Published in final edited form as:

Biochim Biophys Acta. 2009 May ; 1790(5): 326–338. doi:10.1016/j.bbagen.2009.01.012.

Embryonic Lethality of Fortilin-null mutant mice by BMP-pathway overactivation

Yuichi Koide^{a,1}, Tomomi Kiyota², Moltira Tonganunt^{1,3}, Decha Pinkaew^{1,3,4}, Zhihe Liu^{1,4}, Yoichi Kato², Nongporn Hutadilok-Towantana³, Amornrat Phongdara³, and Ken Fujise^{1,4,*}

¹ Research Center for Cardiovascular Diseases, Brown Foundation Institute of Molecular Medicine for the Prevention of Human Diseases, Houston, TX, 77030, U.S.A ² Department of Biomedical Sciences, Florida State University, College of Medicine, Tallahassee, FL, 32306, U.S.A ³ Prince of Songkla University, Hat Yai, Songkla, Thailand ⁴ Division of Cardiology, Department of Internal Medicine, University of Texas Medical Branch at Galveston, Galveston, TX, 77555, U.S.A

Abstract

Background—Fortilin negatively regulates apoptosis and is overexpressed in cancer. However, the role of fortilin in mammalian development is not clear.

Methods & Results—In order to evaluate the physiological role of fortilin *in vivo*, we performed a targeted disruption of the fortilin gene in mice. Fortilin^{+/-} mice have the ability to survive and exhibit normal growth, while fortilin^{-/-} mice are embryonically lethal around the 3.5 days post-coitum (dpc). Cultured blastocysts from fortilin^{+/-} embryos undergo normal outgrowth to produce inner cell mass (ICM) and trophoblasts (TB), while ICM of fortilin^{-/-} embryos either fails to outgrow or prematurely disintegrates. Mouse embryonic fibroblasts (MEF) derived from fortilin^{+/-} embryos are more susceptible to noxious stimuli than are wild type embryos. It has been consistently shown in *Xenopus* embryos that the depletion of fortilin's message severely compromises the formation of neural tissue, including the brain, while overexpression of fortilin induces the partial double body axis in embryos and is capable of blocking BMP4-induced transcription of *Vent1*, *Vent2*, and *Msx1* genes. This suggests that fortilin is an inhibitor of the BMP pathway. Strikingly, when fortilin levels are reduced by siRNA, BMP4 causes MEF to undergo extensive DNA-fragmentation, while DNA fragmentation is minimal in the presence of fortilin. In addition, BMP4 induces more *Msx2* in the absence of fortilin than in its presence. Furthermore, *Msx2* overexpression causes MEF to undergo apoptotic cell death.

Conclusion—We conclude that in early phase of development, fortilin functions as an inhibitor of the BMP pathway. The presence of fortilin in the very early stages of development is required for the survival of embryos.

General Significance—Abnormalities in the fortilin gene may be associated with early pregnancy loss.

*Corresponding author. Division of Cardiology, Department of Internal Medicine, 301 University Blvd, John Sealy Annex, Suite 5.106, Galveston, Texas, 77555, U.S.A. Telephone: +1 409 772 9827. Fax: +1 409 419 1777. E-mail address: E-mail: Ken.Fujise@utmb.edu.

^aPresent Address: The Second Department of Internal Medicine, Yokohama City University, Fukuura 3-9, Kanazawa-ku, Yokohama, 236-0004, Japan

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Keywords

fortilin; knockout; targeted gene disruption; apoptosis; *Msx2*; and BMP

Introduction

Although the nucleotide sequences of mouse and human fortilins, also known as p21 and translationally controlled tumor protein (TCTP)[1,2], were reported in 1988 and 1999, their role in apoptosis regulation has been unknown. In an effort to explain the action of myeloid cell leukemia-1 protein (MCL1) [3], we performed several rounds of yeast-two hybrid screening using MCL1—a Bcl-2-family-member and anti-apoptotic protein—as bait. Fortilin proved to be a molecule that interacts specifically with MCL1 [4]. Since MCL1 is a well-characterized anti-apoptotic molecule [5–7], we reasoned that fortilin—a protein partner of MCL1—is also involved in apoptosis regulation. Fortilin's amino acid sequence is highly conserved in a variety of species such as plants and humans. In addition, fortilin messages are ubiquitous in normal tissue while they are robustly upregulated in tumors [8]. To test whether fortilin blocks apoptosis, we challenged HeLa cells over-expressing fortilin with etoposide. Fortilin over-expression prevented cells from undergoing apoptosis [9]. In addition, over-expression of fortilin prevented caspase-3 activation, while the anti-sense depletion of fortilin induced spontaneous apoptosis [9], suggesting that fortilin is anti-apoptotic [9]. Further studies have suggested that fortilin stabilizes MCL1 [10] and vice versa [4]. Strikingly, fortilin does not require MCL1 in order to function anti-apoptically [11], suggesting that the anti-apoptotic function of fortilin is mediated through both its direct effects on apoptotic pathways [11] and the stabilization of MCL1 [5].

The fact that fortilin is an anti-apoptotic molecule has been validated by Tuynder [8], who showed that fortilin blocks the cleavage of poly(ADP-ribose)-polymerase (PARP)[12,13], a key event in apoptosis. In addition, Lee and others showed that fortilin protects ovarian carcinoma cells against TSC-22-mediated apoptosis [14].

Although fortilin is ubiquitously expressed and powerfully exerts anti-apoptotic activities, its role in mammalian development remains unclear. Here, we report the generation of mice deficient of fortilin. The data presented here suggest that fortilin is a critical molecule in early mammalian development, the lack of which induces embryonic lethality in the very early phases of development—most likely due to the overactivation of BMP pathways.

Materials and Methods

Materials

Proteinase K was obtained from Qiagen (Valencia, CA). Restriction enzymes, dNTP, and Taq polymerase were obtained from New England Biolabs (Ipswich, MA). Betaine was obtained from Sigma-Aldrich (St. Louis, MO). X-gal (5-bromo-4-chloro-3-indoyl β -D-galactosidase) was obtained from Fisher Scientific (Pittsburgh, PA).

Generation of fortilin gene disrupted mice

In the targeting vector, exons 1 through 5 were replaced with a LacZ-Neo cassette (Fig. 1a). Germline transmissions were obtained from three independent ES cell clones.

Blastocyst Outgrowth Assays

7.5 international unit (IU) each of pregnant mare serum gonadotropin (PMSG, Sigma) and human chorionic gonadotropin (hCG, Sigma) were injected 47 hrs apart to 7–9 week old female

fortilin^{+/-} mice. These mice were individually mated with fortilin^{+/-} male mice overnight. The next day, female mice were examined for plugs. Three days after mating, female mice were sacrificed and blastocysts were flushed out of the Fallopian tube and uterus using the M2 buffer (Sigma). Blastocysts were washed 3 times in Dubecco's Modified Eagle Medium (DMEM) supplemented with 15% FCS, 1% penicillin-streptomycin, and 1% glutamine, and seeded into 20 μ L DMEM droplets under mineral oil in 6-cm dishes. Blastocysts were examined and photographed daily. At the end of day 8 *in vitro*, embryos were harvested for a PCR-based genotyping procedure. When appropriate, inner cell masses and trophoblasts of outgrown blastocysts were separately harvested into fresh microcentrifuge tubes, by gentle scraping using an elongated glass pipette tip, and subjected to mRNA isolation and real-time quantitative RT-PCR (qRT-PCR) as described below.

