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REVIEW

Germ Cell Biology

Sex determination and maintenance: the role of DMRT1 and FOXL2

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In many species, including mammals, sex determination is genetically based. The sex chromosomes that individuals carry determine sex identity. Although the genetic base of phenotypic sex is determined at the moment of fertilization, the development of testes or ovaries in the bipotential early gonads takes place during embryogenesis. During development, sex determination depends upon very few critical genes. When one of these key genes functions inappropriately, sex reversal may happen. Consequently, an individual's sex phenotype may not necessarily be consistent with the sex chromosomes that are present. For some time, it has been assumed that once the fetal choice is made between male and female in mammals, the gonadal sex identity of an individual remains stable. However, recent studies in mice have provided evidence that it is possible for the gonadal sex phenotype to be switched even in adulthood. These studies have shown that two key genes, doublesex and mad-3 related transcription factor 1 (*Dmrt1*) and forkhead box L2 (*Foxl2*), function in a Yin and Yang relationship to maintain the fates of testes or ovaries in adult mammals, and that mutations in either gene might have a dramatic effect on gonadal phenotype. Thus, adult gonad maintenance in addition to fetal sex determination may both be important for the fertility.
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INTRODUCTION

Our knowledge of sex determination has evolved over time. The discovery of sex chromosomes and the sex-determining gene sex-determining region Y (*Sry*) firmly established the role of genetics in determining our sex.[1] In mammals, females have two X chromosomes (XX) while males have two distinct sex chromosomes, X and Y. Although genetic sex is determined at the moment of fertilization, phenotypic sex determination, the decision of the bipotential early gonads to develop as either testes or ovaries, takes place during embryogenesis.[2] Sex determination occurs in a narrow window of time and depends on very few critical genes.[2–5] If one of the key genes is disturbed, sex reversal may happen. Consequently, an individual's sex phenotype may not be necessary the same as the sex chromosomes that are carried.[2–5] Disorders of Sex Development (DSD) is a generic definition of all the conditions where the gonads and genitalia, by the time of birth, are atypical in relation to the chromosomes that the individual carries.[6]

For some time, it has been assumed that once the fetal choice is made between male and female in mammals, the sex of an individual remains stable for the rest of life; that males remain male and females remain female. However, it has been known for some time that in some vertebrates, such as fish, sexual phenotype determination is not final even at the adult stage.[7–9] Rather, environmental or social disruptions in such species can cause rapid changes in gonadal and sexual phenotypes.[7–9] Recent studies in mice have provided evidence that this adult switch in gonad sexual phenotype may also be possible in mammals. Indeed, the testis and ovary phenotypes in mice must be actively maintained throughout life, even long after the organs are defined during the fetal period. Recent studies have shown that two genes, doublesex and mab-3 related transcription factor 1 (*Dmrt1*) and forkhead box L2 (*Foxl2*), play critical roles in maintaining sexual phenotype in mice.[10–12] These studies suggest that the concept of adult gonadal maintenance might also be important for the stability of sex identity and fertility after fetal sex determination in other mammals, and indeed that a switch in sexual phenotype might be as simple as mutation of a single key gene.

Herein, we briefly summarize our current understanding of mammalian sex determination in the fetus and sex maintenance in the adult, focusing on the roles of the two key genes, *Dmrt1* and *Foxl2*.

MALE SEX DETERMINATION: GENETIC SIGNALING NETWORKS FOR TESTIS DEVELOPMENT

Male sex determination in most mammals is initiated by the *Sry* gene. In humans, point mutations were found within a particular coding region of Y chromosome in several XY individuals with the female phenotype, indicating that this region which was later termed *SRY* is required for male development.[3,13] We now know that *SRY* is required for testis formation in XY embryos and is sufficient to induce testis differentiation in XX embryos. The trigger for *SRY* expression is not well understood in human although a few candidate genes have been identified based on the studies of mice.[4,5,13] These include Wilms tumor 1 (*Wt1*), GATA binding protein 4 (*Gata4*), zinc fnger protein, fog family member 2 (*Zfpm2*), chromobox homolog 2 (*Cbx2*), mitogen-activated protein kinase 4 (*Map3k4*), and the insulin receptors.[4,5,13] As yet, the only identified target gene that *SRY* activates at the sex determination

