Transcription factors SOHLH1 and SOHLH2 coordinate oocyte differentiation without affecting meiosis I

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Introduction

In contrast to the sex-determining region Y–dependent (SRY-dependent) testis-determining system in early XY gonads, the molecular mechanisms behind XX female gonadal fate are poorly understood. Unlike male germ cells, in which differentiation culminates in meiosis, the oocyte is a unique multitasker, with meiosis and oocyte differentiation proceeding alongside each other. The somatic component plays an important role in XX determination, just as it does in well-characterized XY gonadal differentiation, and forkhead box L2 (Foxl2) expression circa E12 is an important determinant of granulosa cell differentiation and oocyte maintenance (1). However, little is understood regarding oocyte differentiation outside of the somatic sphere of influence.

Previous studies have discovered a group of transcription factors that play a crucial role in postnatal oocyte differentiation; these include spermatogenesis and oogenesis bHLH transcription factor 1 (Sohlh1) (2), Sohlh2 (3, 4), LIM homeobox protein (Lhx8) (5, 6), NOBOX oogenesis homeobox (Nobox) (7), and folliculo-genesis-specific basic helix-loop-helix (Figla) (8) genes. Sohlh1 and Sohlh2 are expressed preferentially in the male and female germline and are critical for male and female germline development. Lhx8, Nobox, and Figla are mainly expressed in the female germline, and their KO’s disrupt only ovarian development, without affecting male germline differentiation. The postnatal phenotypes in ovaries deficient in these transcriptional regulators share many similarities: embryonic gonads look relatively unaffected on gross histology and oocyte counts, and newborn ovaries are similarly unaffected. Within the first few days of postnatal life, the histology reveals significant disruption in follicle formation, with ensuing oocyte death within a few weeks. However, it is clear from ultrastructural studies of embryonic Nobox-deficient gonads that anatomic abnormalities, such as deficient formation of somatic cell projections, commence as early as E16.5 (7). We therefore focused our studies on the prenatal regulation of oocyte differentiation by oocyte-specific transcriptional regulators. Here, we show that SOHLH1 and SOHLH2 protein expression commence in the embryonic gonad and that Sohlh1 and Sohlh2 gene deficiency disrupts embryonic expression of the oocyte differentiation factors NOBOX and LHX8, without an appreciable effect on meiosis I. We conclude that Sohlh1 and Sohlh2 are critical genes in oocyte differentiation, independent of meiosis. Together with findings from previous studies (9), these results indicate that Sohlh1 and Sohlh2 genes are universal regulators of both male and female germline differentiation, with distinct and sex-specific downstream pathways.

Results

Sohlh2 nucleocytoplasmic translocation is dependent on SOHLH1 expression. Little is known regarding embryonic expression of

#### Conflict of interest
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SOHLH1, SOHLH2, LHX8, and NOBOX in embryonic gonads. We evaluated the expression of these proteins at different developmental stages of the ovary, using immunofluorescence and Western blotting. SOHLH2 was expressed as early as E12.5, prior to oocyte entry into meiosis (Figure 1A), and preceded SOHLH1 expression. NOBOX protein and RNA expression had an onset similar to that for SOHLH1 expression in the embryonic ovaries (Figure 1A and Supplemental Figure 1; supplemental material available online with this article; https://doi.org/10.1172/JCI90281DS1). Interestingly, SOHLH2 expression circa E15.5 and earlier was exclusively cytoplasmic (Figure 1B). We did not detect SOHLH1 expression at E12.5 by immunofluorescence or by composite data derived from the NCBI’s Gene Expression Omnibus (GEO) profiles on embryonic ovarian RNA expression (Supplemental Figure 1). At E15.5, oocytes had entered the zygotene stage of meiosis I. At this time, a small subset of oocytes expressed the SOHLH1 protein, which was located in both the cytoplasm and the nucleus (Figure 1B). Western blotting did not detect SOHLH1 at E15.5 (Figure 1A), while immunofluorescence did, perhaps due to epitope conformation or sensitivity. In oocytes that expressed SOHLH1 at E15.5, we found that SOHLH2 expression was mostly confined to the nucleus (Figure 1, B and C). At E17.5 and beyond, 80% of germ cells coexpressed SOHLH1 and SOHLH2, with SOHLH2 expression mainly confined to the nucleus, while SOHLH1 expression was detected in both the nucleus and cytoplasm (Figure 1, B and C). We found that SOHLH2 translocation from the cytoplasm to the nucleus was closely associated with expression of SOHLH1. SOHLH1 and SOHLH2 proteins were also coexpressed postnatally and were detectable mainly in the oocytes of primordial follicles and a few primary follicles (Figure 1B and C) (4, 5).
Immunofluorescence staining with anti-SOHLH1 and anti-SOHLH2 antibodies on ovaries from E16.5 and newborn (E19.5) Sohlh1-deficient animals showed that Sohlh1 deficiency abrogated SOHLH2 translocation into the nucleus (Figure 2B). These results further indicate that SOHLH2 nuclear localization is dependent on SOHLH1 expression.