Purification of Genomic DNA

Adult mice—Mouse tails were digested at 55°C in lysis buffer (10 mM Tris-HCl pH8.0, 25 mM EDTA pH 8.0, 1% SDS, 100 mM NaCl, 200 μ g/mL Proteinase K) overnight. They underwent two round of phenol/chloroform extraction. The DNA was precipitated with 100% isopropanol, and washed with 70% ethanol twice followed by 100% ethanol once before it was suspended in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA).

Outgrown Blastocysts and Embryos—Embryos were digested at 55 °C in lysis buffer (100 mM Tris-HCl pH7.5, 5 mM EDTA pH 8.0, 0.4 %SDS, 200 mM NaCl, 200 μ g/mL Proteinase K) overnight. They underwent one round of phenol/chloroform extraction. The DNA was precipitated with 100% ethanol, and washed with 70% ethanol once before it was suspended in TE buffer.

Southern Blot Analysis of Genomic DNA

Genomic DNA was analyzed by Southern blot analysis with NdeI-digested genomic DNA hybridized with 0.4 kb probe, which detects 3.6 kb fragments in wild type allele and 5.3 kb fragments in mutant allele. This probe was amplified by PCR using PB1 primer (5'-CTGGATACTAGGTATTGTGATGAAGG-3') and PB2 primer (5'-CCCAAACCACTCAAGGAAGGCTCAG-3').

Polymerase Chain Reaction of Genomic DNA

One-step PCR—Fortilin wild type and mutant alleles were distinguished by PCR using the following primer pairs. PCR with wild-type forward primer (5'-AAAGGACACCGTTTGCGACCAAGAGCAGAA-3') and wild-type reverse primer (5'-ACTTACGGCTGATGAGGTCCCGGTAGATGA-3') amplifies 725 bp band from wild allele, while PCR with mutant forward primer (5'-CGT GCT ACT TCC ATT TGT CAC GTC CT - 3') and mutant RV primer (5'-TGA CCA GTG ACC TCA AGA CCC ATT - 3') amplifies 1350 bp band from mutant allele. 1 μ L of DNA solution from a mouse tail was added to 0.4 μ L of 10 mM dNTP, 2 μ L of 10XPCR buffer, 0.4 μ L of FW and RV primer each (50 μ M), 2 μ L of Taq polymerase, 2 μ L of Betaine and adjusted to a total of 20 μ L with H₂O. 34 PCR cycles were performed as follows: 30 sec 94 °C, 1 min 64 °C, 2 min 30 sec 73 °C.

Nested PCR—Embryos were genotyped with nested sets of primes. The first sets for wild type allele were Common FW primer and Wild RV primer. The second sets of wild primers were Nest Wild FW primer (5'-TTTCACTTTCGGAACCCACTAGAGGACCAG-3') and Nest Wild RV primer (5'-TGATGAGGTCCCGGTAGATGATCATGGTG-3'). These sets amplify 673 bp band from wild allele. The first sets for mutant allele were Common FW primer and Mutant RV primer. The second sets of mutant primers were Nest Mutant FW primer (5'-CGTTACCCAACTTAATCGCCTTGCAGCACA-3') and Nest Mutant RV primer (5'-

AAACGGCGGATTGACCGTAATGGGATAGGT-3'). These sets amplify 280 bp band from mutant allele. The first sets of PCR were performed as stated above. The second sets of PCR were performed as follows: 34 cycles, 30 sec 94 °C, 1 min 62 °C, 2 min 30 sec 73 °C.

Beta-galactosidase Staining of Mouse Embryos

Embryos were harvested by blunt dissection, fixed in 0.2% glutaraldehyde, 5 mM EGTA, 2 mM MgCl₂ in PBS at room temperature for 15 min and rinsed three times in 2 mM MgCl₂, 0.01% sodium deoxycholate, 0.02% NP-40 in phosphate buffer pH 7.3 at room temperature for 10 min each. They were stained with 1 mg/mL of X-gal (5-bromo-4-chloro-3-indoyl β-D-galactosidase), 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, 2 mM MgCl₂ in PBS at room temperature for 3 hours and photographed. When appropriate, specimens were embedded in paraffin, sectioned, and counterstained with eosin slightly.

Real time quantitative RT-PCR

Mouse—RNA extraction and real-time quantitative RT-PCR (qRT-PCR) of samples were performed using previously described methods [15–17]. For mouse fortilin, the sequences of forward and reverse primers and probe are as follows: Forward: 5'-TCCGACATCTACAAGATCCGG-3', Reverse: 5'-ATCTTGCCCTCCACCTCCA-3', Probe: 5'-FAM-AGATCGCGGACGGGCTGTGC-TAMRA-3', where FAM = carboxyfluorescein and TAMRA = carboxytetramethylrhodamine. For mouse *Msx2*, the sequences of forward and reverse primers and probe are as follows: Forward: 5'-GCC CAG ACA TAT GAG CCC C-3', Reverse: 5'-CGTGGCTTCCGGTTGGT-3', Probe: 5'-FAM-CCACCTGCACCTGAGGAAACACA-TAMRA-3' (Integrated DNA Technologies). Standard RNAs of mouse fortilin and *Msx2* were made for all assays by the T7 polymerase method (MEGAscript, Ambion, Austin, TX). The correlation between the number of PCR cycles required and the amount of for the fluorescent signal to reach a detection threshold (C_T) standard mRNAs was linear over at least a five-log range of RNA for all assays (data not shown).

Xenopus—The gene expression was tested by real-time qRT-PCR using iCycler Thermal Cycler (Bio-Rad, Hercules, CA) with QuantiTect SYBR Green PCR Kit (QIAGEN). The primers, which were used in this paper, were designed using Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and are as follows. vent-1: 5'-ATTACCCTGGTGGCTTTCCT-3' (u), 5'-CCCAAAGAGTGGGGGATATT-3' (d); vent-2: 5'-AATCCAAGATGGCAGACCAG-3' (u), 5'-GGTGGATGCATGGTATAGGG-3' (d); *Msx1*: 5'-CCAGAACAGGAGAGCCAAAG-3' (u), 5'-CTGTATCCAAGGTGGGCTGT-3' (d); Xba: 5'-GAGCATGGAGCACAAACAGA-3' (u), 5'-CAACTACTCAGGCCAGGAAATA-3' (d); goosecoid: 5'-GTGTTGTGGAGCAGTTCAAG-3' (u), 5'-CAACTGTCAGAGTCCAGGTC-3' (d); Xnr3: 5'-TGAGGCACCATGAAGAGATG-3' (u), 5'-ATGGATCGGCACAACAGATT-3' (d); NCAM: 5'-AGCCCAGAAAAGGAAGAAGC-3' (u), 5'-GCAGTGGAAGAAACCAGCTC-3' (d); sox2: 5'-ACCGCTATGATGTCAGTG-3' (u), 5'-CTGAGGCACTCTGATAGT-3' (d); cardiac actin: 5'-AGGACCTGTACGCCAACAAC-3' (u), 5'-CACCGATCCAGACGGACTAT-3' (d); ODC: 5'-ACATGGCATTCTCCCTGAAG-3' (u), 5'-TGGTCCCAAGGCTAAAGTTG-3' (d). The amount of ODC gene expression was used to normalize the samples.

