The mole genome reveals regulatory rearrangements associated with adaptive intersexuality


Linking genomic variation to phenotypical traits remains a major challenge in evolutionary genetics. In this study, we use phylogenomic strategies to investigate a distinctive trait among mammals: the development of masculinizing ovotestes in female moles. By combining a chromosome-scale genome assembly of the Iberian mole, *Talpa occidentalis*, with transcriptomic, epigenetic, and chromatin interaction datasets, we identify rearrangements altering the regulatory landscape of genes with distinct gonadal expression patterns. These include a tandem triplication involving CYP17A1, a gene controlling androgen synthesis, and an intrachromosomal inversion involving the pro-testicular growth factor gene FGFR9, which is heterochronically expressed in mole ovotestes. Transgenic mice with a knock-in mole CYP17A1 enhancer or overexpressing FGFR9 showed phenotypes recapitulating mole sexual features. Our results highlight how integrative genomic approaches can reveal the phenotypic impact of noncoding sequence changes.

**DIFFERENCES**

In genome sequence and structure provide the molecular foundation for phenotypic diversity across species and enable environmental adaptation. In evolutionary genetics, linking genomic alterations to phenotypic traits has largely relied on candidate gene (I) or linkage-mapping analyses (2). However, the combination of next-generation sequencing with proximity-ligation methods, in particular Hi-C, allows the generation of chromosome-scale genome assemblies (3), introducing ample possibilities for comparative genomics. Hi-C also enables the integration of three-dimensional (3D) genome structure with transcriptional control. Vertebrate genomes are spatially organized into regulatory units, termed topologically associating domains (TADs) (4, 5). Although TADs are generally preserved across species (6, 7), studies of human disease highlighted that alterations in TAD organization can cause changes in gene expression and developmental phenotypes by rewiring enhancer-promoter contacts (8–10). Thus, analytical strategies that consider 3D organization and regulatory potential become essential for a comprehensive annotation of genomes. Here, we introduce a phylogenetic strategy that combines comparative whole-genome, epigenomic, transcriptomic, and chromatin interaction data to identify phenotype-associated genomic changes. We demonstrate the power of this approach by elucidating the molecular underpinnings of generalized intersexuality in female moles, an evolutionary trait distinctive among mammals.

In mammals, sex is determined genetically. Genetic elements direct the differentiation of the bipotential gonad into either testicular or ovarian tissue, which, in turn, leads to the development of sex-specific anatomical, hormonal, and behavioral differences (11). An exception to this paradigm occurs in moles (family Talpidae), in which XX-genotypic females develop unusual gonads composed of an ovarian part (OP) that fully supports sexual reproduction and a testicular part (TP) that lacks fertile germ cells but contains typical male cell populations, such as androgen-producing Leydig cells (12) (fig. S1). As a consequence of increased androgen synthesis, female moles develop masculinized external genitalia, as well as prominent muscles and aggressive behavior (14), traits that likely represent adaptations to a subterranean lifestyle.

**THE IBERIAN MOLE GENOME**

To investigate the molecular origins of mole ovotestes, we generated a chromosome-scale genome assembly for *Talpa occidentalis* based on long- and short-read sequencing and scaffolding using Hi-C data (Fig. 1B and fig. S2). The assembly comprises 2.099 gigabases, and up to 30% is made of transposable elements, with a repeat profile that differs from that of closely related mammals (fig. S3 and supplementary text). Combining RNA sequencing (RNA-seq) datasets and homology-based predictions, we identified 18,751 genes, including 2370 single-copy orthologs. This gene subset was used to determine 1580 one-to-one orthologous genes in nine species and build a phylogenetic tree (Fig. IC). Our analysis confirms moles as a distinct family in the order Eulipotyphla, with shrews and hedgehogs being the most closely related species (15) (supplementary text).