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stage is *SRY*-box 9 (*Sox9*). Similar to *Sry*, *Sox9* is both required for testis formation in XY embryos and sufficient to induce testis differentiation in XX embryos. Loss of function mutations in human *Sox9* causes sex reversal in XY males,[14,15] while gain-of-function mutations, such as gene duplication, can lead to XX female to male sex reversal.[16]

Although *Sox9* is induced by *Sry*, the continued expression of the gene depends on positive regulatory loops with fibroblast growth factor 9 (*Fgf9*) and lipocalin-type prostaglandin D2 synthase (*Ptgds*). Analysis of *Fgf9*$^{-/-}$XY mice revealed male-to-female sex reversal, indicating an important role of this gene in testis determination.[17] *Sry* initiates a feed-forward regulatory loop between *Sox9* and *Fgf9* which results in upregulation of *Fgf9* expression and repression of a female promoting gene, wingless-related MMTV integration site 4 (*Wnt4*), leading the bipotential early gonads to testis differentiation.[18] PTGDS is an enzyme that catalyzes the conversion of prostaglandin H2 (PGH2) to prostaglandin D2 (PGD2). During gonad development, PTGDS has a male-specific pattern of expression, and PGD2 can partially masculinize female embryonic gonads in culture.[19] PGD signaling upregulates *Sox9* in both an autocrine and paracrine manner[20] and facilitates SOX9 translocation to the nucleus.[21] Furthermore, SOX9 can activate PTGDS through binding to a conserved element in its promoter.[22] Thus, SOX9 establishes a feed-forward loop with PGD2 signaling, serving in addition to the SOX9 and FGF9 loop to ensure male development.[22,23] The expressions of SOX9, FGF9, and PTGDS in bipotential embryonic genital ridges determine the fate of Sertoli cells. Differentiation of Sertoli cells drives the development of the bipotential early gonads into testes, which includes mesonephric cell migration, testis cord formation, testis-specific vascularization, and myoid and Leydig cell differentiation. Additional signaling molecules and transcriptional factors were identified in these late stages of testicular development, including WT1, steroidogenic factor 1 (SF1), GATA4, DMRT1, desert hedgehog (DHH), and platelet-derived growth factor (PDGF). Comprehensive reviews on these proteins can be found elsewhere.[4,5,13] This article will only focus on DMRT1 due to its specific roles in the maintenance of testis identity in adult mice.

DMRT1 is involved in sex determination and gonadal development across a broad range of species. DMRT proteins are transcription factors that share a DNA-binding domain that is similar to a zinc finger, called the DM domain.[24] In human, several subtelomeric deletions in the p arm of the chromosome 9 have been associated with sex reversal and gonadal dysgenesis in XY individuals. Since *DMRT1* was identified in this region,[25,26] and was affected by all of these deletions,[25–28] it could be the most likely gene that is responsible for these disorders. The critical role of DMRT1 in the male sex determination in humans is confirmed by a recent study showing that a point mutation in this gene resulted in a complete male-to-female sex reversal at birth.[29] Interestingly, these deletions in the p arm of chromosome 9, and the point mutation, affected only one copy of the *DMRT1* gene, suggesting that DMRT1 is haploinsufficient for testicular development.

Expression of *DMRT1* has been detected in the human undifferentiated XY gonadal primordium at gestation week 6 (GW 6).[30] At the early fetal period (GW 8-20), DMRT1 is predominantly expressed in Sertoli cells, whereas at later gestation (GW 22-40), childhood, and postpuberty, DMRT1 is most abundant in spermatogonia.[30] Expression of DMRT1 was also found in oogonia and oocytes of the early ovary through GW 20 but was completely downregulated following meiotic entry of germ cells.[30] Similarly, DMRT1 is also expressed in the primordial gonads of both sexes at 10.5 days *postcoitum* (dpc) in mice, and subsequently shown to decrease in expression in the ovary and to increase in the testis, in the latter case with high levels of expression maintained until adulthood within Sertoli cells and premeiotic germ cells.[26,31,32] Expression of DMRT1 in germ cells of the fetal ovary may be an important event in generating primordial follicles by activating expression of the meiotic inducer stimulated by retinoic acid 8 (STRA8).[33] Unexpectedly, *Dmrt1*$^{-/-}$mutant mice were reported to not show abnormalities in sex determination and embryonic gonadal development, suggesting that this gene may not play a critical role in the early stages of gonadogenesis in mice.[26,34] Nevertheless, major defects were observed in testicular maturation after birth. Sertoli cells continually proliferated and failed to complete their differentiation during postnatal development. These immature Sertoli cells eventually died, resulting in few seminiferous tubules present in the adult. Germ cells, in turn, did not migrate to the periphery of the seminiferous tubules and died shortly after 7 dpc.[26,34]

