Betaine is accumulated via transient choline dehydrogenase activation during mouse oocyte meiotic maturation

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Taylor McClatchie[‡§1], Megan Meredith[‡§], Mariame O. Ouédraogo[‡§], Sandy Slow[¶], Michael Lever[‖], Mellissa R. W. Mann[‡‡], Steven H. Zeisel[§§], Jacquetta M. Trasler[¶¶‖‖], and Jay M. Baltz[‡§2]**

*From the [‡]Ottawa Hospital Research Institute, Ottawa, Ontario K1H 8L6, Canada, the [§]Departments of Obstetrics and Gynecology and Cellular and Molecular Medicine, University of Ottawa Faculty of Medicine, Ottawa, Ontario K1H 8M5, Canada, the [¶]Department of Pathology, University of Otago, Christchurch 8140, New Zealand, the [‖]Department of Chemistry, University of Canterbury, Christchurch 8041, New Zealand, the **Department of Obstetrics, Gynecology, and Reproductive Sciences, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15213, the [‡‡]Magee-Womens Research Institute, Pittsburgh, Pennsylvania 15213, the [§§]Department of Nutrition, Nutrition Research Institute, Gillings School of Global Public Health and School of Medicine, University of North Carolina, Chapel Hill, North Carolina 27599, the [¶¶]Montréal Children's Hospital and Research Institute of the McGill University Health Centre, Montréal, Quebec H4A 3J1, Canada, and the [‖‖]Departments of Human Genetics, Pediatrics, and Pharmacology and Therapeutics, McGill University, Montréal, Quebec H3A 1B1, Canada*

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Betaine (*N,N,N*-trimethylglycine) plays key roles in mouse eggs and preimplantation embryos first in a novel mechanism of cell volume regulation and second as a major methyl donor in blastocysts, but its origin is unknown. Here, we determined that endogenous betaine was present at low levels in germinal vesicle (GV) stage mouse oocytes before ovulation and reached high levels in the mature, ovulated egg. However, no betaine transport into oocytes was detected during meiotic maturation. Because betaine can be synthesized in mammalian cells via choline dehydrogenase (CHDH; EC 1.1.99.1), we assessed whether this enzyme was expressed and active. *Chdh* transcripts and CHDH protein were expressed in oocytes. No CHDH enzyme activity was detected in GV oocyte lysate, but CHDH became highly active during oocyte meiotic maturation. It was again inactive after fertilization. We then determined whether oocytes synthesized betaine and whether CHDH was required. Isolated maturing oocytes autonomously synthesized betaine *in vitro* in the presence of choline, whereas this failed to occur in *Chdh*$^{-/-}$ oocytes, directly demonstrating a requirement for CHDH for betaine accumulation in oocytes. Overall, betaine accumulation is a previously unsuspected physiological process during mouse oocyte meiotic maturation whose underlying mechanism is the transient activation of CHDH.

Betaine (*N,N,N*-trimethylglycine, glycine betaine) is present in mouse preimplantation embryos at very substantial intracellular concentrations (5–10 mM) that are much higher than in most other cells (1). It serves at least two established roles in embryos, first as a key contributor to cell volume regulation and second as a major source of methyl groups in the blastocyst (1–6).

Many somatic cells utilize organic osmolytes, a diverse class of benign molecules accumulated by cells to maintain normal cell volume (7). Preimplantation embryos similarly rely on organic osmolytes for cell volume regulation, although they employ entirely different mechanisms of osmolyte accumulation from those of somatic cells (8). Betaine is one of two major organic osmolytes present at high levels in early mouse embryos (3, 4, 8), with the other being glycine (9). Decreased cell volume results in arrested embryo development (6, 8–11). Betaine that is accumulated by preimplantation embryos in response to decreased cell volume prevents developmental arrest and restores normal patterns of protein synthesis (4–6, 12). Whereas somatic cells can regulate intracellular betaine to function as an organic osmolyte via BGT1 (SLC6A12 protein) or system A (SLC38A2) transporters (13, 14), early preimplantation embryos instead control betaine levels in response to cell volume perturbations via SIT1[3] (SLC6A20). SIT1-mediated betaine transport is not present in unfertilized eggs but becomes activated several hours after fertilization and persists through the 2-cell stage (3, 4).

The second well-documented role of betaine is as a methyl donor (15). The enzyme betaine-homocysteine methyltransferase (BHMT) catalyzes the transfer of a methyl group from

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[2] To whom correspondence should be addressed: Ottawa Hospital Research Institute, 501 Smyth Rd., Mail Box 411, Ottawa, Ontario K1H 8L6, Canada. Tel.: 613-737-8899 (ext. 79763); E-mail: jbaltz@ohri.ca.

[3] The abbreviations used are: SIT1, betaine transport mechanism (SLC6A20 protein); ALDH, aldehyde dehydrogenase; BHMT, betaine-homocysteine methyltransferase; CHDH, choline dehydrogenase; COC, cumulus–oocyte complex; Bt_2cAMP, dibutyryl cyclic AMP; eCG, equine chorionic gonadotropin; GV, germinal vesicle; hCG, human chorionic gonadotropin; ICM, inner cell mass of blastocyst; KSOM, potassium-supplemented simplex-optimized medium for embryo culture; mKSOM, modified KSOM; MI, first meiotic metaphase; MII, second meiotic metaphase; P, postnatal day; Q-RT-PCR, quantitative PCR after reverse transcription; mHEPES-KSOM, modified HEPES-KSOM; ANOVA, analysis of variance; MEM, minimum Eagle's medium.

betaine to homocysteine to form methionine that is then complexed with adenosine to produce *S*-adenosylmethionine, the universal methyl donor used by an array of methyltransferases (16, 17). Although methyl group generation from betaine via BHMT had been considered to be restricted to the liver in rodents (18), BHMT was unexpectedly found to be active at the blastocyst stage of mouse embryos and highly expressed in the inner cell mass (ICM) that gives rise to the fetus, implying that betaine has a role as a methyl donor specifically in this lineage (1). Suppression of BHMT activity in cultured embryos impairs their ICM development and increases fetal resorptions (1, 2). This is probably related, at least in part, to a requirement for methyl groups for establishing embryonic DNA methylation patterns following the erasure of gamete-specific DNA methylation earlier in preimplantation embryogenesis (19). Suppressing BHMT in conjunction with the parallel methyl-generating folate cycle prevented the normally observed increase in nuclear 5-methylcytosine detected in the ICM, whereas BHMT alone, even with the folate cycle inhibited, was sufficient to support the 5-methylcytosine increase (2). Thus, betaine plays important roles in the preimplantation embryo.

Although the betaine present in preimplantation embryos could potentially be supplied by SIT1-mediated transport during the 1- and 2-cell stages (3), we recently found that endogenous betaine is already present at equivalently high levels in unfertilized mouse eggs (20). Because any betaine in eggs had been accumulated before SIT1 is activated after fertilization (3), a mechanism other than SIT1 must be responsible.

Fully grown oocytes in ovarian follicles are arrested at the germinal vesicle (GV) stage in prophase I of meiosis. When ovulation is triggered, GV oocytes are released from arrest and progress through first meiotic metaphase (MI), become uncoupled from granulosa cells, and are re-arrested in second meiotic metaphase (MII) as mature eggs awaiting fertilization (21–23), a process that is collectively termed meiotic maturation. Although these nuclear changes have been extensively studied, much less is known about other physiological processes during oocyte meiotic maturation that are important for producing a mature MII egg.

As we show in the present study, GV oocytes and early to mid-MI oocytes contain lower endogenous levels of betaine in contrast to the high levels in MII eggs, demonstrating that betaine accumulation must occur during meiotic maturation. Although we recently showed that granulosa cells can take up betaine via the y^+LAT2 transporter (SLC7A6 protein) and transfer some to the enclosed GV oocyte via gap junctions (20), we show here that this is not a likely source of the increased concentration of betaine in MII eggs.