SOHLH1 and SOHLH2 regulate oocyte growth and differentiation. SOHLH1 and SOHLH2 proteins are known to interact in vivo, and we hypothesized that complete elimination of SOHLH1 and SOHLH2 proteins from the ovaries may cause a more severe developmental defect than would single deficiency (9, 10). We generated Sohlh1/Sohlh2 double-deficiency mice (referred to hereafter as S1/2DKO mice) to investigate the effects of combined deficiency on oogenesis. Mice with loss of both Sohlh1 and Sohlh2 in their ovaries showed no histomorphological differences when compared with single-deficiency Sohlh1 and Sohlh2 mice, and both combined and single-deficiency ovaries were substantially smaller when compared with WT ovaries (Figure 3). In newborn mice, we found that Sohlh1- or Sohlh2 single- or double-KO ovarian histology was no different from that of WT littermates (Figure 3, A–D). Sohlh1 or Sohlh2 single-KO ovaries, as well as S1/2DKO ovaries, have remarkably diminished primordial and primary follicles when compared with 1- and 2-week-old WT littermates (Figure 3, E–L) and lose the vast majority of oocytes by the time they reach reproductive maturity at approximately 7 weeks of age (Figure 3, M–P).

Given that Sohlh1-KO, Sohlh2-KO, and S1/2DKO phenotypes are histologically identical despite a different onset of their embryonic expression (4), that SOHLH2 protein precedes SOHLH1 expression, and that SOHLH2 protein translocation to the nucleus is associated with the appearance of SOHLH1 expression circa E15.5, we further tested the hypothesis that the phenotypes observed in Sohlh2-deficient ovaries are solely due to the disruption of SOHLH1 expression. If this is the case, then activating Sohlh1 expression in Sohlh2-KOs may rescue the Sohlh2-KO phenotype and lead to normal oogenesis. To address this question, we engineered a transgenic mouse that expresses Sohlh1-mCherry under the CMV early enhancer–chicken β-actin hybrid (CAG) promoter, when Cre-mediated excision removes the chloramphenicol acetyltransferase (CAT) gene, which lies between the CAG promoter and Sohlh1 (Supplemental Figure 2) (11). We labeled this conditional Sohlh1-expressing construct CCS1. We used DEAD (Asp-Glu-Ala-Asp) box polypeptide–Cre (Ddx4-Cre) (12), whose expression commences between E15.5 and E18.5, after the onset of meiosis, to activate Sohlh1 expression within superoxide dismutase copper chaperone (CCSI). Moreover, we found that Sohlh1 and Sohlh2 deficiencies did not suppress Ddx4 protein expression (Supplemental Figure 3); therefore, Ddx4 promoter–driven Cre will be expressed in the background of Sohlh1- and Sohlh2-deficient ovaries. Ddx4-Cre–driven expression of Sohlh1 from the CCSI transgene rescued ovarian development and fertility in Sohlh2-KOs (Figure 4A). The expression of mCherry and growth differentiation factor 9 (Gdf9) RNA in the CCSI Sohlh1+/− ovary indicated successful expression of the Sohlh1-mCherry transgene in Sohlh1−/− mice (Figure 4B). Moreover, SOHLH2 expression in the CCSI Sohlh1+/− animals was now shifted from the cytoplasm to the nucleus (Supplemental Figure 4). These rescue results are consistent with the appearance of Sohlh1 circa E15.5 and indicate that Sohlh1 does not play a critical role prior to the formation of the embryonic gonad. These results also indicate that Sohlh1 is not essential for meiosis, as meiosis commences around E13.5.