Xenopus embryo manipulation, *in situ* hybridization, and animal cap assay

cDNA for *Xenopus* fortilin (IMAGE clone ID: 5542512) and human Fortilin was subcloned into a pCS2+ vector. Capped RNAs were transcribed *in vitro* from linearized plasmids with SP6 RNA polymerase according to the manufacture's recommended procedure (Ambion).

Eggs were obtained from female *Xenopus laevis*, and embryos were raised in $0.1 \times$ MMR buffer (100 mM NaCl, 2 mM KCl, 1 mM MgSO_4 , 2 mM CaCl_2 , 5 mM HEPES, 1 mM EDTA, pH 7.8). Embryos were staged according to Nieuwkoop and Faber [18]. Injections of RNAs into embryos were done in 3% Ficoll/ $0.1 \times$ MMR buffer. All injections were done at the two-cell stage or four-cell stage, with two blastomeres injected with an equal amount of RNA. Ventral blastomeres at four-cell stage were recognized by their darker pigmentation. Whole mount *in situ* hybridization was performed according to a standard protocol [19], using *in vitro* synthesized antisense probes labeled with digoxigenin-UTP (Roche Applied Science). *Xenopus* fortilin, goosecoid [20] and Xnr3 [21] were used for probes. Animal cap assays were done in at least three independent experiments. For animal cap explants, 500 pg *Xenopus* fortilin, 1 ng BMP4 or 500 pg noggin was injected into the animal pole at two-cell stage. Explants were dissected at stage 8 and cultured in $0.5 \times$ MMR until sibling embryos reached the specified stage. Total RNA was extracted from explants and used for testing the gene expression.

***Xenopus* morpholino oligonucleotides (MO) procedures**

The fortilin MO, 5'-GATCATGTTGGCGGCCTAAGTGTTG-3', which is complementary to the translation region of *Xenopus* fortilin, was designed and synthesized by Gene Tools, LLC (Philomath, OR). The control MO, 5'-CCTCTTACCTCAGTTACAATTATA-3', was obtained from Gene Tools, LLC and used as a negative control.

Cell death and apoptosis assays

MTT assay—Cells were plated at 1×10^4 cells per well in 96-well culture plates. The following day, cells were treated with various cytotoxic agents (Sigma) or recombinant human BMP4 (R&D Systems, Minneapolis, MN). The cells were then treated with MTT labeling reagent at 10 $\mu\text{g}/\text{mL}$ for 4 hr, solubilized in 0.01 N HCl containing 10% SDS overnight before formed formazan was quantified by the spectrophotometer at 600 nm. A cell survival rate was calculated at $(\text{Absorbance}_{600\text{nm}} \text{ of treated cells} - \text{Absorbance}_{600\text{nm}} \text{ of background}) / (\text{Absorbance}_{600\text{nm}} \text{ of untreated cells} - \text{Absorbance}_{600\text{nm}} \text{ of background}) \times 100$.

DNA fragmentation assay—Cells were seeded at 1×10^5 cells per well in 24-well culture plates, treated with siRNA-fortilin or siRNA-luciferase, stimulated with 100–200 ng/mL BMP4 (human BMP4, R&D Systems) for 24 hrs, and subjected to DNA fragmentation assay, according to the manufacturer's instructions (Cell Death Detection ELISA^{PLUS}, Roche, Nutley, NJ). In brief, after a treatment with siRNAs and BMP4, the 1×10^4 cells were lysed and cleared by centrifugation and transferred into streptavidin-coated plates, to which anti-histone antibody conjugated to biotin and anti-nucleosomal-DNA-antibody conjugated to horseradish peroxidase were added. After incubation and wash, 2,2'-azino-bis-[3-ethylbenzthiazoline-6-sulfonic acid] (ABTS) substrate solution was added. The absorbance at 405 nm of each well was measured, with the reference wavelength of 490 nm.

Results

Fortilin^{+/-} mice are healthy and fertile

To investigate the role of fortilin in mammalian development, we mutated the murine orthologue of human fortilin by homologous recombination. The murine gene encoding fortilin is located on the D3 band of the 14th chromosome. There is no other gene in its proximity. Approximately 60 kbp downstream of the fortilin gene is mouse general transcription factor IIF, polypeptide 2 (NP_081092.1). Approximately 58 kbp upstream of the gene is brain mitochondrial carrier protein (BMCP-1)(AK151334). For the generation of fortilin-deficient mice, exons I–V of the fortilin gene were replaced by a LacZ reporter gene that was ligated in frame to the start codon (ATG) of the fortilin gene (Fig. 1A). Three independent fortilin^{+/-} ES

cell clones were then established. ES cells are from Taconic 129S6/SvEvTac mice. Chimeras generated from these clones were all healthy with no obvious abnormalities. Fortilin^{+/-} mice were generated by crossing male chimeras with female C57BL/6J mice (Jackson Laboratories) with genotyping by either Southern blot analysis (Fig. 1B) or PCR analysis (Fig. 1C). Resulting fortilin^{+/-} mice were backcrossed a minimum of 8 times to C57BL/6J mice. Some of the chimeras were also mated to the 129S6/SvEvTac mice to generate fortilin^{+/-} mice on 129 background. All heterozygous knockout mice were also found healthy without obvious abnormalities.

Fortilin null mice do not survive to term regardless of their genetic background

Although animals heterozygous for fortilin are healthy and fertile, no homozygous offspring were obtained from heterozygous intercross of fortilin^{+/-} mice of mixed 129-C57BL/6J background (Tables 1A, 1B, and 1C). This was true for all three groups of mice originating from 3 independent ES cell clones (clones 11A1 [Table 1A], 12F4 [Table 1B], and 13D2 [Table 1C]). We then backcrossed one of three groups of mice—one from ES cell clone 11A1—to C57BL/6 for 8 generations but observed no homozygous mice born alive from heterozygous intercross of 8th generation mice (Table 1D). Furthermore, we backcrossed a male chimera mouse originating from ES cell clone 12F4 to 129S6/SvEvTac females in order to test the viability of these mice in a pure 129 background where we found no homozygous mice born alive from heterozygous intercross of these mice (Table 1E). These data suggest that fortilin^{-/-} mice were embryonically lethal in C57BL/6, 129, and C57BL/6–129-mixed backgrounds.

Fortilin is developmentally regulated with a bimodal expression pattern

To further evaluate if fortilin played a critical developmental role, we isolated RNAs from 3.5 dpc and 9–18 dpc embryos and quantified the amount of fortilin transcripts in each developmental stage. As shown in Fig. 1D, the levels of fortilin transcript per unit total RNA peaked around 9.5 dpc, decreased over the next 3 days to the lower level of 12 dpc before increasing to the level comparable to that of 9 dpc. We then performed qRT-PCR and immunostaining analysis on *in vitro* cultured blastocysts to evaluate the spatial expression patterns of fortilin genes in the blastocysts. As is shown in Figs. 1E&F, fortilin is overexpressed in inner cell masses (ICM, Fig. 1E) as opposed to trophoblasts (TB) both by qRT-PCR (Fig. 1E) and by immunohistochemistry (Fig. 1F). These data suggest that fortilin expression is developmentally regulated and is critical in the development of the embryo proper or inner cell mass.