Another possible source of betaine in mature eggs is through its *de novo* synthesis. The only known mechanism for betaine synthesis in mammalian cells uses choline as the substrate in a two-step reaction where the first and rate-limiting step is catalyzed by choline dehydrogenase (CHDH; EC 1.1.99.1) (24, 25). CHDH converts choline into betaine aldehyde and is specific for betaine synthesis, which is its only known enzymatic function (24). CHDH is also reportedly required for mitophagy, a role that is independent of its enzyme activity and betaine levels (26). Betaine aldehyde produced by CHDH is then oxidized to betaine by an aldehyde dehydrogenase (ALDH; EC 1.2.1.8) identified as ALDH9A1, which accepts a broad range of aminoaldehyde substrates (27–29). CHDH is localized to mitochondria, where betaine aldehyde synthesis occurs.

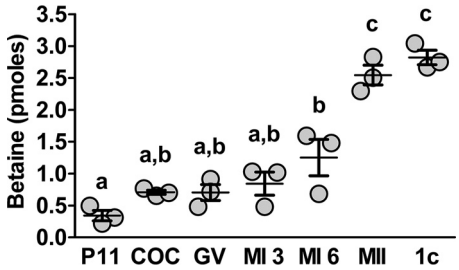

Figure 1. Endogenous betaine in oocytes. Betaine was measured by liquid chromatography tandem mass spectrometry in groups of 250 intact preantral follicles with enclosed oocytes from postnatal (P11) ovaries and groups of 50 cumulus–oocyte complexes with enclosed GV oocytes (*COC*), GV oocytes (*GV*), first meiotic metaphase (*MI*) oocytes undergoing meiotic maturation at 3 (*MI 3*) and 6 (*MI 6*) h post-hCG injection; in mature eggs (*MII*); and in 1-cell embryos (*1c*). The amount of betaine is expressed as pmol per follicle, COC, oocyte, or embryo. The measured background levels of betaine measured in the final wash have been subtracted from each paired measurement. Betaine was essentially undetectable in all paired background samples (<0.05 pmol per oocyte or embryo) except in the paired background for one MII egg sample (0.35 pmol/oocyte). Each point represents an independent repeat (*i.e.* a measurement on one group of 250 P11 follicles or 50 oocytes or embryos). *Horizontal lines*, means ± S.E. (*error bars*). Means that do not share the *same letter* are significantly different ($p < 0.001$ for *a* or *b versus c* and $p < 0.05$ for *a versus b* by ANOVA with Tukey's test).

To investigate the mechanism underlying the production of the high levels of betaine that are present in MII mouse eggs, which are important during early embryonic development, we have tested the hypotheses that CHDH is present and active in oocytes during meiotic maturation, that betaine is synthesized autonomously by oocytes, and that CHDH is required for betaine accumulation.

Results

Higher levels of betaine are present in mouse eggs than in earlier stage oocytes

Substantial betaine is present in preimplantation embryos and ovulated MII eggs of mice (1, 20), but whether betaine is present in oocytes during meiotic maturation was unknown. We measured endogenous betaine by LC-MS/MS in oocytes obtained from CF1 female mice before and during *in vivo* meiotic maturation by collecting them at the stages and times indicated (Fig. 1). Only lower levels of betaine were detectable in preantral follicles containing growing oocytes from postnatal day 11 (P11) ovaries or in fully grown GV oocytes from adult antral follicles. Cumulus–oocyte complexes (COCs) isolated from antral follicles did not contain more betaine than GV oocytes alone, implying that there is no significant reservoir of betaine in cumulus cells. Betaine levels had not increased in MI oocytes at 3 h after ovulation was induced with human chorionic gonadotropin (hCG), and only a small increase (that did not reach significance) was seen in MI oocytes at 6 h. However, significantly higher levels of betaine were present in MII eggs and 1-cell embryos. Thus, betaine is accumulated during meiotic maturation *in vivo*.

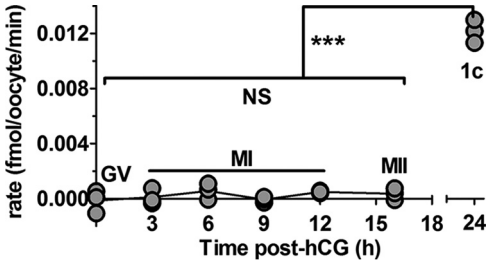

Figure 2. Betaine transport during meiotic maturation. The transport of [^{3}H]-betaine was measured in GV oocytes, oocytes during meiotic maturation through first meiotic metaphase (*MI*, 3–12 h post-hCG), mature eggs (*MII*, 16 h post-hCG), and one-cell embryos (*1c*) that were developed *in vivo* and collected at the times indicated after ovulation had been induced by hCG. Oocytes, eggs, or embryos were incubated with 1 μM [^{3}H]betaine for 30 min, and the rate of transport was expressed as total betaine (fmol) per oocyte, egg, or embryo min^{-1}. No betaine transport was detectable in oocytes or eggs. As expected, substantial betaine transport was found in 1-cell embryos where the SIT1 transporter is active. Each *symbol* represents the rate of transport for one of three independent repeats. There was no significant difference between mean rates for oocytes at each stage or eggs (*NS within bracket*), whereas the mean rate for 1-cell embryos was significantly different (***, $p <$ 0.001 by ANOVA with Tukey's test).

Betaine is not transported during meiotic maturation

Although we previously showed that there was no detectable betaine transport into GV oocytes, MII eggs, or MI oocytes at 4 h after induction of ovulation (3), it was possible that betaine transport could be transiently activated later in MI, which ends 10–12 h after ovulation is triggered in mice (30). Therefore, we measured the transport of [^{3}H]betaine in GV oocytes; in MI oocytes collected during *in vivo* meiotic maturation at 3, 6, 9, and 12 h after ovulation was induced with hCG; and in MII eggs collected at 16 h post-hCG. No saturable betaine transport was detected in oocytes during meiotic maturation (Fig. 2). This implies that betaine transport by oocytes during meiotic maturation did not account for the high levels of betaine in MII eggs.

Choline dehydrogenase is expressed in oocytes

Because betaine was accumulated in oocytes during meiotic maturation despite the apparent absence of betaine transport, we next examined whether betaine is synthesized in oocytes during meiotic maturation, which is assumed to require CHDH. *Chdh* mRNA was present at each oocyte stage tested (Fig. 3*A*), including growing oocytes, GV oocytes, and 1-cell embryos (fertilized eggs). The identity of representative *Chdh* cDNA bands was confirmed by sequencing (not shown). Q-RT-PCR (Fig. 3*A*) showed the highest numbers of *Chdh* transcripts in P11 oocytes, which are ~70% of the diameter of fully grown oocytes (31), and in fully grown oocytes from P21 ovaries. Fully grown GV oocytes from adult females also had a high level of transcripts, which persisted in the 1-cell embryo (fertilized egg) but not in subsequent preimplantation embryo stages. COCs contained an amount of *Chdh* transcript essentially equal to that in GV oocytes, implying that the transcript is restricted to the oocyte rather than cumulus cells. The expected expression patterns of control housekeeping genes *H2afz* and *Ppia* (32) were confirmed in these samples (Fig. 3*B*).

CHDH protein was detected by Western blot analysis as a single band near the predicted size of 66 kDa (UniProtKB Q8BJ64) in GV oocytes and MII eggs as well as in all stages of preimplantation embryo analyzed (Fig. 3*C*). The band detected in oocytes and embryos was at the same position as the single band in *Chdh*$^{+/+}$ mouse kidney lysate, which was absent from *Chdh*$^{-/-}$ kidney lysate (Fig. 3*C*). We further confirmed the identity of the band detected in oocytes as CHDH using GV oocytes obtained from *Chdh*$^{+/+}$, *Chdh*$^{+/-}$, and *Chdh*$^{-/-}$ ovaries (Fig. 3*D*).