We then assessed whether Sohlh1 expression on the Sohlh2-KO background can rescue ovarian development. We assessed fertility parameters such as pups per litter over a 10-month period and performed histomorphometric analyses to determine the presence and types of follicles from 5 pairs of ovaries per genotype (Figure 4, A and C). Sohlh1 expression under the control of Ddx4-Cre did not rescue Sohlh2-KO mice (Figure 4A), despite positive expression of Sohlh1, as evidenced by the presence of the mCherry transcript (Figure 4B). These results indicate that genes other than Sohlh1 are important for Sohlh2 downstream effects or that SOHLH2 expression (absent in the rescue) and heterodimerization (9) with SOHLH1 protein are essential steps for downstream effects and rescue.

We also attempted to rescue the Sohlh2-KO using the Sohlh2 transgene, whose expression is driven by a floxed CAG promoter (CCS2 transgene line, identical to CCSI, except for Sohlh2 expres-
Oocyte-specific transcriptional regulator expression is dependent on Sohlh1 and Sohlh2. We hypothesized that the appearance of SOHLH1 around E15.5, with concomitant nuclear translocation of SOHLH2, correlates with the embryonic appearance of transcripts that encode genes essential for oocyte differentiation. Previous studies have shown that postnatal deficiency of SOHLH1 and SOHLH2 leads to downregulation of multiple oocyte-specific transcriptional regulators, including Nobox, Figla, KIT proto-oncogene receptor tyrosine kinase (Kit), and Lhx8 (5, 14). We used publicly available GEO profiles of ovarian development to assess the expression of Sohlh1, which correlated very well with our experimental data, and chose Sohlh1 profile neighbors. We discovered that Nobox, Figla, Kit, and Lhx8 overlap Sohlh1 expression (Supplemental Figure 1). Western blot analysis using anti-NOBOX antibodies followed the Nobox RNA GEO profile expression pattern and overlapped with SOHLH1 protein and RNA expression (Figure 1A and Supplemental Figure 1). We analyzed coexpression of SOHLH1, NOBOX, and LHX8 proteins in embryonic oocytes using immunofluorescence with affinity-purified antibodies against SOHLH1, NOBOX, and LHX8 (5, 14). We used publicly available GEO profiles of ovarian development to assess the expression of SOHLH1, which correlated very well with our experimental data, and chose Sohlh1 profile neighbors. We discovered that Nobox, Figla, Kit, and Lhx8 overlap Sohlh1 expression (Supplemental Figure 1). Western blot analysis using anti-NOBOX antibodies followed the Nobox RNA GEO profile expression pattern and overlapped with SOHLH1 protein and RNA expression (Figure 1A and Supplemental Figure 1). We analyzed coexpression of SOHLH1, NOBOX, and LHX8 proteins in embryonic oocytes using immunofluorescence with affinity-purified antibodies against SOHLH1, NOBOX, and LHX8 (5, 14). We used publicly available GEO profiles of ovarian development to assess the expression of SOHLH1, which correlated very well with our experimental data, and chose Sohlh1 profile neighbors. We discovered that Nobox, Figla, Kit, and Lhx8 overlap Sohlh1 expression (Supplemental Figure 1). Western blot analysis using anti-NOBOX antibodies followed the Nobox RNA GEO profile expression pattern and overlapped with SOHLH1 protein and RNA expression (Figure 1A and Supplemental Figure 1). We analyzed coexpression of SOHLH1, NOBOX, and LHX8 proteins in embryonic oocytes using immunofluorescence with affinity-purified antibodies against SOHLH1, NOBOX, and LHX8 (5, 14). We used publicly available GEO profiles of ovarian development to assess the expression of SOHLH1, which correlated very well with our experimental data, and chose Sohlh1 profile neighbors. We discovered that Nobox, Figla, Kit, and Lhx8 overlap Sohlh1 expression (Supplemental Figure 1). Western blot analysis using anti-NOBOX antibodies followed the Nobox RNA GEO profile expression pattern and overlapped with SOHLH1 protein and RNA expression (Figure 1A and Supplemental Figure 1). We analyzed coexpression of SOHLH1, NOBOX, and LHX8 proteins in embryonic oocytes using immunofluorescence with affinity-purified antibodies against SOHLH1, NOBOX, and LHX8 (5, 14). We used publicly available GEO profiles of ovarian development to assess the expression of SOHLH1, which correlated very well with our experimental data, and chose Sohlh1 profile neighbors. We discovered that Nobox, Figla, Kit, and Lhx8 overlap Sohlh1 expression (Supplemental Figure 1). Western blot analysis using anti-NOBOX antibodies followed the Nobox RNA GEO profile expression pattern and overlapped with SOHLH1 protein and RNA expression (Figure 1A and Supplemental Figure 1). We analyzed coexpression of SOHLH1, NOBOX, and LHX8 proteins in embryonic oocytes using immunofluorescence with affinity-purified antibodies against SOHLH1, NOBOX, and LHX8. A subset of oocytes within the E16.5 ovary expressed SOHLH1 protein, which was localized in both the nucleus and cytoplasm. We found that NOBOX and LHX8 expression commenced sole-
that SOHLH1 and SOHLH2 and other oocyte-specific transcription factors are codependent on one another for their appropriate expression in the embryonic gonad (4, 5, 7, 14).