Fortilin^{-/-} embryos die in the very early stage of development

In order to evaluate when the lethality of fortilin^{-/-} embryos occurred, we genotyped embryos from heterozygous intercrosses ranging from 6.5 dpc to 9.5 dpc. We found no fortilin^{-/-} embryos during this time period, suggesting that embryonic lethality due to fortilin deficiency occurs in the very early phase of development, earlier than 6.5 dpc (Table 2).

Blastocyst analysis shows the halted development in fortilin^{-/-} embryos

We then tested whether fortilin^{-/-} embryos are viable in their blastocyst stage. Harvested blastocysts from heterozygous intercrosses were individually incubated in 50 μ L DMEM with 15% FCS and photographed daily (Fig. 2). One day after the harvest (1st day of *in vitro* growth), 18.8% of blastocysts were fragmented or abnormal in morphology. However, none of these blastocysts were fortilin-null upon the nested PCR genotyping. The growth pattern for the rest of the blastocysts was followed for 8 days. Two out of fifty-two genotyped blastocysts were fortilin^{-/-}. In the first 1–3 days, all blastocysts appeared normal with inner cell masses (ICMs) present (Fig. 2A). In days 5–7, 71.5% of fortilin^{+/-} blastocysts displayed overgrowth of two

distinct components: inner cell mass (ICM) and giant trophoblasts (GTB) (Fig. 2B). Two *fortilin*^{-/-} blastocysts hatched without problems. The first *fortilin*^{-/-} blastocyst (Fig. 2C, top row) displayed abrupt disintegration and fragmentation of the cell mass after attaching and outgrowing for one day. Its ICM was significantly smaller than those of *fortilin*^{+/-} blastocysts, with a limited number of giant trophoblasts (GTB) (Fig. 2C, 3rd and 4th rows). The second *fortilin*^{-/-} blastocyst (Fig. 2C, 2nd row) failed to attach and remained floating for the entire course of the *in vitro* culture. In summary, neither of the two *fortilin*^{-/-} blastocysts survived up to day 8 whereas 36 of 50 *fortilin*^{+/-} blastocysts did survive ($P = 0.0305$, two-tailed Chi-square analysis). These data (Table 2 and Fig. 2) suggest that some *fortilin*^{-/-} embryos survive beyond 3.5 dpc, but none survive to 6.5 dpc. It is likely that *fortilin*^{-/-} blastocysts either undergo premature cell death, manifesting themselves in disintegration and fragmentation, or are unable to attach and outgrow.

Fortilin gene is induced in the neural and mesenchymal tissues in both early and late stages of development

In order to mechanistically investigate the lethality of *fortilin*^{-/-} embryos, we followed the expression pattern of the LacZ reporter gene inserted into the *fortilin* locus (Fig. 1A). In the 8.5 dpc, there was significant reporter gene activation in neural crest-fold and somites (Fig. 3A). The same expression pattern continued to the 9.5 dpc when LacZ signals were evident in the mid-hind brain, somites, and distal neural tube (Fig. 3B). In the later stage of embryonic development, at 16.5 dpc, LacZ reporter signals remained abundant in the neural and mesenchymal tissues, including the ventricular zone of the mid brain (Figs. 3C&D), the spinal cord (Fig. 3E), bones and cartilages (Figs. F&G), and muscles (Figs. H–K). These data suggested the role of *fortilin* in the development of tissues from the ectoderm and mesoderm.

Fortilin^{+/-}MEF are more sensitive to apoptotic stimuli than fortilin^{+/+}MEF

In order to investigate the anti-apoptotic role of *fortilin* in embryonic tissue, we established mouse embryonic fibroblasts (MEF) from *fortilin*^{+/-} and *fortilin*^{+/+} mice. MEF from *fortilin*^{+/-} embryos expressed lesser quantities of *fortilin* compared to *fortilin*^{+/+} embryos (Fig. 4A). We challenged these MEF (N = 3 for *fortilin*^{+/+}, N = 3 for *fortilin*^{+/-}) with TNF- α , 5-fluorouracil (5-FU), or thapsigargin and evaluated their sensitivity to these cytotoxic agents. As is shown in Fig. 4B–D, MEF-*fortilin*^{+/-} were more sensitive to all 3 agents than were MEF-*fortilin*^{+/+} (% survival after the indicated treatment = *fortilin*^{+/+} vs. *fortilin*^{+/-} for TNF- α , 55.2 ± 17.6 vs. $16.5 \pm 11.6\%$ [$P = 0.05$]; for 5-FU, 80.0 ± 6.0 vs. $62.2 \pm 1.7\%$ [$P < 0.05$]; for thapsigargin, 56.4 ± 7.4 vs. $35.5 \pm 7.3\%$ [$P < 0.05$]). These data suggest that the lack of *fortilin* in embryonic cells causes them to be more susceptible to noxious stimuli and that *fortilin* deficiency may result in the early embryonic loss due to apoptosis of vital tissue within embryos. At this point, we hypothesized that *fortilin*^{-/-} embryos die due to excessive apoptosis in response to a gene(s) induced in their tissue during early development.

Fortilin is an inhibitor of the BMP pathway in a *Xenopus* development system

In order to further define the sequence of events leading to the early embryonic death of *fortilin*^{-/-} embryos, we turned to a *Xenopus* system. We found a *Xenopus* homologue of *fortilin* by database mining analysis. The homology of the *fortilin* amino acid sequence between human and *Xenopus* was 68%. We first examined the temporal and spatial expression of *Xenopus fortilin* by whole mount *in situ* hybridization (Fig. 5). The *fortilin* expression was observed in the animal hemisphere of the embryo at the early embryonic stage (Figs. 5A, B, & C). In the sagittal section of stage 10 embryo, the *fortilin* expression is detected in the epithelial and sensorial layer cells of the animal cap and marginal zone (Fig. 5D). Subsequently, the expression of *fortilin* was observed in the anterior part of the embryo at stage 12 and in the neural crest and the edge of the neural plate at stage 16 (Figs. 5E&F)—consistent with the

pattern seen in mouse embryos (Fig. 3). To dissect the function of *Xenopus* fortilin during early embryogenesis, we then performed gain-of-function study of *Xenopus* fortilin. We injected *Xenopus* fortilin RNA into the ventral marginal zone of four-cell stage embryos (Fig. 6). Interestingly, ectopic expression of *Xenopus* fortilin in the ventral side of the embryo induced the partial secondary axis (Fig. 6B). In addition, human fortilin also induced the partial secondary axis in *Xenopus* embryos (Fig. 6C). However, *Xenopus* fortilin could not induce the ectopic expressions of *Goosecoid*, a dorsal mesoderm and Spemann Organizer marker, and *Xnr3*, a direct downstream target of Wnt signaling, at the ventral side of embryos (Figs. 6G&H), suggesting that the function of *Xenopus* fortilin functions as a BMP inhibitor during embryogenesis. To perform a loss-of-function study, we depleted the endogenous *Xenopus* fortilin protein utilizing the Morpholino Oligonucleotides (MO) approach. We first examined whether MO against *Xenopus* fortilin (x-fortilin MO) depleted the endogenous *Xenopus* fortilin. We found that the amount of *Xenopus* fortilin protein was reduced by fortilin MO, but not by the control MO (Fig. 6I). Fortilin MO was then injected into the dorsal marginal zone of four-cell stage embryos. The head formation of embryos was inhibited (dorsoanterior index [22], DAI = 4) and this inhibition was rescued by co-injection with human fortilin whose translation initiation sequence is different from *Xenopus* one (Fig. 6E&F). These data also support that *Xenopus* fortilin inhibits the BMP signaling pathway. To test this hypothesis, we performed an animal cap assay. As we expected, the expression of *Vent1*, *Vent2*, and *Msx1* induced by the overexpression of BMP4 was inhibited by co-injection with *Xenopus* fortilin (Figs. 7A, B, &C) and *Xenopus* fortilin alone induced neural markers, *NCAM* and *Sox2* (Figs. 7E&F). On the other hand, *Xenopus* fortilin did not induce pan-mesoderm markers, *Xbra*, early dorsal mesoderm marker, *Goosecoid*, the downstream targets of Wnt signaling, *Xnr3* (Fig. 7D), and late dorsal mesoderm marker, cardiac actin (Fig. 7G). These data collectively showed that fortilin functions as a BMP inhibitor in the early stage of development in *Xenopus* embryos.