CHDH is transiently active in oocytes during meiotic maturation

To determine whether CHDH was active in oocytes, we adapted and validated a CHDH enzyme activity assay (24, 33) for use with small samples. The modified assay exhibited increasing activity with increasing kidney inner medulla protein until saturation (Fig. 4*A*). The CHDH inhibitor 3,3-dimethylbutanol completely blocked activity above background, where background was determined as the counts/min obtained when reaction mix to which no kidney lysate had been added was processed. After subtracting paired 3,3-dimethylbutanol controls, the assay was linear up to ~0.15 mg of total kidney protein (Fig. 4*B*). Using a saturating amount of kidney protein (0.9 mg), CHDH activity was detected in kidney lysate but not above the background level in negative controls consisting of the same amount of boiled kidney lysate, lung lysate (which has <1% of the activity of kidney) (24), or BSA instead of tissue lysate (Fig. 4*C*).

We then assessed whether CHDH activity was present in oocyte and embryo lysates (100 oocytes or embryos pooled). CHDH activity began to increase in oocytes as meiotic maturation progressed *in vivo* with maximal activity present in MI and MII oocytes from 3–16 h after meiotic maturation was induced with hCG (Fig. 5*A*). CHDH activity then decreased after fertilization with only low levels of CHDH activity remaining in 1-cell embryos through blastocysts. The period of maximal CHDH activity coincided with the period during which endogenous betaine was accumulated (Fig. 5*B*). A similar activation was evident with GV oocytes removed from the ovary and allowed to undergo spontaneous meiotic maturation *in vitro* (Fig. 5*C*). Intact COCs exhibited the same lack of CHDH activity as denuded GV oocytes (109 ± 178 cpm for COCs *versus* 67 ± 69 for GVs, mean ± S.E., $n = 3$, $p = 0.83$ by t test), indicating that cumulus cells do not possess CHDH activity and thus are not likely to be a site of betaine synthesis from choline.

To confirm that the measured activity in lysate from maturing oocytes was indeed due to CHDH, we used MI oocytes obtained 9 h after meiotic maturation had been triggered *in vivo* with hCG. As predicted, activity was inhibited by either 3,3-dimethylbutanol or betaine aldehyde added to the lysate, and the inhibition was to the same extent as when the lysate had been boiled (Fig. 5*D*).

The CHDH activity measured in oocytes is comparatively high. Kidney inner medulla, along with liver, has the highest CHDH activity reported in tissues assessed previously (24). Kidney inner medulla lysate activity in our assay was ~7.6×10^3 cpm/mg of total protein (calculated from the slope in Fig. 4*B*). For oocytes, peak measured activity was ~800 cpm/100 oocytes (Fig. 5). Because total protein is ~3×10^{-5} mg/oocyte (34), this corresponds to ~2.7×10^5 cpm/mg, or about 35-fold higher specific activity in oocytes compared with kidney inner

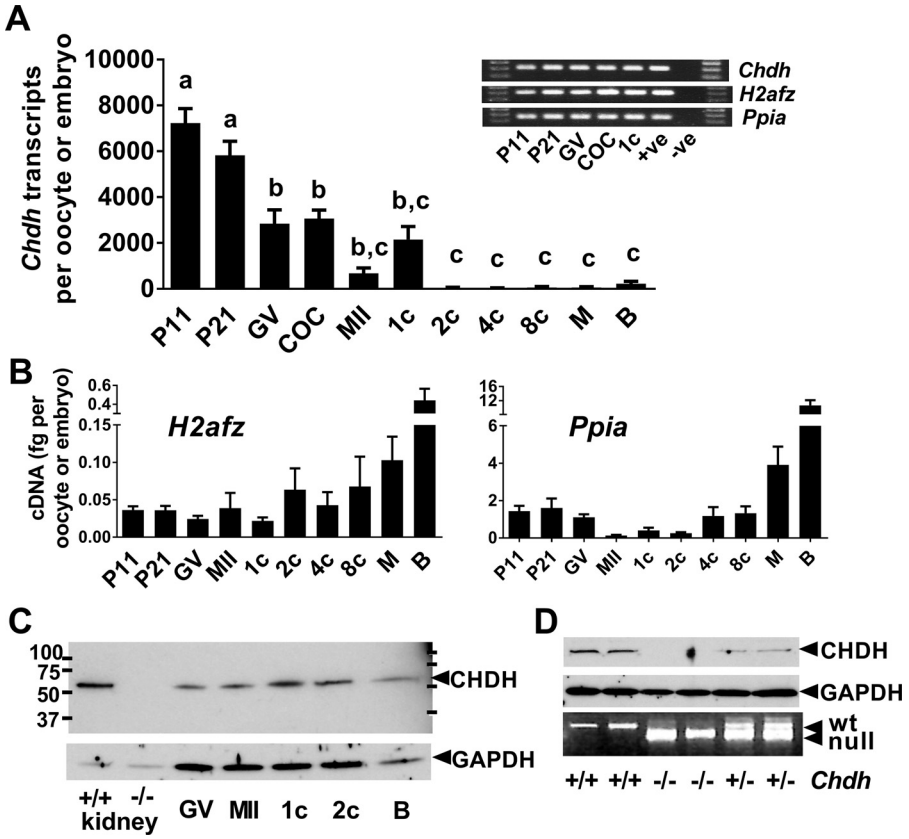

Figure 3. Expression of choline dehydrogenase. *A*, *Chdh* transcripts were detectable by conventional RT-PCR (*top right*, 40 cycles) in growing oocytes from P11 ovaries, fully grown oocytes from P21, GV oocytes from adult females, COCs containing GV oocytes, and 1-cell embryos. Kidney served as a positive control (+*ve*), whereas water added to reaction mix was a negative (−*ve*). Transcripts for *H2afz* and *Ppia* controls were detected as expected. The gels shown represent one example of three similar repeats. Markers visible in the *left* and *right lanes* are 100, 200, and 300 bp. Quantitative RT-PCR (*bottom left*) confirmed the presence of *Chdh* in growing (P11) oocytes and fully grown (P21) oocytes from neonatal ovaries, GV oocytes, and 1-cell embryos, but not in 2-cell (*2c*), 4-cell (*4c*), 8-cell (*8c*), morula (*M*), or blastocyst (*B*) stage embryos. *Bars*, mean ± S.E. (*error bars*) of five independent repeats, except 10 repeats for GV oocytes. *Bars* that do not share the *same letter* are significantly different ($p < 0.001$ for *a* or *b versus c* and $p < 0.05$ for *a versus b* by ANOVA with Tukey's test). A fixed amount (1 pg) of *Gfp* cRNA was added to four sets of selected samples, and Q-RT-PCR was performed to assess the efficiency of recovery. Mean recovery was similar for each repeat and ranged from 79 to 84% (not shown). Thus, the transcript levels reported here probably represent ~80–85% of the actual levels at each stage. *B*, Q-RT-PCR was performed to detect *H2afz* and *Ppia* as controls. The relative expression levels are essentially as reported previously (32). Transcript numbers were not calculated for these, and the results are expressed in fg of amplicon. *C*, Western blotting of GV oocytes, MII eggs, and 1-cell (*1c*), 2-cell (*2c*), and blastocyst (*B*) stage embryos showing CHDH (*top*) and GAPDH as a loading control (*bottom*). Fifty oocytes or embryos were loaded in each lane. Kidney lysate (250 ng of total protein) from $Chdh^{+/+}$ and $Chdh^{-/-}$ neonates demonstrates antibody specificity. Positions of molecular weight markers are indicated and *labeled* at the *left*. The example shown is representative of three independent repeats. *D*, Western blotting of GV oocytes (60 oocytes/lane) isolated from $Chdh^{+/+}$, $Chdh^{+/-}$, and $Chdh^{-/-}$ P21 neonatal ovaries. The oocytes were cultured for 6 h post-isolation in MEMα, at which point maximal CHDH activity would have developed. CHDH (*top*) and GAPDH loading controls (*middle*) are shown, with genotyping by PCR (*bottom*). The positions of the bands were essentially identical to those shown in *C*, and therefore only the immediate region of the gel is shown. One of three similar independent repeats is shown.