Sohlh1 and Sohlh2 deficiencies do not disrupt meiosis I. Meiosis in mouse ovaries commences circa E13.5 and arrests in the diplo-tene stage before birth. By E16.5, most oocytes show zygotene and pachytene stages of meiosis, whereas at E18.5, pachytene and diplotene stages predominate. The substages of meiosis I prophase are defined by the following chromosome configurations and structures: pairing, which occurs during the leptotene and zygotene stages; synapsis, which is completed at the onset of the pachytene stage; and desynapsis, which occurs during the diplotene stage (15). Rapid oocyte loss occurs when meiosis I components are perturbed, as exemplified by KO models for mutS homolog 5 (Msh5) (16), REC8 meiotic recombination protein (Rec8) (17), and DNA meiotic recombinase 1 (Dmc1) (18). SOHLH2 expression precedes SOHLH1 expression in the embryonic gonad and overlaps the initiation of meiosis I. SOHLH1 and SOHLH2 ovaries lose oocytes rapidly after birth, at the time when most oocytes have entered the diplotene stage of meiosis I. We studied whether gross perturbations in meiosis account for rapid oocyte loss in Sohlh1- and Sohlh2-deficient ovaries.

We performed RNA-sequencing (RNA-seq) on Sohlh1- and Sohlh2 single-KOs as well as on S1/2DKO newborn ovaries (Table 1) to assess the expression profiles of genes encoding well-char-
acerted meiotic factors. The relative transcript levels of Dmc1, Msh5, SPOI11 meiotic protein covalently bound to DSB (Spo11), Rec8, and other well-characterized meiotic genes were not significantly different between the WT and Sohlh1, Sohlh2, or S1/2DKO ovaries (Figure 9). These results indicate that meiotic components currently known to disrupt early oogenesis are not affected by Sohlh1 or Sohlh2 deficiency and that oocyte differentiation can be governed by factors independent of meiosis.

SOHLH1- and SOHLH2-independent gene expression profile in the ovary. We performed RNA-seq analysis on single-KO or S1/2DKO newborn mouse ovaries to determine the molecular perturbations that precede a frank pathology in double-KO versus single-KO mouse ovaries. SOHLH1 and SOHLH2 were coexpressed in the vast majority of oocytes in newborn ovaries, and the histology of newborn ovaries was equivalent between WT and KOs. Analysis of the RNA-seq data revealed that 63 genes were significantly downregulated and 154 genes were markedly upregulated in Sohlh1+/− mice; 53 genes were downregulated and 155 were upregulated in Sohlh2+/− mice; and 94 genes were downregulated and 140 were upregulated in double-KO ovaries (Figure 10). Sohlh1- and Sohlh2-regulated networks were mainly repressed in the presence of Sohlh1 and Sohlh2, but a substantial number of genes were also activated (downregulated in KOs). There was a significant overlap in genes misexpressed between Sohlh1 and Sohlh2 single-KO ovaries. Among downregulated genes, Sohlh1 and Sohlh2 single-KOs shared 44 genes of a total of 63 among Sohlh1-KOs (70%) and 53 genes among Sohlh2-KOs (83%). Among upregulated genes, Sohlh1 and Sohlh2 shared 135 genes (90%). S1/2DKO ovaries showed a substantially higher number of downregulated genes, 94, while the number of upregulated genes was not significantly different. The significantly higher number of genes downregulated in S1/2DKOs argues that Sohlh1 and Sohlh2 may have synergistic effects at the molecular level, despite a lack of effect on the histology phenotype. SOHLH1 and SOHLH2 proteins are known to heterodimerize (9), and heterodimers may have additional effects on gene expression.