The lack of fortilin leads to the lethal over-activity of BMP-4 pathway in MEF

The data derived from the above experiments using *Xenopus* embryos (Figs. 5–7) collectively suggest that fortilin is an inhibitor of BMP-4 pathway. How does the lack of the inhibition by fortilin of the BMP pathway in fortilin^{-/-} embryos lead to embryonic lethality in the very early stage of the development? BMPs have been shown to induce apoptotic cell death in developing embryos [23–25], possibly through *Msx2* [23]. Based on these observations, we hypothesized that the lack of fortilin resulted in the overactivity of BMP4 pathway, leading to excessive and lethal apoptosis during the early developmental phase. First, we tested whether the lack of fortilin is associated with increased cell death in MEF upon BMP4 challenge. We treated MEF with either siRNA-fortilin or siRNA-luciferase, incubated them with recombinant BMP4 or vehicle and measured the degree of DNA fragmentation. As is shown in Fig. 8A, siRNA-fortilin treatment lowered the fortilin levels to an undetectable range (lanes 1 and 2, Western). In the presence of fortilin (lanes 3 and 4), the addition of BMP4 did not increase the amount of DNA fragmentation (PBS vs. BMP4 = 0.039 ± 0.004 vs. 0.027 ± 0.003 , NS, N = 3; lanes 3 vs. 4). In a fortilin-deficient state, however, the addition of BMP significantly increased the amount of DNA fragmentation (PBS vs. BMP4 = 0.059 ± 0.016 vs. 0.106 ± 0.032 , $P < 0.05$, N = 3; lanes 1 vs. 2). These data suggest that BMP4 induces apoptosis in the absence of fortilin in MEF.

Secondly, we treated MEF with either siRNA-fortilin or siRNA-luciferase, incubated them with BMP4 and measured the *Msx2* message levels by real-time qRT-PCR assays. Treatment with siRNA_{Fortilin} drastically reduced the fortilin message levels (the left panel, lanes 1, 3, 5, Fig. 8B). In the absence of BMP stimulation, *Msx2* expression was minimal irrespective of the presence of fortilin (columns 7 vs. 8; siRNA_{Fortilin} vs. siRNA_{Luciferase} = 3.87 ± 0.99 vs. 4.04 ± 0.64 , NS). Upon BMP4 stimulation, *Msx2* expression robustly increased (columns 7 & 8 vs. columns 9–12). In this system, *Msx2* levels were significantly higher in the absence of fortilin than in its presence, at both concentrations (100 and 200 ng/mL) of BMP4

(siRNA_{Fortilin} vs. siRNA_{Luciferase} = 207.7 ± 19.7 vs. 179.5 ± 23.3 for BMP4 100 [ng/mL]; 227.0 ± 15.6 vs. 192.0 ± 6.52 [ng/mL] for BMP 200 [ng/mL], both $P < 0.05$ by Fisher's pairwise comparison, Fig. 8B).

Msx2 has been shown to mediate apoptosis during development [26], potentially through a pathway involving Bax activation [24]. In order to test whether *Msx2* is directly involved in the induction of apoptosis in MEF, we overexpressed *Msx2* in MEF using pBMN-GFP, a bicistronic expression vector in which 5'-LTR drives *Msx2* expression (pBMN-*Msx2*-GFP; or no gene expression in the control vector, pBMN-*Empty*-GFP) and internal ribosome entry site (IRES) drives green fluorescent protein expression (Fig. 8C). A real-time qRT PCR of *Msx2* showed that MEF transfected with pBMN-*Msx2*-GFP had a far higher amount of *Msx2* than those transfected with pBMN-*Empty*-GFP (Control vs. *Msx2*, 16.2 ± 21.7 vs. 9184.0 ± 767.5 [fg/ μ g of total RNA], $P < 0.001$). Forty-eight hours after transfection, the number of GFP-positive and rounded (dead) cells was significantly greater in cells transfected with pBMN-*Msx2*-GFP than those with pBMN-*Empty*-GFP (Control vs. *Msx2*, 11.9 ± 1.4 vs. 20.2 ± 4.4 , $N = 3$, $P < 0.05$, Dead Cell in Fig. 8C). Finally, we quantified the amount of fragmented DNA in MEF treated with either pBMN-*Empty*-GFP or pBMN-*Msx2*-GFP and found that pBMN-*Msx2*-GFP-treated MEF had a significantly larger amount of DNA fragmentation (Control vs. *Msx2*, 1.31 ± 0.09 vs. 1.96 ± 0.19 , $N = 6$, $P < 0.001$, DNA Fragmentation in Fig. 8C). Together, these data suggest that fortilin blocks BMP4-induced apoptosis in MEF, that fortilin attenuates BMP4-induced induction of *Msx2*, and that *Msx2* causes MEF to undergo apoptosis. It is likely that fortilin^{-/-} embryos died in the very early phase of development because of the unopposed activation of the BMP4 pathway and the induction of *Msx2*, a proapoptotic gene (Fig. 8D).

Discussion

In the current work, we report that fortilin is critical for normal mammalian development and that the lack of it leads to embryonic lethality regardless of the genetic backgrounds (Tables 1A – 1E). The lethality occurred very early in the development—around 3.5 dpc (Table 2). Fortilin expression was developmentally regulated (Fig. 1D) and more abundant in the inner cell mass or embryo proper than trophoblastic cells (Figs. 1E&F), suggesting its critical role in organogenesis and development. In order to explore the events around 3.5 dpc *ex vivo*, we performed blastocyst outgrowth assays where mouse embryos were harvested at their blastocyst (or 3.5 dpc) stage (Fig. 2A), incubated individually in tissue culture media, and observed for the following: expansion, hatching from zona pellucida, attachment to plastic, and outgrowth into inner cell mass and trophoblasts (Fig. 2B). While fortilin^{+/-} blastocysts rapidly hatched, attached to plastic, and outgrew into inner cell mass and trophoblasts by day 5, fortilin^{-/-} blastocysts either disintegrated after trivial outgrowth or failed to attach to plastic (Figs. 2C&D), supporting that fortilin deficiency results in lethal developmental derangement around 3.5 dpc. We then evaluated the pattern of fortilin gene induction in fortilin^{+/-} embryos using β -galactosidase (LacZ) staining (Figs 3A–K). The data collectively showed that fortilin gene is induced in neural (both central nervous system and spinal cord) and mesenchymal (somite, bone and cartilage) tissues (Figs 3A–K). We also found that the decreased fortilin levels make MEF susceptible to noxious stimuli, suggesting that the lack of fortilin may make neural and mesenchymal cells more susceptible to developmental apoptotic stimuli (Fig. 4). In order to identify pathways regulated by fortilin, we then turned to the *Xenopus* embryo system. A series of experiments using *Xenopus* embryos showed that fortilin functions as a BMP signaling pathway inhibitor in the early stages of development (Figs 5–7). Finally, we tested whether BMP4 would induce more apoptosis in fortilin-deficient MEF, and found that it did (Fig. 8A). We further showed that BMP4 was capable of inducing more *Msx2* message in the absence of fortilin, but not in its presence (Fig. 8B). Finally, we showed that *Msx2* causes MEF to undergo apoptosis (Fig. 8C). Taken together, these data suggest that the death of fortilin^{-/-} embryos was caused by the BMP4-induced, *Msx2*-mediated apoptosis unopposed