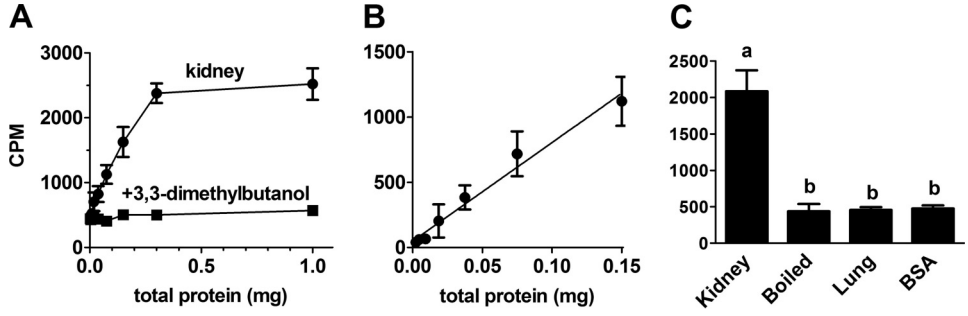

Figure 4. Choline dehydrogenase enzyme activity assay development. *A*, kidney inner medulla lysate was used to validate the choline dehydrogenase activity assay. [^{3}H]Betaine production from [^{3}H]choline was measured and expressed as counts/min of ^{3}H eluted from the column. Activity increased with increasing total kidney lysate protein and reached a plateau, whereas 3,3-dimethylbutanol (10 mM) completely eliminated measured activity above the background seen with no lysate added (at 0 mg). Each point represents the mean ± S.E. (*error bars*) of three independent repeats. *B*, background in the presence of 3,3-dimethylbutanol was subtracted from total activity (data from *A*) and fit by linear regression for the linear range ($R^2 = 0.83$). *C*, activity measured in kidney inner medulla lysate (*Kidney*, 0.9 mg total protein) compared with boiled kidney lysate or lung lysate or when only BSA rather than lysate was added (each at 0.9 mg). *Bars*, mean ± S.E. of three independent repeats. *Bars* that do not share the *same letter* are significantly different ($p < 0.001$ by ANOVA with Tukey's test).

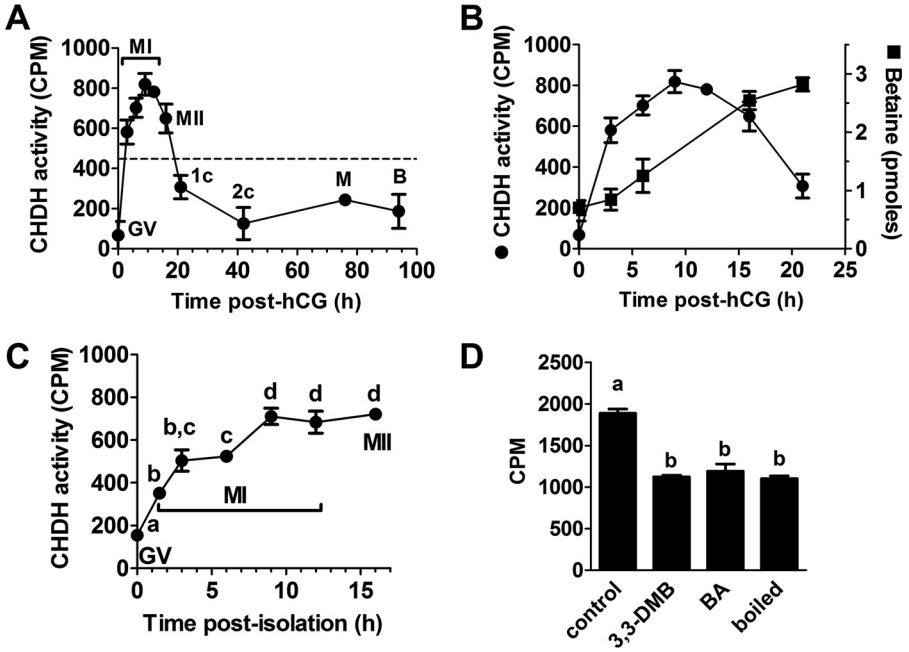

Figure 5. Choline dehydrogenase enzyme activity in mouse oocytes and preimplantation embryos. *A*, choline dehydrogenase activity measured in *in vivo*-developed oocytes and preimplantation embryos as a function of time post-hCG. Activity in GV oocytes was not significantly different from 0 ($p = 0.43$, one-sample *t* test). Activity peaked during meiotic maturation in maturing oocytes (*MI*) and eggs (*MII*). Significantly lower activity was present after fertilization in 1-cell (*1c*), 2-cell (*2c*), morula (*M*), and blastocyst (*B*) stage embryos. Each point represents the mean ± S.E. (*error bars*) of three independent repeats. Points lying *above* the *dashed line* are significantly different from those *below* ($p < 0.05$ by ANOVA with Tukey's test). *B*, the subset of choline dehydrogenase activity data (*circles*) for oocytes, eggs, and 1-cell embryos in *A* is replotted with the endogenous betaine content (*squares*, from Fig. 1), to show the temporal relationship between choline dehydrogenase activity and accumulation of betaine *in vivo*. *C*, choline dehydrogenase activity measured in oocytes undergoing spontaneous meiotic maturation *in vitro* after removal from antral follicles. Each point represents the mean ± S.E. of three independent repeats. Points that do not share the *same letter* are significantly different ($p < 0.05$ by ANOVA with Tukey's test). Stages are indicated as in *A*. *D*, choline dehydrogenase activity measured in MI oocytes isolated from the ovaries at 9 h post-hCG was equally inhibited by 3,3-dimethylbutanol (*3,3-DMB*; 10 mM) or betaine aldehyde (*BA*; 5 mM) or when the oocyte sample was boiled. *Bars*, mean ± S.E. of three independent repeats. *Bars* that do not share the *same letter* are significantly different ($p < 0.001$ by ANOVA with Tukey's test). For *A–C*, background in the presence of 10 mM 3,3-dimethylbutanol has been subtracted. Total counts/min are shown in *D*.

medulla. Thus, the mouse oocyte's capacity to produce betaine during meiotic maturation is substantial.

Development of CHDH activity requires meiotic maturation and global protein synthesis

To determine whether development of CHDH activity in oocytes depends on their meiotic progression, *in vitro* meiotic maturation was prevented using Bt_2cAMP (300 μM). Maintaining meiotic arrest essentially prevented the development of CHDH activity in oocytes (Fig. 6*A*). Although oocytes are transcriptionally silent during meiotic maturation, many proteins are synthesized from stored mRNA (35). To determine whether translation in general was required for CHDH activation, GV oocytes were cultured for 3 h in the presence of cycloheximide at a level (50 μM) that we have previously shown was sufficient to nearly completely inhibit global protein synthesis in oocytes (36). When global protein synthesis was inhibited, choline dehydrogenase enzyme activity was essentially abolished (Fig. 6*B*). To determine whether CHDH levels had been affected, GV oocytes were similarly treated with cycloheximide for 3 h and then analyzed by Western blotting. However, CHDH protein was only slightly decreased after the identical time with cycloheximide (Fig. 6*C*). Furthermore, the amount of CHDH protein did not appear to increase from GV oocytes, where there is no detectable CHDH activity, to MI oocytes at 6 h post-hCG, where there is maximal CHDH activity (Fig. 6*D*). There also did not appear to be more CHDH protein in MII eggs than GV oocytes (Fig. 6*E*), despite the high activity in MII eggs compared with the lack of measurable activity in GV oocytes (Fig. 5). Thus, increased CHDH activity, while requiring global protein synthesis, was apparently not mediated by *de novo* synthesis of CHDH itself.