The defects in germ cell migration and survival may relate to the losses of the gene in both somatic and germ cell types. Unlike many other sex-determining genes, *Dmrt1* is unusual in that it is expressed by both Sertoli and germ cells, and its loss affects the development of both cell types.[26] Germ line-specific deletion of the gene indicated that expression of *Dmrt1* in germ cells, but not Sertoli cells, is important for the migration of gonocytes to the basal lamina, for their mitotic reactivation, and for germ cell survival during the first postnatal week. DMRT1 activity in Sertoli cells apparently is essential for the survival and differentiation of germ cells beyond the second postnatal week.[34] Overall, these data indicate that in human, DMRT1 is critically required for the development of the testis during fetal period,[27] while in mice, the gene played more important roles for the maintenance and/or growth of the testis in the postnatal and adult period.[26,34]

FEMALE SEX DETERMINATION: GENETIC SIGNALING NETWORKS FOR OVARY DEVELOPMENT

In females, whose cells do not contain the Y chromosome and *SRY* gene, the ovary-forming pathway is activated by a different set of signaling molecules and takes a different developmental sequence. Ovary formation for many years was considered to be a "default" gonad development pathway resulting from the absence of *SRY* expression. We now know that a number of essential ovary-specific factors exist (β-catenin, follistatin [Fst], FOXL2, R-spondin [*RSPO1*], and WNT4), without which ovary development cannot take place. Mutation of these genes may result in aberrant ovary development. Like the testis, the ovary also contains three principal cell lineages: the germ cells, which enter meiosis and become primary oocytes in the developing ovary; the granulosa cells, which support germ cell development (analogous to Sertoli cells); and the theca cells, which produce steroid hormones (analogous to Leydig cells).[35]

In humans, duplications of the *WNT4* gene in males or loss of functional mutations in females resulted in diverse sexual anomalies including cryptorchidism, XY male-to-female sex reversal, or XX female-to-male sex reversal.[36–38] In mice, the role of *Wnt4* in the XX gonad is to inhibit important testis-specific processes, including migration of endothelial cells from the mesonephros[39] and steroidogenesis, either by repressing *Sf1*[40] or precluding the recruitment of steroidogenic cell precursors.[39] These results indicate that *Wnt4* is a pro-ovary (or an anti-testis) gene. The critical role of WNT signaling in ovary development is further supported by the observation that in humans, mutations in *RSPO1*, a ligand for canonical WNT signaling,[41,42] resulted in female-to-male sex reversal.[43] Although overexpression of *Rspo1* in XY mice did not disturb testis development,[44] its absence in null mutant XX mice partially reproduced the XX female-to-male sex reversal in human.[38] This suggests that RSPO1 suppresses the male

pathway in the absence of SRY by activating WNT4 signaling. It may be the factor that tips the balance toward the female pathway during sex differentiation in XX mammals.[45] Since β-catenin is the common effector of both RSPO1 and WNT4 signaling, its effect on male sex development was tested. The constitutive stabilization of β-catenin in the somatic cells of the gonads of XY mice is sufficient to induce XY male-to-female sex reversal, as indicated by downregulation of testis-specific gene markers such as *Sox9* and anti-Mullerian hormone (*Amh*), activation of ovary-specific markers such as *Foxl2*, bone morphogenetic protein 2 (*Bmp2*), *Wnt4*, and *Fst*, and the lack of testicular cords.[46] Since *Wnt4* expression is upregulated in the gonads of XY mice with null mutations of either *Sox9*[44,47] or *Fgf9*,[18] it was hypothesized that *Wnt*4 and *Fgf9* act as mutually antagonistic signals during the process of sex determination.[18,48] These findings suggest that the male fate depends not only on whether male genes, such as *Sox9*, are expressed, but also on the active repression of female genes such as *Wnt4*.[49]