by fortilin, in the very early stage of development (Fig. 8D). We propose fortilin to be a novel inhibitor of the BMP pathway during mammalian development, which has not been reported to our knowledge. Intriguingly, fortilin functions as an intracellular inhibitor of the BMP4 pathway while *Noggin* and *Chordin* are secreted molecules binding to BMP4 and preventing it from accessing BMP4 receptors [27]. The *Msx1* expression in MEF was found very poor even with BMP4 stimulation and its quantification was not technically possible (data not shown). There are no mammalian analogues of *Vent1* and *Vent2* described.

BMPs are multi-functional growth factors that structurally belong to the TGF β superfamily [28]. The fact that the overactivation of the BMP pathway leads to apoptosis during mammalian development has been reported. Using chick embryos, Graham and others observed that apoptotic rhombomeres 3 and 5 over-expressed BMP4 and *Msx2* and that the addition of BMP4 robustly induced *Msx2* in *ex vivo* culture of rhombomere 3 [23]. Their observation was the first to show that BMP4 induces neuronal apoptosis most likely via the induction of *Msx2* [23]. Marazzi and others found that the overexpression of *Msx2* was associated with increased cell death in P19 embryonal carcinoma cells [29]. Gomes and others reported that BMP4 induced cell death in cultured sympathetic neuroblasts and that cell death was aborted by depleting *Msx2*, suggesting that BMP4 induces cell death in these cells through *Msx2* [24]. In addition, Ferrari and others showed that the ectopic *in vivo* overexpression of *Msx2* in chick limbs severely impairs limb morphogenesis by inducing apoptosis and reducing cell cycle proliferation [26]. Finally, Panchision and others showed that BMP-receptor IB overactivation in the early embryonic period causes increased apoptosis in the brain [30]. These data suggest that the lack of adequate and timely BMP4 pathway inhibition leads to apoptotic loss of embryonic tissue and are entirely consistent with the data presented in Fig. 8. The novelty of this portion of our work lies in the finding that fortilin blocks BMP4-induced apoptosis (Fig. 8A), that fortilin suppresses BMP4-mediated induction of *Msx2* (Fig. 8B) and that *Msx2* induces apoptosis in non-neuronal cells (Fig. 8C). The limitation of the study includes the use of MEF—instead of embryos themselves—in some of the functional assays (Figs. 4 and 8). Thus, the model shown in Fig. 8D may not fully represent the role of fortilin in the BMP pathway of developing mouse embryos.

In the current study, the systematic analyses of *Xenopus* embryos allowed us to determine that the BMP pathway is inhibited by fortilin during embryogenesis (Figs. 5–7). In the *Xenopus* system, the silencing of *Xenopus* fortilin by morpholino oligonucleotides (MO) resulted in phenotypes similar to those caused by the lack of BMP inhibitors (Fig. 6), the most prominent of which was the underdevelopment of neuronal tissue manifesting itself in small heads (Fig. 6E)—rescuable by human fortilin expression (Fig. 6F). The disruption of BMP signals in the ectoderm is required for neural induction [31–33]. BMP4, originally identified by its osteoinductive capabilities [34], acts within the ectoderm to induce epidermal development and suppress neural development [35–37]. Further analyses here showed that fortilin blocks the BMP4-induced expression of BMP4 target genes, such as *Msx1*, *Vent1* and *Vent2* (Fig. 7).

In the *Xenopus* system, fortilin behaved very similarly to *Noggin* and induced *NCAM* and *Sox2*. During *Xenopus* embryogenesis, the BMP pathway plays a crucial role for Dorsal-Ventral (D–V) patterning [38]. At the gastrula stage, BMP4 is expressed in the ventral mesoderm and ectoderm, and ventralizes embryos [39,40]. At the same time, BMP antagonists, such as *Noggin* [41] and *Chordin* [42], are secreted from the Spemann-Mangold Organizer, which is the dorsalizing center in the dorsal mesoderm, and bind to BMPs in the extracellular space to block the interaction between BMPs and BMP receptors. The overexpression of *Noggin* and *Chordin* in the ventral side of embryos therefore induces a secondary body axis [43,44]. *Noggin* and *Chordin* are also found critical for neural induction in mammalian development [27,45]. Beside BMP antagonists, *Nodal*, from the TGF- β family, and *Wnt* signals have been shown as dorsal inducers and ectopic expression of them in the ventral side of

Xenopus embryos can also generate a secondary axis [46,47]. We have demonstrated that ectopic expression of fortilin in the ventral side of embryos induces a partial secondary body axis but not the expression of *Goosecoid*, a target gene of *Nodal* [48], and *Xnr3*, a target gene of *Wnt* [49], in the ventral side (Fig. 7). This suggests that fortilin blocks BMP signaling and inhibits the ventralization of embryos. In fact, fortilin could inhibit the function of BMP4 in animal cap explants and induced neural differentiation without inducing dorsal mesoderm markers similar to *Noggin*, a BMP antagonist (Fig. 7). However, the mechanism of fortilin blocking BMP signaling during embryogenesis remains unknown. Interestingly, fortilin is expressed in both dorsal and ventral sides of the ectoderm and mesoderm marginal zones at the gastrula stage (Fig. 5). These findings indicate that a dorsalizing signal may activate fortilin and block an intracellular component of BMP signaling at this stage. Elucidating this question remains central to understand the function of fortilin during embryogenesis.

The targeted disruption of the fortilin (TCTP) gene has been reported. Chen and others deleted exons 3 and 4 of the fortilin gene and generated the fortilin deletion-mutant allele consisting of exons 1, 2, 5, and 6 [50]. Consistent with our findings, their fortilin^{-/-} mice were embryonically lethal. Their fortilin^{-/-} embryos exhibited increased TUNEL-positive cells around 6.5 dpc and died around 9.5–10.5 dpc, while our fortilin^{-/-} embryos died much earlier—around 3.5 dpc. Shortly before death, fortilin^{-/-} embryos were smaller in size with a severely disorganized structure in Chen's study. Although the timing of death differs, both studies suggest that the loss of embryos was caused by excessive apoptosis, which is likely to be due to the lack of appropriate inhibition by fortilin of the BMP pathway, according to our data (Fig. 8). Exons 1, 2, 3, 4, and 5 of fortilin gene consist of 28, 74, 191, 106, and 117 nucleotides respectively, while exon 6 consists only of a stop codon (TAA). The deletion of exons 3 and 4 would result in a chimeric protein of exons 1 (9 amino acids) and 2 (25 amino acids), with amino acids from exon 5. Since the anti-fortilin antibody used in Chen's study to detect fortilin protein was raised against the 11th–172nd amino acids of fortilin as antigen [10] and since the chimeric protein may have been very small in size, it is possible that the chimeric protein could not be detected by the antibody. If that is the case, it is likely that the chimeric protein, being partially functional, prevented embryos from undergoing apoptotic death. It is also possible that the chimeric protein protected MEF against apoptosis in their tissue-culture-based apoptosis assays [50]. Nevertheless, both sets of data complementarily support the anti-apoptotic role of fortilin not only in developed cells [4,8–11,51] but also in developing cells and organisms. Conditional knockout strategies would be necessary to evaluate in more detail the role of fortilin in both developing and developed animals.