Choline is converted to betaine via CHDH in isolated oocytes during meiotic maturation

The results reported above, including the presence of active CHDH and its correlation with the appearance of betaine during oocyte maturation *in vivo*, implied that the increase in betaine could be due to choline conversion to betaine by CHDH. However, the possibility that betaine was instead taken up by maturing oocytes via a mechanism that we failed to detect could not be ruled out. To directly demonstrate that oocytes were capable of producing betaine during meiotic maturation, we tested whether the amount of betaine in isolated oocytes would increase if they were matured *in vitro* with no betaine present in the medium. Isolated GV oocytes were cultured for 18 h in modified KSOM (mKSOM) medium in the presence of choline (0.5 mM), and the amount of betaine in the resulting MII eggs was then determined by LC-MS/MS. This revealed that MII eggs that had matured *in vitro* in the presence of choline contained significantly more betaine than freshly isolated GV oocytes or oocytes matured *in vitro* in the absence of choline (Fig. 7*A*), indicating that choline was converted to betaine within maturing oocytes.

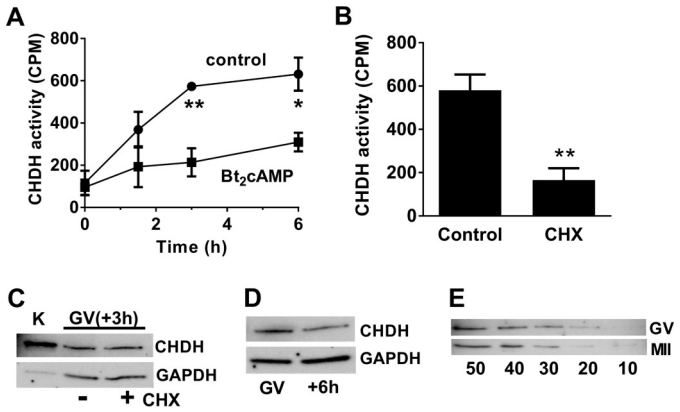

Figure 6. Dependence of choline dehydrogenase activity development on meiotic progression and protein synthesis. *A*, choline dehydrogenase activity was measured as a function of time following the removal of GV oocytes from antral follicles, either undergoing spontaneous meiotic maturation *in vitro* (control) or maintained in GV arrest with Bt_2cAMP (300 μM). Both the effects of Bt_2cAMP and that of time are significant by two-way ANOVA ($p = 0.0002$), with Bt_2cAMP inhibiting the development of choline dehydrogenase activity. *, $p < 0.05$; **, $p < 0.01$ for effect of Bt_2cAMP at the indicated time points by Bonferroni post hoc test. *B*, inhibition of protein synthesis with cycloheximide (*CHX*; 50 μM) significantly inhibits the development of choline dehydrogenase activity in oocytes measured at 3 h after removal from the follicle (**, $p = 0.01$ by *t* test). In *A* and *B*, each *symbol* or *bar* represents the mean ± S.E. (*error bars*) of three independent repeats. *C*, CHDH was assessed by Western blotting in GV oocytes that had been cultured for 3 h (*GV(+3h)*), when maximal choline dehydrogenase activity has developed, in the absence (−) or presence (+) of cycloheximide. Mouse kidney lysate (*K*) was run to indicate the position of the CHDH band. GAPDH served as a loading control. The example shown is representative of two independent repeats. *D*, CHDH protein did not increase at 6 h post-hCG relative to GV oocytes. The example shown is representative of three independent repeats. *E*, CHDH protein was not higher in MII eggs than GV oocytes. *Lanes* contained the lysate of the number of oocytes or MII eggs indicated at the *bottom*. The example shown is representative of three independent repeats. In *C–E*, the positions of the bands relative to molecular weight markers (not shown) are essentially identical to those shown in Fig. 3*C*.

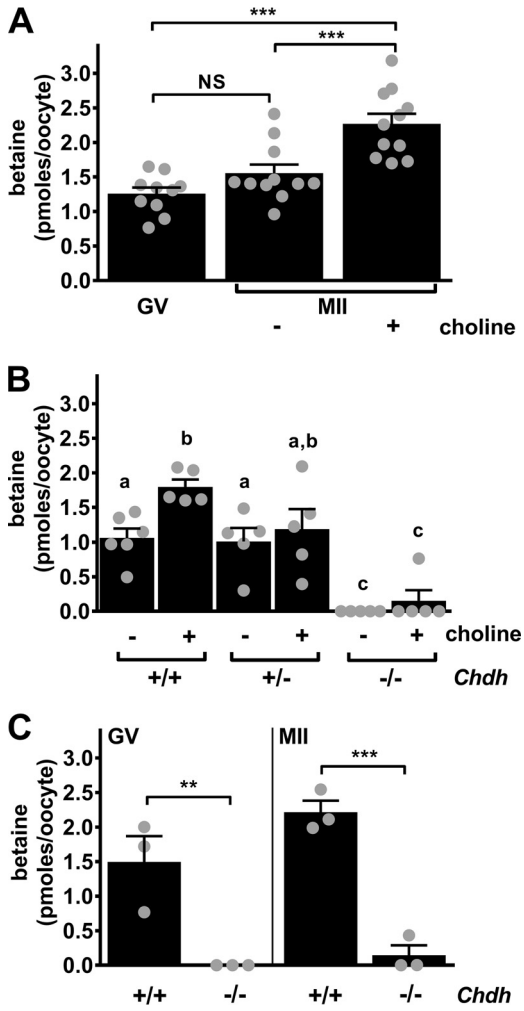

Figure 7. Dependence of betaine accumulation in oocytes on choline and *Chdh*. *A*, GV oocytes were isolated immediately (*GV*) for analysis or cultured overnight to mature to MII eggs in the presence (+) or absence (−) of choline (0.5 mM) in mKSOM medium and then processed for betaine measurement by LC-MS/MS. Each point represents the measured betaine of a group of 25–30 oocytes or eggs, expressed as pmol/oocyte or egg. *Bars* indicate means ± S.E. (*error bars*) ($n = 10$ for GV, $n = 11$ for MII); *symbols* indicate individual measurements. Means that do not share the *same letter* are significantly different ($p < 0.01$ by ANOVA with Tukey's test). Eggs cultured in the presence of choline contained significantly higher betaine than GV oocytes, indicating that betaine was synthesized during meiotic maturation. *B*, GV oocytes from $Chdh^{+/+}$, $Chdh^{+/-}$, and $Chdh^{-/-}$ females were *in vitro*-matured to MII eggs in the presence (+) or absence (−) of choline and then were processed as in *A*. Indistinguishable levels of betaine were present in wild-type (+/+) and heterozygous (+/−) GV oocytes, whereas betaine was not detected in null (−/−) oocytes. Only wild-type oocytes accumulated betaine during meiotic maturation, whereas there was no significant increase in betaine in null or heterozygous oocytes. Means that do not share the *same letter* are significantly different ($p < 0.05$ by ANOVA with Tukey's test). *C*, the amount of betaine present in GV oocytes and MII eggs that developed *in vivo* in $Chdh^{+/+}$ or $Chdh^{-/-}$ females was determined. GV oocytes and MII eggs from wild-type females contained significantly higher levels of betaine than those from null females (**, $p < 0.01$; ***, $p < 0.001$ by ANOVA with Bonferroni test). Because of the difficulty of obtaining ovulated eggs from these females, only a limited data set was obtained ($n = 3$ pools of 30 oocytes or eggs for each genotype), and the analysis was restricted to comparing $Chdh^{+/+}$ with $Chdh^{-/-}$.