**EPIGENETIC AND TRANSCRIBATIONAL LANDSCAPE OF MOLE GONADAL DEVELOPMENT**

We generated epigenetic and transcriptomic profiles of mole gonads at 7 days postpartum (FP), processing TP and OP separately (fig. S2). Specifically, we produced chromatin immunoprecipitation sequencing (ChiP-seq) datasets against histone modifications (H3K4me1, H3K4me3, H3K27ac, and H3K27me3) to segment the mole genome into functional states for each tissue. Additionally, we performed assays for transposable-accessible chromatin using sequencing (ATAC-seq) (fig. S2) and intersected both datasets to predict active enhancers in each tissue (22,105 in total) (Fig. 1D and data S1). Although the TP and testis shared a higher number of putative enhancers than the TP and OP, the large number of TP-specific putative
Fig. 1. Mole genome and epigenetic and transcriptional study of ovotestis development.
(A) An Iberian mole (Talpa occidentalis) and an adult mole ovotestis. Scale bar, 500 μm.
(B) Genome assembly of T. occidentalis and gene annotation statistics. (C) Phylogenetic tree, based on fourfold degenerate sites, between selected species. mya, million years ago.
(D) Venn diagram of active enhancers (data S1).
(E) Principal component (PC) analysis of RNA-seq datasets of P7 mole gonads.

Fig. 2. Identification of genes with altered 3D chromatin regulatory landscapes.
(A) Strategy used to identify genes with altered 3D chromatin organization as a result of species-specific rearrangements.
(B) Strategy used to assign regulatory elements to candidate genes. Number of active enhancers is correlated to gene expression levels for each tissue.
(C) Correlation between the percentage of active enhancers and gene expression per tissue (orange, ovary part; green, testis part; blue, male testis) for selected candidates (full gene dataset in fig. S4 and data S7). STRA6, FGF9, and CYP17A1 display the highest positive correlation; ATM shows negative correlation.
enhancers (2726) indicates a distinct molecular profile from the testis and OP. Principal component analysis of RNA-seq data further confirmed the distinctive nature of the TP (Fig. 1E).

An analytical framework for evolutionary analyses

We combined our functional datasets with comparative genomics analyses and focused evolutionary analyses (8, 9). Rearrangements can be identified in genome comparisons as synteny breaks, defined here as alterations on the conserved colinear order of loci between species. To identify mole-specific rearrangements, we compared the mole genomes (T. occidentalis and C. cristata) with full-chromosome assemblies from human, mouse, and shrew, as the closest taxonomical outgroup with normal ovarian development. Although both genes are essential for gonadogenesis (16, 17), they show a similar expression pattern in moles and mice and are likely not relevant for mole ovotestis formation (fig. S6).

Because no relevant candidates were found, we searched for changes in 3D chromatin organization, on the basis of the hypothesis that rearrangements can alter regulatory domains and affect gene expression (8, 9). Rearrangements can be identified in genome comparisons as synteny breaks, defined here as alterations on the conserved colinear order of loci between species. To identify mole-specific rearrangements, we compared the mole genomes (T. occidentalis and C. cristata) with full-chromosome assemblies from human, mouse, and shrew, as the closest taxonomical outgroup with normal ovarian development (Fig. 2A). We used our Hi-C domain predictions to identify genes located in TADs affected by a synteny break, for a total of 2595 candidate genes.
genes considered to be susceptible to altered regulation (data S6). We filtered these candidates according to GO terms related to sex differentiation, restricting the list to 39 genes (table S1 and data S4).

Using our functional datasets, we then searched for footprints of altered gene regulation that might be the consequence of mole-specific rearrangements. We considered the nature of each rearrangement (Fig. 2B and supplementary text) and our TAD predictions, to delimit a potential region of new interactions for each candidate gene and determine the number of active regulatory elements contained within. Of the 39 candidate genes, only 17 were predicted to gain de novo interaction with regions containing active enhancers. Furthermore, we ranked the candidate genes by correlating the number of active elements with the expression levels for each tissue, as an indicative parameter of potential effects of the rearrangement on transcription (Fig. 2B, fig. S7, and data S7). A positive correlation between active enhancers and gene expression was found for 10 genes. Among the top-ranked candidates, we selected those displaying higher expression for subsequent functional validation: the androgen-related gene CYP17A1 and the pro-testicular growth factor gene FGF9.