The downregulated genes include well-known oocyte-specific genes, such as Lhx8, Nobox, Zp3, and Kit (Figure 10 and Supplemental Table 1). Thirty genes were exclusively downregulated in S1/2DKO ovaries (Figure 10 and Supplemental Table 2). The downregulated gene list is enriched in the histone transcripts H2a, H4, H2a, H4, H2a, H4, H2a, H4, and H2a, although the significance of this is unclear. One hundred twenty-six genes were upregulated in both single- and double-KO ovaries (Figure 10 and Supplemental Table 3) and include stimulated with retinoic acid gene 8 (Sta8) as well as testis-specific genes such as histone cluster 1, H1 (Hist1h1); histone cluster 1, H2a (Hist1h2a); histone cluster 1, H2b (Hist1h2b); testis-expressed gene 16 (Test16); and testis-expressed gene 101 (Test101). Multiple members of the reproductive homeobox cluster (Rhox) family of homeobox genes, important in male infertility (19), are also upregulated and, in part, account for the preponderance of misregulated X chromosome genes (39%) in Sohlh1- and Sohlh2-deficient ovaries. The overexpression of testis-specific genes has been previously observed in other oocyte-specific KOs of Nobox (20) and Figla (21) genes and signifies the importance of oocytes in repressing male germine differentiation.

**Discussion**

Oocyte differentiation is closely intertwined with the onset of meiosis I, but the drivers of oocyte differentiation are unknown. The role of somatic factors is well established in embryonic male gonadal differentiation. Moreover, we and others have previously shown that Sohlh1 and Sohlh2, two unique and germine-specific basic helix-loop-helix transcriptional regulators, coordinate male spermatogonial differentiation (9, 10, 22, 23). Much less is known regarding female gonadal differentiation, though somatic proteins encoded by Wnt4 and Foxl2 genes occupy a prominent role in the
female pathway. Even less is known regarding oocyte-expressed genes that drive oocyte differentiation independently of meiosis. We and others have previously shown the importance of the transcriptional regulators SOHLH1 (2, 5), SOHLH2 (3, 4), LHX8 (5, 6), NOBOX (7), FIGLA (8), and TATA-box–binding protein–associated factor 4b (TAF4B) (24) in postnatal oocyte differentiation. Postnatal studies have shown that these factors are critical in primordial follicle formation, primordial follicle–to–primary follicle transition, and oocyte survival. However, the embryonic onset of their expression and action in the embryonic ovary is poorly understood. SOHLH1 and SOHLH2 are exclusively expressed in the male and female germline (2, 3). We hypothesized that SOHLH1 and SOHLH2 are important factors in driving oocyte differentiation in the embryonic ovary.

The Sohlh2 gene was initially identified as a homolog of Sohlh1 (3), with their limited homology confined to the bHLH domains (~50%). Our data presented here, as well as the GEO expression profile data (Supplemental Figure 1), show that Sohlh2 RNA and protein expression commences relatively early, as early as E12.5, and precedes Sohlh1 expression. Interestingly, we observed that SOHLH2 protein was predominantly located in the cytoplasm prior to the appearance of SOHLH1 protein (Figure 1). SOHLH1 protein’s appearance, circa E15.5, led to the translocation of SOHLH2 into the nucleus and coincided with the appearance of LHX8 and NOBOX proteins (Figure 2). Sohlh1–/– ovaries, examined at E16.5 and in newborns, showed that SOHLH2 protein expression was confined to the cytoplasm (Figure 2), reinforcing the conclusion that SOHLH2 cellular localization depends on SOHLH1 expression. Single deficiency of Sohlh1, Sohlh2, Lhx8, or Nobox disrupts the pattern of expression of other genes, implying an interdependence of these factors (Figure 8).

We do not know whether cytoplasmic SOHLH2 has a specific function. Transcription factors like p53 have posttranscriptional roles and bind transcripts to regulate their translation (25, 26). We hypothesize that cytoplasmic SOHLH2 is inactive and that translocation and heterodimerization with SOHLH1 are necessary for activity, however, further studies are needed to address this hypothesis.