To confirm that CHDH was required for the increase in betaine in oocytes, we utilized oocytes lacking CHDH. Because *de novo* synthesis of CHDH during meiotic maturation did not appear to be required (above), and thus an antisense knockdown approach was not feasible, we used oocytes lacking the *Chdh* gene. GV oocytes were isolated from $Chdh^{+/+}$, $Chdh^{+/-}$, and $Chdh^{-/-}$ females to determine whether betaine was produced in these oocytes when they were matured to MII eggs *in vitro* in the presence *versus* absence of choline. Betaine levels were higher in oocytes from $Chdh^{+/+}$ females matured to MII eggs with choline as expected. In contrast, little or no detectable betaine was present in MII eggs from $Chdh^{-/-}$ oocytes matured to MII eggs either in the presence or absence of choline (Fig. 7*B*). The level of betaine in heterozygous GV oocytes from $Chdh^{+/-}$ females was indistinguishable from that in wild-type oocytes. However, heterozygous oocytes did not significantly accumulate betaine during meiotic maturation.

The lack of detectable betaine in MII eggs from $Chdh^{-/-}$ females that had been matured *in vitro* implied that GV oocytes from null females would lack even the lower level of betaine in wild-type GV oocytes. We therefore measured endogenous betaine in GV oocytes from $Chdh^{+/+}$ and $Chdh^{-/-}$ females. No betaine was present in GV oocytes from $Chdh^{-/-}$ females (Fig. 7*C*). When oocyte maturation was induced *in vivo* with hCG, betaine was found in mature MII eggs isolated ~16 h post-hCG from $Chdh^{+/+}$ females as expected, but little or no betaine was detected in $Chdh^{-/-}$ eggs (Fig. 7*C*). These results indicate that CDHD is essential for any betaine to be present in oocytes.

Discussion

Our results indicate that the substantial quantity of betaine accumulated in oocytes during meiotic maturation and then used by the early embryo is synthesized from choline by CHDH within the oocyte. The developmental window of CHDH activity during oocyte maturation coincided with endogenous betaine accumulation in oocytes *in vivo*, implicating CHDH-mediated betaine synthesis as the likely mechanism. This was directly confirmed by the detection of increased betaine levels in GV oocytes that had matured *in vitro* to MII eggs in the presence of choline. Because these isolated oocytes were cultured in the absence of other cells or sources of betaine, the increased betaine must have been produced by oocytes themselves, establishing that the production of betaine is autonomous to maturing oocytes. The failure of *Chdh*$^{-/-}$ oocytes to produce betaine when similarly cultured with choline points to an essential role for CHDH in betaine accumulation in oocytes.

Choline is actively taken up by mouse oocytes (21, 37). The dominant route of choline transport into GV oocytes is through uptake into cumulus granulosa cells followed by transfer into the enclosed oocyte, which continues during maturation until ~6 h post-hCG, when the cumulus and oocyte become uncoupled (21, 22). The minor component due to transport by the oocyte itself then becomes the sole route of choline uptake in oocytes (21), which persists through the end of maturation (38). We propose that the robust choline transport in COCs and the continued uptake of choline by oocytes after uncoupling serve to supply the maturing oocyte with the choline that is used for betaine synthesis.

We found that betaine is present in GV oocytes even before CHDH is activated, although at a lower level than in MII eggs. Furthermore, because GV oocytes from *Chdh*$^{-/-}$ females lacked detectable betaine, the betaine present in wild-type GV oocytes must have been synthesized by CHDH outside of oocytes and delivered to the follicle via the circulation. Circulating betaine is probably derived primarily from CHDH-mediated synthesis in tissues, mainly liver (25, 39, 40), except when dietary betaine is substantially supplemented (41). Thus, we propose that the betaine already present in GV oocytes is primarily synthesized elsewhere by CHDH before being taken up by cumulus cells via the SLC7A6/y$^+$LAT2 transporter and transferred to the cumulus-enclosed oocyte via gap junctions (20). This can continue until the oocyte and cumulus granulosa cells become uncoupled during meiotic maturation (21, 22). Further investigations, to determine whether betaine could be restored to GV oocytes in *Chdh*$^{-/-}$ females by dietary betaine supplementation, would be needed to test this hypothesis.

The mechanism by which CHDH is activated during meiotic maturation of the oocyte is not known. Meiotic progression, which depends on activation of M-phase-promoting factor, is required, because CHDH activity did not develop in oocytes maintained in GV arrest. ERK-type MAPK is also activated during meiotic maturation (43). This activity, in addition to mediating the physiological arrest of the mature egg in metaphase II, also acts during meiotic maturation to regulate meiotic spindle integrity, oocyte cytoskeletal dynamics, and transporter activity in oocytes (44–46). The possibility that MAPK might also regulate CHDH remains to be investigated.

Protein synthesis is also required, although relatively equivalent levels of CHDH protein in GV oocytes and MII eggs and the lack of a substantial effect of inhibiting protein synthesis on CHDH protein levels are inconsistent with the hypothesis that CHDH itself is synthesized during meiotic maturation. The effect of general protein synthesis inhibition might instead indicate a requirement for synthesis of a regulatory protein. However, very little is known about the regulation of CHDH enzyme activity, at least in part because active mammalian CHDH has not been successfully purified or produced (47), and so there are no established candidates for this role. Alternatively, it could reflect a secondary effect through general inhibition of meiotic progression, because inhibition of protein synthesis blocks meiosis at some point after chromosome condensation but before MII (48). Regulation through betaine aldehyde dehydrogenase activity is unlikely, because this is mediated by a broad-spectrum aldehyde dehydrogenase. Finally, choline transport into mitochondria, which is mediated by specific transporters (49), could control betaine production by mitochondrial CHDH. Further investigations will be required to determine how CHDH is regulated during meiosis.

Thus, substantial betaine accumulation is a previously unsuspected feature of meiotic maturation that is mediated principally by the transient activation of betaine synthesis via CHDH. Based on our present results and previous reports (3, 4, 20), it appears that betaine accumulation in mouse eggs and preimplantation embryos, where it functions both in cell volume regulation and as a methyl source, is subject to complex developmental regulation. First, the lower amount of betaine that is already present in GV oocytes is probably contributed by transport into cumulus granulosa cells via the SLC7A6/y$^+$LAT2 transporter and its subsequent transfer into the enclosed oocytes via gap junctions (20). Second, the major portion of intracellular betaine present in MII eggs is due to its synthesis from choline that is catalyzed by a transient activation of CHDH during meiotic maturation and inactivated after fertilization. Third, following fertilization, betaine levels are likely to be additionally regulated in response to cell volume perturbations by the SIT1 transporter in 1- and 2-cell stage embryos (3), and the betaine is then stored until it is metabolized for its methyl groups by newly synthesized BHMT in blastocysts, the stage at which intracellular betaine returns to low levels (1, 2).

Despite these developmentally choreographed mechanisms for betaine accumulation and regulation in oocytes and preimplantation embryos, *Chdh* null females are fertile (25), indicating that the activation of CHDH during meiotic maturation is not an absolute requirement for producing a fertilizable egg or for subsequent development. We speculate that this is a consequence of parallel mechanisms for cell volume homeostasis and methyl pool synthesis. In the absence of available betaine, its functions are probably backed up by other mechanisms, including glycine that acts as a major organic osmolyte in eggs and early preimplantation embryos (9, 36) and the contribution of the folate cycle to the methyl pool in embryos (2). Consistent with this interpretation, severe phenotypes were revealed when the betaine-dependent mechanisms were perturbed under *in*

vitro conditions where the putative parallel mechanisms were not functional (2, 5). Further work will also be required to determine whether a lack of CHDH-mediated betaine accumulation in the egg *in vivo* affects its ability to survive stress or impairs the long-term health of the embryo. Nevertheless, our current results indicate that developmentally regulated betaine synthesis from choline via CHDH is the normal physiological source of most betaine in mouse eggs and preimplantation embryos, where it plays key roles in the embryo's unique embryonic mechanisms of cell volume control and in the synthesis of the cellular methyl pool.