A tandem triplication at the CYP17A1 locus is linked to increased androgen production and strength

We detected an intra-TAD tandem triplication at the mole CYP17A1 locus that creates two additional copies of the gene. Through comparative genomics analysis, we confirmed the exclusive presence of the rearrangement in the mole lineage and its absence in other mammals (Fig. 3A and fig. S8). CYP17A1 encodes for aromatase, an enzyme that converts androgens to estrogens (18), suggesting a role in female mole masculinization.

The triplication was associated with high CYP17A1 expression in testis and TP, both substantially exceeding the expression levels in mice (Fig. 3B and data S8). Searching for other genes of the steroidogenic pathway, we observed that CYP19A1, located downstream of CYP17A1, is not expressed in the TP (fig. S9). CYP19A1 encodes for aromatase, an enzyme that converts androgens to estrogens (18), and is expressed exclusively in the OP (fig. S9). It is thus expected that the high levels of CYP17A1 in the OP do not impede estrogen production and reproductive function, because of the protective effect of aromatase in degrading androgens locally. The absence of CYP19A1 expression in the TP, in combination with high CYP17A1 expression, provides a plausible explanation for the masculinization observed in female moles. We used gas chromatography–mass spectrometry to quantify serum levels of male hormones and found high levels of circulating androgens in female moles. Androgen levels were similar to those in male individuals (fig. S9), contrary to the general pattern among mammals in which males display higher levels than females.

To examine the contributions of the additional CYP17A1 alleles to increased androgen production, we analyzed RNA-seq data from mole testes and TP. The three CYP17A1 paralogues have sufficiently diverged to enable unambiguous mapping of RNA-seq reads. The two newly emerged CYP17A1-2 and CYP17A1-3 jointly contribute less than 5% of the CYP17A1 transcript (Fig. 3C). Furthermore, sequence conservation analyses revealed that they diverge more from the human sequence than CYP17A1 (fig. S10). These findings suggest that the triplication of the CYP17A1 gene itself does not explain the increased androgen levels in moles. Instead, the triplication also caused the duplication and fusion of two predicted enhancer elements, termed “enhancer A” and “enhancer B” (Fig. 3C). As the triplication does not affect any TAD boundary, the duplicated enhancers and CYP17A1 genes locate within the same regulatory domain (fig. S11). The duplicated fusion element, “enhancer A-B,” shows a high degree of sequence conservation with the original elements (84 and 90%, respectively) and high abundance of active enhancer marks (Fig. 3C). Computational predictions of binding affinities showed that enhancer A-B maintains significant binding affinities for TFs found in the original enhancer A, as well as previously uncharacterized binding affinities for TFs that are expressed in the TP (data S9). Together, these observations suggest that duplication and functional changes in regulatory sequences, rather than amplification of coding sequence, cause the observed phenotypic adaptations.

To confirm this hypothesis, we inserted the mole enhancer A-B sequence into the Cyp17a1 locus.
We identified a rearrangement at the FGF9 locus, which is exclusive to the mole lineage and not present in any other mammals examined (Fig. 4A and figs. S13 and S14). FGF9 is a known testis-determining gene that functions in positive feedback with SOX9 and inhibits the ovary-determining WNT4/β-catenin pathway (11) (fig. S15). Consequently, loss of Fgf9 in XY gonads results in down-regulation of testicular markers and male-to-female sex reversal. Comparative analyses against human, representative of the ancestral organization at the locus, revealed a large inversion that relocates a distant genomic region (26 Mb away in the human genome) to the mole FGF9 locus (Fig. 4B). Hi-C data showed that the synteny break occurs in the human FGF9 TAD, disrupting its 3D organization. Thus, the mole locus is reorganized, with most of the FGF9 TAD remaining conserved but extending beyond the synteny breakpoint on the centromeric side. This extended interaction domain is delimited by the presence of two CTCF binding sites with divergent orientation, a genomic signature associated with TAD boundaries (7) (Fig. 4B). A closer examination of the newly interacting region revealed several elements enriched for active epigenetic marks, some of them specific for the mole oovtestis (fig. S16). This interaction pattern was also validated through independent circular chromosome conformation capture sequencing (4C-seq) experiments (Fig. 4B), which confirmed the association of the FGF9 gene with the region containing the putative tissue-specific enhancers. We tested one element (fig. S16) in mouse LacZ reporter assays. Although this element displayed enhancer activity in tissues such as the eye (fig. S17), we did not observe gonadal staining, which could be due to specific requirements for additional trans-acting factors in the mole. Alternatively, it may reflect known limitations of the reporter assay that might be intensified by the interspecies nature of the experiment (19).