Because SOHLH1 and SOHLH2 show limited homology, we originally hypothesized that these 2 proteins play independent roles and that double deficiency will produce synergistic effects in the ovary. However, the onset and extent of pathology in mice lacking both Sohlh1 and Sohlh2 is identical to that in single-KO

Figure 6. Sohlh1 overexpression with Gdf9 promoter–driven Cre rescues Sohlh1–/– fertility but not the primordial follicle reserve. (A and B) Conditional expression of Sohlh1 from the CC51 transgene with Gdf9-Cre rescued Sohlh1–/– folliculogenesis. Histology sections were stained with anti–NOBOX antibodies to identify oocyte nuclei. The WT ovary showed various stages of folliculogenesis, while the Sohlh1–/– ovary lacked oocytes at 5 weeks. CC51 Sohlh1–/– mice showed normal folliculogenesis from primary to large antral follicles at 5 weeks and 8 months, but had an unusually low number of primordial follicles (n = 3 per group). (C) Primordial follicle numbers in WT and CC51 Sohlh1–/– mice at 5 weeks and 8 months (n = 5 per group). (D and E) Fertility testing of CC51 Sohlh1–/– mice over a period of 6 months (n = 3 per group). (F) Expression levels of Sohlh1, Sohlh2, mCherry, and Gdf9 in WT, Sohlh1-deficient (Sohlh1–/–), and Sohlh1–/––rescued ovaries (CCS1 Sohlh1–/–). **P < 0.01 and ***P < 0.001, by 2-tailed Student’s t test. Data represent the mean ± SEM and are representative of at least 3 independent experiments.
The lack of a synergistic effect is consistent with the interpretation that Sohlh1 and Sohlh2 share a common pathway that is enabled at E15.5, when the appearance of SOHLH1 induces SOHLH2 nuclear translocation. Previous studies have shown that SOHLH1 and SOHLH2 physically interact and can form homo- and heterodimers (9, 10). Consistent with KO studies, which indicate that SOHLH1 and SOHLH2 could regulate common pathways, RNA-seq analyses of gene expression in Sohlh1- and Sohlh2-deficient ovaries showed a large overlap in misexpressed genes. Double mutants had more profound changes in gene expression, consistent with Sohlh1 and Sohlh2 synergism (Figure 10). SOHLH1 and SOHLH2 heterodimers may regulate genes involved in later steps of oocyte differentiation rather than those involved in the earlier steps of development.

Meiosis is a big event in the female embryonic ovary, commencing circa E13.5. Meiosis is dissociable from oocyte differentiation, as shown in studies of postnatal phenotypes in Stra8-deficient ovaries (27). Stra8, one of the genes stimulated by retinoic acid, is essential for oocyte entry into meiosis. Stra8 has a narrow window of expression around E14.5, after which it rapidly declines (Supplemental Figure 1). SOHLH1 has been proposed, in somatic cell lines, to play a role in Stra8 downregulation (28). Despite the lack of a meiotic prophase, a subset of Stra8-deficient oocytes survive fetal life, can synthesize zona pellucida, can organize surrounding somatic cells into follicles, are ovulated in response to hormonal stimulation, undergo asymmetric cell division to produce a polar body, and cleave to form 2-cell embryos upon fertilization (29). Nonetheless, Stra8-deficient oocytes are highly abnormal, with a large loss of oocytes commencing in fetal ovaries and oocyte depletion being completed by 6 weeks’ postnatal age, with resulting sterility (30).

Deficiency of Sohlh1 and Sohlh2 is associated with persistent expression of Stra8 (Figure 10J and Supplemental Table 3). Because Stra8 expression persists in both Sohlh1- and Sohlh2-KOs, it is likely that SOHLH1:SOHLH2 heterodimers are important in regulating Stra8 expression. Despite persistent Stra8 expression, meiosis I is grossly normal, and expression of other meiotic gene transcripts is unaffected (Figure 9). Other meiotic gene KOs support the notion that abnormal meiosis does not disrupt oocyte differentiation. For example, HORMA domain-containing 1 (Hormad1) is a major pachytene checkpoint, and our previous work revealed that Hormad1-deficient oocytes have unsynapsed chromosomes and lack pachynema, yet have a normal number of primordial follicles with normal oogenesis and folliculogenesis (31, 32). Hormad1 deficiency does not affect oocyte differentiation (33), nor does it perturb the expression of SOHLH1 (Supplemental Figure 3).