The data presented here are also consistent with the recent work by Hsu and others where the deletion of the entire drosophila fortilin (d-fortilin or dTCTP) coding sequence resulted in 100% lethality of the larvae [52]. Hsu's data also suggest that d-fortilin is required for the activation of Rheb (Ras homologue enriched in brain), a Ras superfamily GTPase [53], and S6K (ribosomal S6 kinase)[54]. Further investigation is necessary to evaluate the relative importance of fortilin in the regulation of the Rheb-S6K and BMP-*Msx2* pathways.

Conclusion

In this work, we generated mice deficient of fortilin, an anti-apoptotic molecule [4,8–11], to determine the role of fortilin *in vivo*. We conclude that fortilin is required early in development. This is based on the finding that homozygous fortilin knockout mice are stillborn and that embryonic lethality occurs on or before its blastocyst stages (3.5 dpc). Systematic analyses using *Xenopus* embryos show that fortilin is a functional inhibitor of BMP pathways. Using MEF, we then show that BMP4, in the absence of fortilin, causes MEF to undergo apoptosis through the induction of *Msx2*, a proapoptotic gene. In addition to the fact that fortilin blocks apoptosis through the stabilization of MCL1 [10], our current data reveals another facet of

fortilin—its anti-apoptotic activity through the functional antagonism of the BMP4 pathway during the early mammalian development. Further investigation is needed to determine the exact molecular mechanism by which fortilin inhibits BMP-4 signal transduction *in vivo*.

Acknowledgments

We are grateful to Eric Vu for his expert help in the genotyping of mice.

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Fig. 1. The generation of fortilin KO (heterozygote) mice

A: targeting vector construction. LacZ (β -galactosidase) gene was fused in frame to the start codon of the fortilin gene. Neo^r = Neomycin resistant gene, Thick arrows = PCR probes, Thin arrows = direction of translation, Blue rectangle = the probe for Southern blot analysis. **B: Southern blot analysis.** Southern blot analysis performed on adult mouse tail DNAs shows that 1, 3, 5, 6, 7, 9, and 10 are heterozygotes while the rest are wild type. **C: PCR-based genotyping.** Wild, wild type allele; LacZ, mutant allele containing beta-galactosidase gene; W, wild type control validated by Southern blot analysis; H, heterozygous control validated by Southern blot analysis; Lanes 2, 5, 6, 8, 9, 11 represent wild type mice while lanes 1, 3, 4, 7, 10, 12, and 13 are heterozygous. There are no homozygotes seen in mice older than 6.5 dpc (see Table 1A–1E). **D. Fortilin expression in intrauterine development.** Day, days post-coitum (dpc). A real-time quantitative RT-PCR (qRT-PCR) was performed to quantify the amount of fortilin transcripts in RNA isolated from mouse embryos aging 3.5 through 18 dpc. A polynomial regression line shows the temporal trend of fortilin messages between 3.5 and 18 dpc. **E. Fortilin expression in outgrown blastocysts.** ICM, inner cell mass; TB, trophoblasts; ***, $P < 0.005$, $N = 3$. Fortilin transcripts were more abundant in ICM than in TB in outgrown blastocysts. Fortilin may play a critical role in the development of embryo proper around 3.5 dpc. **F. Fortilin immunostaining of outgrown blastocysts.** Arrow, inner cell mass; scale bar, 50 μ m. Fortilin protein was more abundant in ICM than in TB in outgrown blastocysts.

Fig. 2. Fortilin^{-/-} blastocysts disintegrate or fail to attach in blastocyst outgrowth assays

A. Normal blastocyst. ICM, inner cell mass; BCC, blastocyst cavity; PTB, polar trophoblast; MTB, mural trophoblast. Scale bar = 100 μ m. **B. Outgrown blastocyst.** ICM, inner cell mass; GTB, giant trophoblasts; RDC, round and dead cells. Scale bar = 100 μ m. **C. Blastocyst outgrowth assay.** Blastocysts were harvested from 5 fortilin^{+/-} female mice mated individually with fortilin^{+/-} males, plated individually, and allowed to outgrow. Although both fortilin^{+/-} and fortilin^{-/-} blastocysts hatched without problems, fortilin^{-/-} blastocysts either failed to attach to culture dish or disintegrated shortly after attachment. Genotype was determined by nested PCR (see the Materials and Methods section) on outgrown blastocysts after 8th day *in vitro*.

Fig. 3. LacZ staining of *fortilin*^{+/-} embryos on 8.5, 9.5, and 16.5 PDC

A: 8.5 dpc embryo. yellow arrows, neural fold; red arrows, somites. Scale bar = 1mm. **B: 9.5 dpc embryo.** white arrows, brain; yellow arrows, hind brain; red arrows, somites; blue arrows, distal neural tubes. Scale bar = 1mm. **C–K: Sagittal section of mouse embryo (16.5 dpc) with lacZ staining and neural red counterstaining.** **C:** brain, npc: neopallial cortex (future cerebral cortex), iz: intermediate zone, vz: ventricular zone, lv: lateral ventricle. (25×), **D:** brain, magnified view of (a) (100×), **E:** spinal cord (sc), sm: skeletal muscle, vb: vertebral body (100×), **F:** rib and rib cartilage, rb: rib bone (100×), **G:** rib and intracostal muscles (200×), **H:** oropharynx (op), tm: tongue muscle, tg: tongue (100×), **I:** tongue (200×), **J:** skeletal muscle (sm) (100×), **K:** skeletal muscle (200×).

Fig. 4. MEF analysis shows that MEF^{Fortilin^{+/-}} express less fortilin and more susceptible to noxious stimuli than does MEF^{Fortilin^{+/+}}

A: Western blot analysis of MEF clones. MEF, mouse embryonic fibroblasts. **B:** TNF- α -induced cell death. 1, 2, 3 represents 3 independent clones of MEF^{Fortilin^{+/-}} and MEF^{Fortilin^{+/+}}; Av, average; *, $P < 0.05$. **C:** 5-fluorouracil(5-FU)-induced cell death. *, $P < 0.05$. **D:** Thapsigargin-induced cell death. *, $P < 0.05$. The Y-axis represents % survival after the indicated treatment.

Fig. 5. *Xenopus* embryos exhibit robust fortilin expression in animal pole

A: Stage 6 lateral view, **B:** Stage 8 animal pole view; Circle, the area of fortilin expression, **C:** Stage 10 lateral view; an, animal pole; ve, vegetal pole. **D:** Stage 10 sagittal section; an, animal pole; ve, vegetal pole. **E:** Stage 12 dorsal view; a, anterior; p, posterior; arrowheads, the area of fortilin expression. **F:** Stage 15 dorsal view; a, anterior; p, posterior; arrowheads, the area of fortilin expression.

