEFLEAVGGLMWSVQPHHIDADVSKET-----ENKTKQFVLLKQAGGQHSMLVARAFDGLNWSPPRCQOJSSA  
Danio rerio 529 QISAVHIEFAKQCELEAIVAGQCHQDHPHILPELEPS-----ENSOQFRLLOAABAGDPSMLVARAFDGLNWSPPRCQOJSSA  
Strongylocentrotus purpuratus 504 MSAVHIEFAKQCELEAIVAGQCHQDHPHILPELEPS-----ENYNEGMDVMEAAEAGDPSMLVARAFDGLNWSPPRCQOJSSA  
Crassostrea gigas 508 WSAVHIEFAKQCELEAIVAGQCHQDHPHILPELEPS-----DEHTNRQVDMVBAEAGDPSMLVARAFDGLNWSPPRCQOJSSA  
Ixodes scapularis 485 QSAVHIEFAKQCELEAIVAGQCHQDHPHILPELEPS-----ESVSRGVELEOARAGDPSMLVARAFDGLNWSPPRCQOJSSA  
Caenorhabditis elegans 569 KQSAVHIEFAKQCELEAIVAGQCHQDHPHILPELEPS-----EYVNEGMDVMEAAEAGDPSMLVARAFDGLNWSPPRCQOJSSA  
Hydra magnipapillata 452 FSSAVHIEFAKQCELEAIVAGQCHQDHPHILPELEPS-----HDLGNFMIEAARNDKDMKTRAFDGLNWSPPRCQOJSSA  
Amphimedon queenslandica 397 LDASVHIEFAKQCELEAIVAGQCHQDHPHILPELEPS-----PDNEAMCKVLLKSAKTDQOAMVYGRILPEGEN---SERDWEA

Mus musculus 637 LHYNTLAETDQDGG-----GEYDGIQDEPQALLAREMELLTGGCFGLDKDFORSQDLYTONAFAAAMEAMKGRLANOYVEKAEAWAQNDE  
Danio rerio 619 VHYDSDAKMTDYDDEG-----GEFDMQDEPRVLLARAEYQEGCNLDNDPQORAGDLPTEAAWAAMEAMKGRLANOYVYKAEAWAQNDE  
Strongylocentrotus purpuratus 595 MHYDEAVNTEKVDDEG-----GHYDSTNDPQVLLARAEYQEGCNLDNDPQORAGDLPTEAAWAAMEAMKGRLANOYVYKAEAWAQNDE  
Crassostrea gigas 598 VHYDEAVNTEKVDDEG-----GDFCTNDPQVLLARAEYQEGCNLDNDPQORAGDLPTEAAWAAMEAMKGRLANOYVYKAEAWAQNDE  
Ixodes scapularis 575 CLHYNTLAETDQDGG-----GEYDGIQDEPQALLAREMELLTGGCFGLDKDFORSQDLYTONAFAAAMEAMKGRLANOYVEKAEAWAQNDE  
Caenorhabditis elegans 679 LHYNTLAETDQDGG-----GEYDGIQDEPQALLAREMELLTGGCFGLDKDFORSQDLYTONAFAAAMEAMKGRLANOYVEKAEAWAQNDE  
Hydra magnipapillata 539 LHYNTLAETDQDGG-----GEYDGIQDEPQALLAREMELLTGGCFGLDKDFORSQDLYTONAFAAAMEAMKGRLANOYVEKAEAWAQNDE  
Amphimedon queenslandica 483 AHYNTLAETDQDGG-----V-----PLEDDYIMAKLGNVNTGAFGLSDYENAGQYQEAADLAMEAMKGRLANOYVYKAEAWAQNDE

**Figure S6. Elongation factor 2 kinase is conserved across eukaryotes, related to Figure 4.**

Protein alignment of elongation factor 2 kinase across diverse eukaryotes. Genbank accession numbers for protein sequences shown: *M. musculus*, AAH55361.1; *D. rerio*, AAH76410.1; *S. purpuratus*, XP\_788087.1; *C. gigas*, EKC40554.1; *I. scapularis*, EEC15469.1; *C. elegans*, CCD65313.1; *H. magnipapillata*, XP\_002162308.1; *A. queenslandica*, XP\_003385502.1. Black shading indicates identical amino acids and gray shading indicates >50% consensus. Alignment performed via ClustalW algorithm and Boxshade 3.21.

## **Supplemental Experimental Procedures**

### **The complete loss of eEF2 phosphorylation in eEF2K<sup>-/-</sup> mice.**

To investigate the physiological role of eEF2K, we generated eEF2K<sup>-/-</sup> mice by disrupting the *Eef2K* gene in mouse embryonic stem cells. A targeting vector was used in which exon 7 and the majority of exon 8 of the *Eef2K* gene were replaced with the neomycin resistance gene (Figure S2A). This strategy resulted in elimination of a portion of the catalytic domain of eEF2K (Figure S2B). The mouse genotypes were analyzed by the PCR reactions (Figure S2C). The eEF2K<sup>+/-</sup> mice produced progeny of eEF2K<sup>+/-</sup>, eEF2K<sup>+/+</sup> and eEF2K<sup>-/-</sup> mice with the expected ratio of 2:1:1. We analyzed the phenotype of eEF2K<sup>-/-</sup> mice and found that they were overtly normal, fertile and had a normal growth rate, food intake, and glucose and insulin tolerance tests (Figure S2F-I). Western blotting showed that no eEF2K protein was detected and no phosphorylation of eEF2 was observed in cell extracts from eEF2K<sup>-/-</sup> mice (Figure S2D). We also performed a kinase assay using purified rabbit eEF2 as a substrate and found that purified eEF2 was unable to be phosphorylated in eEF2K<sup>-/-</sup> cell lysates, indicating a complete lack of eEF2K activity in eEF2K<sup>-/-</sup> mice (Figure S2E).