The expression of many oocyte-specific transcriptional regulators, such as LHX8, NOBOX, and FIGLA, is initiated in the embryonic ovaries and overlaps with SOHLH1 expression and SOHLH2 nuclear translocation. Moreover, Sohlh1 and Sohlh2 gene deficiencies disrupt LHX8 and NOBOX protein embryonic expression without disrupting meiosis. The loss of oocytes at the very early ages in single and combined Sohlh1 and Sohlh2 deficiencies is therefore unlikely to be due to meiosis but rather to disruption in the regulation of genes such as Lhx8 and Nobox (5, 7, 14). Our results are consistent with the interpretation that SOHLH1 and SOHLH2 are important regulators of oocyte differentiation, independent of meiosis, whose molecular inter-
play begins circa E15.5 with the appearance of SOHLH1 and nuclear translocation of SOHLH2.

Recent human studies have shown that SOHLH1 and SOHLH2 loss-of-function mutations are involved in a subset of XX and XY gonadal dysgenesis cases (34, 35) and show the importance of this pathway in human gonadal differentiation (34, 35). Our results show a dynamic interaction between oocyte-specific transcriptional regulators in the embryonic gonad and

Figure 8. Transcriptional regulator deficiencies disrupt coexpression of oocyte-specific transcriptional regulators. (A) Immunofluorescence staining with SOHLH1 (magenta), LHX8 (yellow), and NOBOX (cyan) in ovaries from WT, Sohlh1−/−, Sohlh2−/−, S1/2DKO, and Lhx8−/− mice. Scale bars: 100 μm (top) and 50 μm (enlargements). Arrows indicate oocytes expressing only LHX8 or NOBOX but not both (n = 3 per group). DNA was stained with DAPI (gray). (B) Quantitation of NOBOX-only, LHX8-only, SOHLH1-only, and LHX8/NOBOX-coexpressed oocytes in A. Data are representative of at least 3 independent experiments.
expression and orchestrate sex-specific germline differentiation remain to be determined.

**Methods**

**Animal breeding and transgenic lines.** Pups were weaned at 3 weeks of age, and breeding pairs were set up at 6 weeks of age. One mating pair was placed per cage and inspected daily for the presence of litters. Sohlh1, Sohlh2, Lhx8, and Hormad1 mice used in the current studies were previously described (2, 3, 14, 31). The CCS1- and CCS2-transgenic mice were generated by integrating the CAG-loxp-CAT-loxp Sohlh1-mCherry (CCS1) transgene or the CAG-loxp-CAT-loxp Sohlh2-EGFP (CCS2) transgene into the pronuclei of zygotes of FVB × FVB mice. Founder CCS1 or CCS2 mice were bred with FVB mice to

Table 1. Meiosis gene expression

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<tr>
<th>Gene</th>
<th>WT RPKM</th>
<th>Sohlh1-KO RPKM</th>
<th>Sohlh2-KO RPKM</th>
<th>S1/2DKO RPKM</th>
<th>P value</th>
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</tr>
<tr>
<td>Smc1a</td>
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<tr>
<td>Smc3a</td>
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<tr>
<td>Syca1</td>
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<td>299.07</td>
<td>338.24</td>
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Atm, ataxia telangiectasia mutated; Atr, ataxia telangiectasia and Rad3-related; Mlh1/3, mutL homolog 1; Smc1a, structural maintenance of chromosomes 1A; RPKM, reads per kilobase per million.

Figure 9. Sohlh1 and Sohlh2 deficiency and meiosis. Quantitative PCR analysis was conducted for Dmc1, Msh5, Spo11, Rec8, and synaptonemal complex protein 3 (Sycp3) transcripts in WT, Sohlh1-/-, Sohlh2-/-, and S1/2DKO mice (n = 6 per group). Gapdh was used for normalization, and the ΔΔCt was calculated for the fold change. Error bars indicate the mean ± SD. A 2-tailed Student’s t test was used to calculate P values (compared with WT). Data are representative of at least 3 independent experiments. A P value of less than 0.05 was considered statistically significant. No significant differences in expression were found between different genotypes (P value was greater than 0.05).
Confocal microscopy (Nikon A1) was used to detect immunofluorescence. RNA-seq was performed in WT and Sohlh1−/− and Sohlh2 single- or double-KO mouse ovaries. Total RNA was isolated from pools of 5 newborn ovaries, using the RNeasy Mini Kit (QIAGEN) and following the manufacturer’s protocol. A cDNA library was made using a SMARTer Stranded Total RNA Sample Prep Kit (Clontech), and next-generation sequencing was performed on an Illumina NextSeq 500 (Illumina) with the Mid Output Kit (Illumina). Approximately 40 million reads were generated per sample (WT control and single and double knockouts cDNA).