Experimental procedures

Chemicals and media

Chemicals were obtained from Sigma-Aldrich unless otherwise stated. [*methyl*-^{3}H]Betaine (2960–3145 GBq/mmol; catalogue no. ARC1903) was purchased from American Radiolabeled Chemicals (St. Louis, MO). [*methyl*-^{3}H]Choline (2220–3330 GBq/mmol; catalogue no. NET109001MC) was purchased from PerkinElmer Life Sciences. mKSOM and HEPES-KSOM (mHEPES-KSOM, pH 7.4) embryo culture media (50) were used in which glutamine was omitted and polyvinyl alcohol (1 mg/ml; M_r 30,000–70,000) was substituted for bovine serum albumin. Where specified, oocytes were cultured for up to 16 h in MEMα culture medium (Life Technologies/Gibco (Burlington, Canada), catalogue no. 12561) to which polyvinyl alcohol (1 mg/ml) was added.

Animals

All animal protocols were approved by the Animal Care Committee of the Ottawa Hospital Research Institute or the University of Ottawa Faculty of Medicine and followed the guidelines of the Canadian Council on Animal Care. CF1 strain females (Charles River Canada, St-Constant, Canada) 5–6 weeks of age were used except where otherwise specified. Neonatal CF1 females from which growing oocytes were isolated were obtained from litters with lactating dam (Charles River). BDF1 (B6D2F1) strain males (Charles River) were used for mating of CF1 females to obtain embryos. *Chdh* null mice on a C57Bl/6 background have been described previously (25). $Chdh^{+/+}$ offspring were obtained here from $Chdh^{+/+} \times Chdh^{+/+}$ or $Chdh^{+/-} \times Chdh^{+/-}$ (female × male) matings, and $Chdh^{+/-}$ and $Chdh^{-/-}$ offspring were obtained from $Chdh^{-/-} \times Chdh^{+/-}$ or $Chdh^{+/-} \times Chdh^{+/-}$ matings. Oocytes, eggs, and kidneys were obtained from females at 3–4 weeks of age. Some genotyping of *Chdh* wild-type, heterozygous, and null C57Bl/6 mice was done by multiplex PCR using primers described previously (25) that yield amplicons of 2.4 kb for wild type and 1.6 kb for null. For most genotyping, a set of primers designed to produce smaller amplicons was used, where the forward (sense) primer for wild type was 5′-GTGTGAGGCTGTCACGAGAA-3′ and that for null was 5′-ACGCGTCACCTTAATATGC-3′ (within the neomycin resistance cassette). A common reverse (antisense) primer was used for both: 5′-AGCCAAGAGTCGGGGTATCT-3′. These yielded amplicons of 525 bp for wild type and 478 bp for nulls. The identity of the amplicons was confirmed by direct sequencing (Ontario Genomics Innovation Centre, Ottawa, Canada) (not shown).

Adult mice were maintained on Teklad Global 18% Protein Rodent Diet 2018 (Envigo, Indianapolis, IN). Lactating females were maintained on Teklad Global 19% Protein Extruded Rodent Diet 2019 (Envigo). Both chows include choline supplementation (1200 mg/kg) but do not contain added betaine. Neonates were also provided with DietGel Recovery Purified Dietary Supplement (ClearH_2O, Portland, ME) immediately after weaning as needed to support weight gain. All animals had free access to food and water.

Oocyte, cumulus–oocyte complex, and embryo isolation

Isolation of oocytes, eggs, and embryos was carried out in mHEPES-KSOM. For GV oocytes or COCs, adult CF1 females were primed with equine chorionic gonadotropin (eCG; Merck Folligon, Intervet, Kirkland, Canada; 5 IU i.p.), and the ovaries were removed and minced ~44 h post-injection, releasing COCs. GV oocytes were isolated from surrounding cumulus cells by repeatedly pipetting through a narrow-bore pipette. Growing oocytes and follicles were obtained from neonatal females on P11 (yielding oocytes ~45–50 μm in diameter) and fully grown neonatal oocytes (~70–75 μm) on P21 as described previously (31).

To obtain *in vivo*-maturing MI oocytes, mature MII eggs, or preimplantation embryos, adult females were injected with hCG (Merck Chorulon, Intervet; 5 IU, i.p.) 47 h post-eCG. *In vivo*-maturing oocytes were obtained from ovaries at specified times up to 12 h post-hCG. Ovulated MII eggs were flushed from oviducts at ~16 h post-hCG. For embryos, CF1 females were caged overnight with BDF1 males beginning immediately after hCG injection. One-, 2-, 4-, and 8-cell embryos were flushed from oviducts at ~22, 42, 56, and 66 h post-hCG, respectively. Morulae were flushed from the utero-tubal junction at ~76 h. Blastocysts were flushed from uteri at ~93 h. For MII eggs and 1-cell embryos, a brief exposure to mHEPES-KSOM supplemented with 300 mg/ml hyaluronidase was used to remove the adherent cumulus matrix.

To obtain *in vitro*-matured oocytes, GV oocytes were cultured in MEMα (or mKSOM where stated) for up to 16 h, as specified. Where maintenance of meiotic arrest was required, GV oocytes were cultured in medium supplemented with Bt_2cAMP (300 μM). For experiments in which translation was inhibited, cycloheximide was present at a concentration (50 μg/ml in MEMα) previously shown to inhibit protein synthesis by >85% (36).

Betaine measurements

For measurement of endogenous betaine in CF1-derived oocytes during *in vivo* oogenesis, three independent sets of 50 COCs, GVs, maturing oocytes (3, 6, and 16 h post-hCG), and 1-cell embryos as well as three sets of 250 follicles from P11 neonates were collected and processed exactly as described previously (1, 20). For GV oocytes and *in vivo*-matured MII eggs from $Chdh^{+/+}$ or $Chdh^{-/-}$ females, three independent sets of 30 were collected. For measurement of betaine content after *in vitro* maturation with or without choline, 5–11 independent sets (as specified) of 30 freshly obtained GV oocytes were pro-

cessed immediately for betaine measurement or cultured in mKSOM for 12 h in the presence or absence of choline (0.5 mM), and the amount of betaine was then measured in the *in vitro*-matured MII eggs.

Betaine was measured in coded samples by LC-MS/MS (51). Measurements done at the Canterbury Health Laboratories (Christchurch, New Zealand) (reported in Fig. 1) used an Agilent 1290 high-performance liquid chromatograph (0.3-ml/min flow rate) with an Agilent 6490 Triple Quadrupole mass spectrometer (Agilent Technologies). Separation was by a Cogent 100 mm × 2.1-mm, 4-μm Diamond Hydride silica column (Microsolv Technologies). The mobile phase gradient went from 50% line A (1:1 distilled water/acetonitrile with 10 mmol/liter each ammonium formate and formic acid) and 50% line B (1:9) to 100% line A over 8 min. Measurements done at the Pharmacokinetics Laboratory of the Clinical Investigations Unit, Ottawa Hospital (reported in Fig. 7) used a Thermo Accela HPLC system (0.4-ml/min flow rate) with a Thermo TSQ Quantum Access Max Triple Quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, MA). Separation was by a Thermo Accucore HILIC column (100 × 2.1 mm, 2.6 μm). The mobile phase was line A (0.1% formic acid) and line B (acetonitrile + 0.1% formic acid) with a gradient of 10–90% B in 2 min and back to 10% B in 0.5 min and then holding at 10% B for another 2 min, for a total run of 4.5 min.