### **Cell viability assay**

For the MTT assay,  $3 \times 10^3$  cells/well (100  $\mu$ l) were grown overnight in 96-well plates with DMEM containing 10% fetal bovine serum. Cells were treated with doxorubicin for 24h, then incubated with 20  $\mu$ l of 1 mg/ml MTT (3-(4,5-dimethylthiazol)-2,5-dipheyltetrazolium bromide) at 37°C for 4h. Media was removed and DMSO was added into each well. The number of viable cells was determined by measuring the absorbance at 550 nm.

### **Caspase inhibition by QVD-Oph**

Pan-caspase inhibitor QVD-Oph [quinoline-Val-Asp(OME)-CH<sub>2</sub>-PH] was purchased from

Calbiochem. When cells achieved a confluency of 70%, 20  $\mu$ M QVD-Oph was added 30 min before the apoptotic stimuli. Consequently, cells were incubated in 1  $\mu$ M doxorubicin (Sigma, MO) for 24h in the presence or absence of QVD-Oph to induce apoptosis. Cells were harvested and cell lysates were used for Western blotting analysis.

### **Analysis of mouse estrous cycle**

Female mice were separated according to their genotypes. Vaginal smears were obtained every morning and stained with Giemsa stain (Sigma, MO). Light microscopy was used to identify the different stages of the estrous cycle based on smear cell morphologies. The approximate duration of each phase was estimated.

### **Hormone measurements**

To measure hormone levels, estrus cycles were analyzed by vaginal smears in 3-month-old wild-type and eEF2K-deficient female mice. Mice were sacrificed on the estrus day and blood samples were collected by heart puncture. Whole blood samples were transferred into 1.5 mL centrifuge tubes and allowed to clot at room temperature for 90 min. Samples were centrifuged at 2,000g for 15 min, after which the serum was stored at -20°C. Mouse FSH-RIA, LH-IRMA, Progesterone-RIA, and Estradiol-ELISA assays were conducted by The University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core Facilities

### **Glucose and insulin tolerance test**

For glucose tolerance test, 2-month-old eEF2K<sup>+/+</sup> and eEF2K<sup>-/-</sup> male mice were fasted overnight, and IP injected with 20 mg/ml glucose at a dose of 2 g/kg body weight. For insulin tolerance test, mice were starved for 5h and IP injected with 0.75 U/kg body weight recombinant insulin (Invitrogen, CA). Blood was obtained from the tail at time points 0, 15, 30, 60, 120 min for glucose measurement.

### **Kinase assay**

The eEF2 kinase assay was performed as described (Dorovkov et al., 2002). Mouse cell lysates were incubated with purified rabbit reticulocyte eEF-2 in reaction buffer (50 mM HEPES–KOH with pH 6.6, 10 mM magnesium acetate, 5 mM DTT, 100  $\mu$ M  $\text{CaCl}_2$ , 0.5  $\mu$ g calmodulin, 60  $\mu$ M ATP, and 1  $\mu$ Ci [ $\gamma$ - $^{32}$ P]-ATP (3000 Ci/mmol)). Reactions were incubated at 30 °C for 10 min and were terminated by incubation on ice with addition of Laemmli buffer. Samples were analyzed by 8% SDS–PAGE and autoradiography.

### **Measurement of protein synthesis with $^{14}\text{C}/^3\text{H}$ double labeling**

For  $^{14}\text{C}/^3\text{H}$  double labeling,  $2 \times 10^4$  fibroblasts/well were cultured in 12-well plate in the low leucine medium (L-leucine 6.6 mg/L) labeled with 5  $\mu$ Ci/ml of  $^3\text{H}$ -Leucine for 24 hours. After the treatment with 1.6  $\mu$ M doxorubicin for 6 hours or 9 hours in  $^3\text{H}$ -labeled medium, cells were washed with PBS and were supplemented with  $^{14}\text{C}$  labeled medium (2  $\mu$ Ci/ml of  $^{14}\text{C}$  amino acids mixture) for another 3h. Subsequently, cells were lysed and radioactivity was determined as previously described (Dorovkov et al., 2002). The rate of protein synthesis was determined by calculating  $^{14}\text{C}/^3\text{H}$  ratio.

### **Supplemental References**

Dorovkov, M.V., Pavur, K.S., Petrov, A.N., and Ryazanov, A.G. (2002). Regulation of elongation factor-2 kinase by pH. *Biochemistry* *41*, 13444-13450.