Fig. 6. Fortilin overexpression enhances, but fortilin deficiency retards, the head development in *Xenopus*

The overexpressions of *Xenopus* (xFortilin) and human (hFortilin) fortilin induced the partial secondary body axis indicated by arrows (**B** and **C**) but not the ectopic expressions of *goosecoid* (*gsc*) and *Xnr3* indicated by arrow heads (**G** and **H**). Controls are shown in **A**. Fortilin RNA was injected into the ventral marginal zone of four-cell stage embryos, and the phenotype was observed at stage 35 or whole mount *in situ* hybridization was performed using stage 10 embryos. Fortilin MO inhibited the head formation (**E**) and this phenotype was rescued by co-injection with human fortilin (**F**). MO was injected into the ventral marginal zone of four-cell stage embryos and control MO was used as a negative control. Table showed the summary of injection experiments. Fortilin MO partially depleted endogenous *Xenopus* fortilin protein in stage 10 embryos (**I**). DAI, dorsoanterior index.

Fig. 7. Fortilin is a BMP4 inhibitor in *Xenopus* development

In the animal cap assay, *Xenopus* fortilin (xFortilin) inhibited the expression of direct targets of BMP signaling, *Vent1*, *Vent2* and *Msx1* (A, B, C), and induced the expression of neural markers, *NCAM* and *Sox2* (E, F). In addition, other markers, *Xbra*, *goosecoid*, *Xnr3* and *cardiac actin* (D and G), were not induced by xFortilin. The gene expression was tested by quantitative real time RT-PCR at stage 10 (A, B, C, D) and stage 18 (E, F, G), and *ODC* (ornithine decarboxylase) expression was used to normalize the samples. Noggin was used as a positive control of a BMP inhibitor. The same experiments were performed three times, the means of which are depicted along with standard deviations (SDs).

Fig. 8. Fortilin deficiency leads to *Msx2* induction and apoptosis in MEF

A. Assay of the DNA fragmentation of fortilin-depleted MEF stimulated by BMP4.

siRNA_{Fortilin}, small interfering RNA against fortilin; siRNA_{Luciferase}, small interfering RNA against luciferase (control); PBS, phosphate buffered saline; BMP4, bone morphogenic protein 4; GAPDH, glyceraldehyde-3-phosphate dehydrogenase (loading marker); *, $P < 0.05$; NS, not statistically significant.

B. Assay of *Msx2* message levels in the presence and absence of fortilin. Lanes 1–6, effective silencing of fortilin by siRNA_{Fortilin} in MEF; lanes 7–12, the suppression by fortilin of BMP4-induced *Msx2* induction in MEF; ***, $P < 0.005$; **, $P < 0.01$; *, $P < 0.05$. The lack of fortilin was associated with the induction by BMP4 of significantly larger amount of *Msx2*.

C. Cell death and DNA fragmentation assays of MEF overexpressing *Msx2*. Yellow arrow, 5'-LTR; blue arrow, internal ribosomal entry site; GFP, green fluorescent protein; Phase, photomicrograms of MEF; GFP, GFP-positive cells; white arrows, rounded and dead cells; *, $P < 0.05$; ****, $P < 0.001$. Scale bar = 10 μ m.

D. Fortilin—a novel anti-BMP molecule in early development. Blue arrow, facilitative roles; black line, inhibitory role. Fortilin prevents BMP4 from inducing *Msx2*, a proapoptotic protein. The

lack of fortilin in very early development leads to embryo loss, most likely due to the unopposed, excessive activation of BMP4 pathway and *Msx2*-induced apoptosis.

Table 1

Table 1A. Results of Hetero-Intercross at 4–6 Weeks of Age: 129-C57BL/6J mixed genetic background (Clone = 11A1)				
Sex	Fortilin^{+/+}	Fortilin^{+/-}	Fortilin^{-/-}	Total
Male	31 (28.7)	77 (71.3)	0 (0)	108 (100)
Female	32 (26.2)	90 (73.8)	0 (0)	122 (100)
Total	63 (27.4)	167 (72.6)	0 (0)	230 (100)
Table 1B. Results of Hetero-Intercross at 4–6 Weeks of Age: 129-C57BL mixed genetic background (Clone = 12F4)				
Sex	Fortilin^{+/+}	Fortilin^{+/-}	Fortilin^{-/-}	Total
Male	6 (37.5)	10 (62.5)	0 (0)	16 (100)
Female	10 (41.7)	14 (58.3)	0 (0)	24 (100)
Total	16 (40.0)	24 (60.0)	0 (0)	40 (100)
Table 1C. Results of Hetero-Intercross at 4–6 Weeks of Age: 129-C57BL mixed genetic background (Clone = 13D2)				
Sex	Fortilin^{+/+}	Fortilin^{+/-}	Fortilin^{-/-}	Total
Male	4 (22.2)	14 (77.8)	0 (0)	18 (100)
Female	8 (36.4)	14 (63.6)	0 (0)	22 (100)
Total	12 (30.0)	28 (70.0)	0 (0)	40 (100)
Table 1D. Results of Hetero-Intercross at 4–6 Weeks of Age: C57BL/6J genetic background (after 8 backcrossing, 11A1)				
Sex	Fortilin^{+/+}	Fortilin^{+/-}	Fortilin^{-/-}	Total
Male	5 (17.9)	23 (82.1)	0 (0)	28 (100)
Female	5 (29.4)	12 (70.6)	0 (0)	17 (100)
Total	10 (22.2)	35 (77.8)	0 (0)	45 (100)
Table 1E. Results of Hetero-Intercross at 4–6 Weeks of Age: 129 genetic background (Clone = 13D2)				
Sex	Fortilin^{+/+}	Fortilin^{+/-}	Fortilin^{-/-}	Total
Male	12 (54.5)	10 (45.5)	0 (0)	22 (100)
Female	8 (30.8)	18 (69.2)	0 (0)	26 (100)
Total	20 (41.7)	28 (58.3)	0 (0)	48 (100)

Table 2

Fortilin^{-/-} mouse embryos are lethal around 3.5PCD.

Age (PCD)	Methods	Fortilin ^{+/+}	Fortilin ^{+/-}	Fortilin ^{-/-}	Non- conclusive	Conclusive total
9.5	SB	13 (21)	50 (79)	0 (0)	5	63 (100)
8.5	SB	12 (25.0)	36 (75.0)	0 (0)	15	48 (100)
	PCR	3 (11.0)	25 (89.0)	0 (0)	2	28 (100)
	PCR+SB	15 (19.7)	61 (80.3)	0 (0)	2	76 (100)
7.5	N-PCR	17 (22.4)	59 (77.6)	0 (0)	10	76 (100)
6.5	N-PCR	14 (32.6)	29 (67.4)	0 (0)	8	43 (100)
3.5	N-PCR	9 (17.3)	41 (78.8)	2 (3.8)	12	52 (100)

PCD: post coitus date, Parenthesis: percentage (%), PCR: single-step polymerase chain reaction, SB: Southern blot analysis, PCR+SB: combined results from PCR and SB, N-PCR: nested PCR (see Methods for details), ND: not determined.