Another critical gene in ovary development is *FOXL2*. Mutations in the *FOXL2* gene in humans are associated with Blepharophimosis Ptosis Epicanthus Inversus Syndrome (BPES), a condition that affects development of the eyelids. One of the phenotypes associated with type 1 BPES is the development of premature ovarian failure in females.[50] Deregulated *FOXL2* expression has also been described in the Goat Polled Intersex Syndrome in which XX female-to-male sex reversal occurs.[51] Further characterization of the model confirmed the critical role of *FOXL2* in goat sex determination.[52] In contrast to mice, in which *Foxl2* was found to play a role in maintaining ovarian functions postnatally, *FOXL2* is a bona fide female sex-determining gene in goat. Without it, XX females develop testes.[52] In the mouse gonad, *Foxl2* expression is detected in both granulosa cells and theca cells.[53] Global deletion of the gene during development was found to lead to ovary dysgenesis and infertility. The null mutation of the gene in XX mice led to defective ovaries in which primordial follicles contained granulosa cells unable to undergo the squamous-to-cuboidal transition. These follicles underwent subsequent atresia, leading to infertility due to progressive follicular depletion.[53–55] Hence, in mice, *Foxl2* may not be involved in early XX female-to-male sex reversal but is necessary for correct follicle development and female fertility maintenance in postnatal animals. More recent studies in the mouse have shown that sustained *Foxl2* expression is necessary for repressing genetic reprogramming of the postnatal ovarian somatic cells to testicular cell types, and thus for the maintenance of the adult female phenotype.[12]

FOXL2 AND OVARY MAINTENANCE IN THE ADULT

Defects in ovary development, including female-to-male sex reversal, can result from gain of function mutations in genes that promote testis development, such as *Sry* or *Sox9*, or loss of function mutations in genes that actively promote ovary development, such as *Wnt4*, *Rspo1*, and *Foxl2*. In these experimental circumstances, the defects occur as a result of mutations in cells during development. Is there a possibility that the differentiated ovary can become testis-like in the adult long after cell fates are determined during development? Uhlenhaut and colleagues[12] demonstrated that it was indeed possible. They showed that upon conditional deletion of *Foxl2* in the adult mouse ovary, the two major female-specific somatic cell lineages switched their cell fates into male counterparts; granulosa cells were reprogrammed into testis-specific Sertoli-like cells, while the steroidogenic theca cells were transformed into testosterone-producing Leydig-like cells. Reprogramming of the granulosa cells was followed by other aspects of testis differentiation, including development of seminiferous tubule-like structures. Reprogramming of the theca cells resulted in male-like levels of testosterone in the blood. This "transdifferentiation" process was accompanied by the activation of testis-promoting genes, including *Sox9*, and the silencing of ovary-promoting genes including *Rspo1* and *Wnt4*. FOXL2 is considered to directly repress the testis-specific enhancer of *Sox9*[56] through synergistic interaction with estrogen receptors-α and -β (ER-α-β).[12] This suggests that *Foxl2* expression is necessary to actively suppress *Sox9* expression in the ovary throughout life. Therefore, in the event of loss of *Foxl2*, *Sox9* expression is de-repressed in granulosa cells which then transdifferentiate into Sertoli-like cells. It is well known that during ovary development, *Sox9* is inhibited by WNT/β-catenin signaling. However, in reported cases of sex reversal resulting from loss of WNT/β-catenin, *Foxl2* was still expressed, suggesting that FOXL2 may not function in response to the WNT/β-catenin pathway, but rather must independently regulate *Sox9* activity. To develop and maintain the ovary phenotype, *Sox9* expression must be continuously repressed in XX mice. Apparently, this repression is exerted first by the WNT signaling pathway during the embryonic stage, and then by FOXL2/ER-α-β throughout adulthood.[57]