Samples were resuspended in 1 ml of 10% methanol, 90% acetonitrile with deuterated d_9-betaine (10 μmol/liter) internal standard. The injection volume was 10 μl, and the oven temperature was 40 °C. Betaine was detected in positive ion mode by electrospray ion source with a mass transition 118 → 59 (127 → 68 for d_9-betaine). The betaine content of an equal volume of final wash drops was also determined to ensure that there was no contamination.

Betaine transport measurements

Betaine transport was measured in groups of 6–11 oocytes, MII eggs, or 1-cell embryos exactly as described previously (20, 52). Briefly, oocytes, eggs, or embryos were incubated in KSOM at 37 °C for 30 min with 1 μM [*methyl*-^{3}H]betaine and washed, and intracellular ^{3}H was quantified by liquid scintillation counting. Non-saturable transport was determined with 5 mM unlabeled betaine present during incubation. Rates were expressed as fmol/embryo or oocyte min^{-1}.

RT-PCR and Q-RT-PCR

Primer pairs to detect *Chdh* (OligoPerfect, Invitrogen) transcript were 5′-CTC ATC CGG ACA TCC AGT TC-3′ (forward, bases 1472–1491 of NM_001136240) and 5′-CCT CGA CAT CGG TTT CTG TT-3′ (reverse, 1672–1653), producing a 201-bp amplicon. Control genes *H2afz* and *Ppia* were detected using primers described previously, yielding 202- and 150-bp amplicons, respectively (20, 32). *Gfp* primers were 5′-CCT GAA GTT CAT CTG CAC CA-3′ (forward) and 5′-GGT CTT GTA GTT GCC GTC GT-3′ (reverse, 196 bp). *Gfp* cRNA was produced from *Gfp* in linearized pcDNA3 plasmid as described previously (45).

Sets of 30 oocytes, eggs, COCs, or embryos were pooled and stored at −80 °C until RNA extraction. Just prior to extraction, 1 pg of *Gfp* cRNA was added to selected samples to assess sample loss during processing. Total RNA was extracted (RNeasy Micro Kit, Qiagen) and reverse transcribed into cDNA (RETROscript Kit, Ambion). The conventional PCR program was 95 °C (15 min) and 40 cycles of 94 °C (60 s), 60 °C (30 s), and 72 °C (60 s). Sequences of selected amplicons were confirmed (Ontario Genomics Innovation Centre). Quantitative PCR (LightCycler thermocycler, Roche Applied Science) was performed on cDNA after reverse transcription (Q-RT-PCR) with 20 μl of reaction mixtures with 0.15 oocyte, COC, or embryo-equivalent cDNA template, 500 nM primers, and LightCycler 480 SYBR Green I Master Mix (Roche Applied Science). The quantitative PCR program was 5 min at 95 °C and 50 cycles of 95 °C (15 s), 60 °C (20 s), and 72 °C (30 s), with melting curve analysis after each run. Duplicate samples were averaged (*i.e.* a technical repeat) within each independent repeat. Standard curves were constructed by serial 7-fold dilutions of quantified amplicon. The mRNA copy number was determined by the absolute quantification method according to the manufacturer's instructions.

Western blots

A rabbit polyclonal IgG directed against CHDH (17356-1-AP, Proteintech, Chicago, IL) was used. Band intensity was linearly related to the number of GV oocytes from 20–100 oocytes (not shown). Generally, 50 oocytes or embryos were used per lane except where specified otherwise. Antibody validation is described under "Results." After SDS-PAGE and transfer, the nitrocellulose membrane was blocked with 5% milk (1 h, room temperature) and incubated overnight with primary antibody (1:1000) in 5% milk, followed by goat anti-rabbit HRP-conjugated IgG secondary antibody (1:5000; catalogue no. 1706515, Bio-Rad) in 5% milk (1 h, room temperature). Detection was with the Amersham Biosciences ECL Prime detection kit (RPN2232, GE Healthcare, Mississauga, Canada). Loading control was GAPDH (~36 kDa), done on the lower portion of the membrane (cut at ~50 kDa) using GAPDH FL-355 antibody (sc-25778, Santa Cruz Biotechnology, Inc., Dallas, TX) and the Thermo Scientific Pierce ECL kit (catalogue no. 32209, Thermo Fisher Scientific, Burlington, Canada). For Western blots that included oocytes from offspring of $Chdh^{+/-} \times Chdh^{-/-}$ matings, immunoblots were performed blind, after which the genotype was determined.

CHDH enzyme activity assay

A CHDH enzyme activity assay (24, 33) was adapted for small samples. The major changes from the published assay were an increased incubation period from 4 to 24 h and omission of unlabeled choline. Kidney inner medulla (33) was used for assay optimization and positive control, and lung was used as a negative control (24). Both were obtained from CF1 female mice.

For oocytes or embryos, 100 were pooled in 15 μl of homogenization solution (250 mM sucrose, 50 mM Tris, 0.1 mM EDTA, pH 7.8) and lysed with five freeze-thaw cycles before adding 10 μl of reaction solution (1 μCi of [*methyl*-^{3}H]choline, 50 mM sodium phosphate, 50 mM Tris, 0.1 mM $CaCl_2$, and 2.5 mM phenazine methosulfate, pH 7.5) and incubating in the dark in a rotating water bath at 37 °C. After 24 h, 5 μl of 1 N NaOH and 10 μl of 30% H_2O_2 were added to terminate the reaction

and completely oxidize betaine aldehyde to betaine (1 h, room temperature).

Samples were loaded onto a 3 × 1-cm column of Dowex AG50W-X8 (200–400 mesh) resin beads that had been equilibrated with 1 M LiOH for 2 h and extensively washed with deionized water until the eluent reached stable nearly neutral pH (at least 1 week) and stored in water. [^{3}H]Betaine was eluted with 6 ml of deionized water that was collected in 500-μl aliquots. Unreacted [^{3}H]choline was retained on the column. Pilot experiments using kidney lysate indicated that the third 500-μl aliquot had the highest signal/background ratio (not shown). Therefore, 100 μl of the third 500-μl eluate was subjected to liquid scintillation counting as a measure of [^{3}H]betaine production. CHDH activity is reported as counts/min in 100 μl of eluate. Bound [^{3}H]choline was removed with 3 ml of 1.5% HCl followed by 3 ml of deionized water, and the column was reused.

Compounds that mimic choline competitively inhibit CHDH (42). Here, 3,3-dimethylbutanol (10 mM 3,3-dimethyl-2-butanol) was added to the reaction mix where specified. Betaine aldehyde (5 mM), which blocks CHDH activity by product inhibition (24), was added to the reaction mix where indicated. For samples in which enzyme activity was eliminated by boiling, sample tubes were immersed in boiling water for 10 min before the assay.

Data analysis

Statistical analysis and graphing were done with Prism version 5 or 7 (GraphPad Software, La Jolla, CA). Means were compared by ANOVA (more than two means) followed by Tukey's (comparing all pairs) or Bonferroni's (comparing subset of means) multiple-comparison tests, two-tailed *t* test (two means), or one-sample *t* test (mean against constant value), as specified. Two-way ANOVA with Bonferroni post hoc test was used to determine the significance of dependence on two variables. Data are presented as mean ± S.E. $p < 0.05$ was considered significant. Where indicated, linear regression was performed, and R^2 for the fit was calculated using Prism.

Author contributions—J. M. B., J. M. T., and M. R. W. M. conceived the study. J. M. B. and T. M. wrote the manuscript. J. M. B. designed and supervised the CHDH activity assay experiments. T. M. carried out transport measurements, validated protein expression analysis and antibody specificity, and prepared figures. M. M. developed and validated genotyping methodology. M. M. and M. O. O. carried out expression analyses and prepared figures. S. S. and M. L. performed the measurements of endogenous betaine by mass spectroscopy. S. H. Z. produced the Chdh null line and provided assistance in establishing the colony and genotyping and experimental design. All authors reviewed the results and approved the manuscript.

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