Sohlh1−/− and Sohlh2 single- or double-KO RNA-seq reads were mapped and analyzed using TopHat (37), Cufflinks (38), and Cuffdiff (39). The raw RNA-seq data from this study expand the colony. Ddx4-Cre mice were purchased from The Jackson Laboratory, and Gdf9-Cre mice were a gift of Austin Cooney (University of Texas at Austin, Austin, Texas, USA) (13).

Histology and whole-mount immunostaining. Ovaries were fixed in 10% buffered formalin (Sigma-Aldrich). Fixed tissues were embedded in paraffin, serially sectioned (5-μm thickness), and stained with hematoxylin (Sigma-Aldrich) and periodic acid–Schiff (PAS). Germ cell cysts and primordial, primary, and secondary follicles were defined as described previously (5). WT and mutant oocytes were stained concurrently with the same mixture of antibodies.

For whole mounting, ovaries were fixed in 4% paraformaldehyde and serially washed with 25%, 50%, 75%, and 100% methanol. Oocytes were labeled with anti-SOHLH1 (2); anti-SOHLH2 (3); anti-DDX4 (ab13840; Abcam); anti–c-KIT (a gift of Tasumi Hirata, National Institute of Genetics, Mishima, Japan) (36); anti-NOBOX (7); and anti-LHX8 antibodies (14). Confocal microscopy (Nikon A1) was used to detect immunofluorescence.

RNA-seq. RNA-seq was performed in WT and Sohlh1- and Sohlh2 single- or double-KO mouse ovaries. Total RNA was isolated from pools of 5 newborn ovaries, using the RNeasy Mini Kit (QIAGEN) and following the manufacturer’s protocol. A cDNA library was made using a SMARTer Stranded Total RNA Sample Prep Kit (Clontech), and next-generation sequencing was performed on an Illumina NextSeq 500 (Illumina) with the Mid Output Kit (Illumina). Approximately 40 million reads were generated per sample (WT control and single and double knockouts cDNA). Sohlh1- and Sohlh2 single- or double-KO RNA-seq reads were mapped and analyzed using TopHat (37), Cufflinks (38), and Cuffdiff (39). The raw RNA-seq data from this study

Figure 10. Sohlh1 and Sohlh2 deficiencies show significant overlap in affected genes. Venn diagrams show overlap and differences in the number of downregulated (A) and upregulated (B) transcripts as well as transcript heatmaps (C and D) derived from Sohlh1−/−, Sohlh2−/−, and S1/2DKO mice when compared with the WT newborn ovary transcriptomes. Transcript data were derived from RNA-seq, and genes that showed a greater-than 2-fold change compared with WT were included. (E–J) Quantitative PCR analyses derived from RNA-seq of select genes such as Sohlh1 (E), Sohlh2 (F), Lhx8 (G), Nobox (H), Gdf9 (I), and Stra8 (J) in WT, Sohlh1−/−, Sohlh2−/−, and S1/2DKO mice (n = 6 per group). Gapdh was used for normalization, and ΔΔCt was calculated for the fold change. Error bars indicate the mean ± SD. *P < 0.05, by 2-tailed Student’s t test (compared with WT). Data are representative of at least 3 independent experiments.
have been deposited in the NCBI’s Sequence Read Archive (accession no. PRJNA293873). RNA-seq data for specific transcripts were validated by quantitative PCR as previously described (4).

**Statistics.** Data are presented as the mean ± SEM. Results were analyzed using a 2-tailed Student’s t test. A P value of less than 0.05 was considered statistically significant.

**Study approval.** All experimental and surgical procedures complied with the NIH Guide for the Care and Use of Laboratory Animals (National Academies Press, 2011) and were approved by the IACUC of the University of Pittsburgh, under protocol number 14094558.

**Author contributions**

YHS, KJG, VM, HS, and HWA conducted the experiments. YHS, KJG, YR, and AR analyzed and interpreted the data. YHS, YR, and AR wrote the manuscript. YR and AR reviewed and revised the manuscript. AR supervised the study.

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