DMRT1 AND TESTICULAR MAINTENANCE IN THE ADULT

The involvement of DMRT1 in testis development in the fetus is well established. Deletion or inactivation of *DMRT1* gene in human resulted in XY male-to-female sex reversal.[25–29] In mice, XY animals lacking *Dmrt1* were born as males with testes, but these gonads underwent rapid deformation during the postnatal period.[26,34] These observations suggest that in mice, this gene may play a more important role in sex maintenance than in sex determination during fetal development. In studies of how DMRT1 may affect male reproduction system differently depending on the development stage, Zarkower and colleagues[10] deleted *Dmrt1* and examined the effects of doing so on testis determination and maintenance. When *Dmrt1* was knocked out during gestation, testes were formed and testis phenotypes were established by the time of birth. However, by 4 weeks after birth, structures similar to follicles were seen rather than normal seminiferous tubules, and the tubules contained cells that expressed the female-specific transcription factor Foxl2, which is normally only expressed in granulosa and theca cells.[53,54]

As discussed above, knockout of *Foxl2* in the adult ovary was found to lead to the transdifferentiation of ovarian cells into testicular-like cells.[12] One intriguing question could be that will the loss of *Dmrt1* in the adult testis do the same to the testicular cells as what *Foxl2* knockout did to ovarian cells? Interestingly, that was exactly what was found. One month after the deletion of *Dmrt1* in adult testis,[10] some Sertoli cells had been converted into granulosa-like cells expressing the female gene *Foxl2* instead of male gene *Sox9*, while others maintained their morphology as Sertoli cells but began to express *Foxl2* in addition to *Sox9*. The conversion of Sertoli to granulosa-like cells was also supported by the finding that the mRNA and protein levels of two proteins involved in estrogen synthesis, HSD17β1 and aromatase, were strongly expressed in mutant gonads. Consistent with this, estradiol levels rose in the serum of the mutants relative to control adult males. The mutant gonads also contained cells closely resembling theca cells, and expressed both *Foxl2* and smooth muscle actin (*Acta2*). The remodified gonad also responded to exogenous gonadotropin stimulation.

Loss of *Dmrt1* expression in the adult testis can trigger testicular cell fate reprogramming into ovary-like cells. Will forced expression of this gene in the adult ovary, by itself, reprogram the ovary cells into testicular-like cells? To test this, Zarkower and colleagues[32] made a conditional *Dmrt1* transgenic animal in which the gene is

expressed in somatic cells of the ovary. *Dmrt1* expression in the ovary silenced the female sex maintenance gene *Foxl2* and reprogrammed adult granulosa cells into Sertoli-like cells, triggering formation of structures resembling male seminiferous tubules. Associated with the morphological changes, expression of *Dmrt1* also activated multiple testicular genes and downregulated ovarian genes. The results indicated that DMRT1 can silence *Foxl2* and key ovary genes even in the absence of the testis-determining genes, such as *Sox9*. The study provided an *in vivo* example of how activation of single-gene can reprogram cellular sex identity by adult.[32]

FOXL2 AND DMRT1; YIN AND YANG RELATIONSHIP IN THE MAINTENANCE OF GONAD SEX IDENTITY

During the critical window of fetal development, there are two distinguishable signaling networks to guide the differentiation of the bipotential embryonic genital ridge into the testis or ovary. To ensure that one or the other is formed, the two signaling networks must work in a mutually exclusive way. In the male, the positive feedback regulatory loops (*Sox9-Fgf9* and *Sox9-Ptgds*) not only reinforce the activation of the male signaling network but also inhibit the key members of the female network members (*Wnt4, Rspo1,* and *Foxl2*). Similarly, activation of female signaling molecules has negative effects on the expression of male genes. Apparently, maintenance of these mutually exclusive expressed signaling networks not only is important in the establishment of the gonads during the fetal period but also is critical for the maintenance of the differentiated sex organs in the adult. In the mice, the two key genes that guide the two signaling networks in the adulthood are *Foxl2* and *Dmrt1*. Like Yin and Yang, DMRT1 and FOXL2 maintain male and female gonadal sex phenotypes, respectively, by promoting their own signaling networks and also by blocking the other. Thus, antagonism between these sex-specific transcriptional regulators is central to gonadal phenotypic stability in both sexes throughout reproductive life. Loss of either gene, even in the adulthood, can trigger a transdifferentiation of cell fates involving extensive reprogramming of sex-specific gene regulation.[10,12] The two proteins appear to anchor mutually antagonistic regulatory networks that lock in sexual differentiation and then continuously maintain appropriate cell fates.

An intriguing question is: what is the physiological reason that bipotential sexual fates are maintained long after sex is specified? At this time, there is no clear answer. One possibility is that it could be a consequence of evolution. Heterosexual reproduction, adopted universally by higher species, requires the involvement of two sexes. However, in the event of dire circumstances such as one sex becoming unavailable, sex reversal in adults might be used as "a backup plan" to ensure the continuation of the species. Indeed, there is evidence from lower species that this occurs; and as discussed above, this can occur even in some vertebrates, such as fish.[7–9] Maintenance of bipotential sexual fate could relate to the unique nature of organogenesis. The gonads are distinct between the two sexes. However, testes and ovaries develop from a common group of precursor cells of the bipotential embryonic genital ridge. These precursor cells may maintain the imprints (epigenetic configurations, for example) required for male or female development. Sex determination in fetal gonads is triggered by very few key genes (*SRY* for example). The differentiated cells specified during the fetal period may retain their "imprints," enabling them to become cells of opposite systems. To keep their fates stable, specific signaling cascade must be inhibited or enforced continuously through adulthood.

A recent study provides clues as to why DMRT1 is required for a stable testis phenotype. It is well known that retinoic acid (RA) signaling between Sertoli and germ cells is essential for adult mammalian spermatogenesis.[58] In the absence of DMRT1, however, RA signaling may also activate genes that can drive male-to-female transdifferentiation. Thus, DMRT1 allows Sertoli cells to participate in RA signaling while avoiding consequent cell fate reprogramming. How does RA signaling relate to sex-determining networks? Two hypotheses have been proposed.[11] First, DMRT1 binds to chromatin near many of the key feminizing genes and inhibits their expression, including *Foxl2* and *ERβ*.[10] Thus, the simplest model for sexual fate maintenance is that DMRT1 and retinoic acid receptor (RARα) are antagonistic regulators of feminizing genes whose inappropriate expression in the testis can cause transdifferentiation. By silencing this subset of RARα targets, DMRT1 allows Sertoli cells to use RA signaling that is essential for spermatogenesis while avoiding transdifferentiation. Another possibility is that RARα may act together with feminizing genes to cooperatively regulate target gene expressions. For example, ER and RARα are nuclear hormone receptors that cooperate to bind DNA and regulate gene expression in breast cancer cells.[59] These two nuclear receptors may cooperate by a similar molecular mechanism in *Dmrt1* mutant Sertoli cells to promote female transdifferentiation.

CONCLUSIONS

Sex determination in mammals is based on a genetic cascade that controls the fate of the gonads. The sex-determining genes control whether bipotential gonadal primordia differentiate into testis or ovary. Gonads will then direct the establishment of phenotypic sex through the production of hormones. Different types of sex reversal are expected if mutations occur in the sex-determining genes or their signaling cascades that disrupts one of the three steps in gonadal differentiation: formation of the gonadal primordia, sex determination, and testis or ovary development. Gonad sex identity may be able to switch at adult stage in some fish species. In mammals, however, it has been assumed that once the fetal choice is made between male and female, the gonadal phenotype remains stable for the rest of life. Recent studies in mice, however, suggest that the gonads in mammalian species may also have some degrees of plasticity in the adult. Ablation of *Foxl2* from the adult ovary causes transdifferentiation of granulosa cells to Sertoli-like cells and transformation of ovarian follicles into testis-like structures. Similarly, depletion of *Dmrt1* in the adult testis resulted in the conversion of testis structures into ovarian ones. FOXL2 and DMRT1 are functioning in a Yin and Yang relationship to maintain gonad sex identity in both organs. The realization that gonads are able to transform throughout an individual's lifetime by changing only single genes may have broad implications. For example, some of the frequently diagnosed reproductive disorders, such as sex differentiation disorders in children and premature failure in reproductive function in adulthood (ovarian failure and female menopause, for example), may have similar underlying causes as gonadal reprograming. The significance of these findings and their impacts on the reproductive field just begin to emerge.

AUTHOR CONTRIBUTIONS

SH and HC drafted the manuscript. LY and HC revised the article. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declare no competing interests.

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