We then explored possible alterations on the dimorphic FGF9 expression pattern observed in other mammals, which is essential for suppressing germ cell meiosis (20). In mice, Fgf9 is first expressed in the bipotential gonad of both sexes and becomes progressively restricted to the testis and turned off in ovaries, allowing the initiation of female meiosis at embryonic day 13.5 (E13.5). In mole gonads, however, FGF9 expression is maintained after sex determination in both sexes and becomes restricted to the OP at later stages (Fig. 4C). Immunostaining analyses showed that FGF9 expression persists in the OP across the entire prenatal period and becomes confined to a thin rim at postnatal stages (P7). The spatial reduction in FGF9 expression is concomitant with the initiation of meiosis (Fig. 5A and fig. S18), which is considerably delayed in female moles until birth, an exceptional feature among mammals (21). Consequently, the observed heterochrony on mole FGF9 expression, compared with mouse, and its potential effects are suggestive of a contribution to the masculinization of female mole gonads.

We hypothesized that FGF9 expression during early XX mole gonadogenesis might prevent germ cells from entering meiosis in the OP, allowing the TP to develop further. To test this hypothesis, we engineered a bacterial artificial chromosome (BAC) construct to overexpress Fgf9 in somatic ovarian populations and generated transgenic mice through PiggyBac transgenesis and morula aggregation. Highly chimeric animals displayed early embryonic lethality, likely due to Fgf9 misexpression in other organs, an effect that precluded their study in later stages. Nevertheless, RNA-seq analysis of E13.5 ovaries demonstrated an inhibition of the meiotic process, manifested by down-regulation of meiosis markers (Fig. 5B and data S10). By contrast, low-chimera individuals composed of XX wild-type and XY mutant cells were viable and displayed Fg/f9 expression during the entire ovarian development. These animals showed a complete female-to-male sex reversal, defined by gonadal morphology and expression of male-specific factors such as SOX9 (Fig. 5C). These results directly confirm the potential of altered FGF9 expression to induce masculinization in mammalian XX females.

**Fig. 5. FGF9 sustained expression delays meiosis and promotes XX gonadal masculinization.**

(A) Spatiotemporal expression of FGF9 and the meiotic marker SYCP3 [immunostaining, green; 4′,6-diamidino-2-phenylindole (DAPI), blue]. Insets display zoomed regions from OP. Scale bars, 100 μm. (B) Volcano plot from RNA-seq of XX mutant versus wild-type gonads (E13.5) and GO analysis. (C) Hematoxylin and eosin staining of XX gonads of adult mutants and wild-type controls. Scale bars, 200 μm.

**An inversion at the FGF9 locus is associated with delayed meiosis and gonadal masculinization**

We identified a rearrangement at the FGF9 locus, which is exclusive to the mole lineage and not present in any other mammals examined (Fig. 3D). Adult knock-in mice carrying the additional mole enhancer displayed a three- and twofold up-regulation of Cyp17A1 expression in females and males, respectively. The increased expression occurs in the same cell type (steroidogenic cells) as in wild-type controls (fig. S12). A similar effect was observed at embryonic stages (fig. S12), thus confirming the increased in vivo activity of the mole-specific fusion enhancer in gonadal tissue. The up-regulation of Cyp17A1 expression was accompanied by an increase in circulating testosterone in females and males (two- and threefold respectively) (Fig. 3E). Because androgens have potent anabolic effects in muscle, we performed a grip-strength test that revealed a significant increase in muscle, we performed a grip-strength test that revealed a significant increase in muscle strength in mutants compared with wild-type controls (Fig. 3F). Thus, the regulatory nature of the CYP17A1 rearrangement offers a plausible molecular mechanism for the observed shift in hormone levels and the corresponding phenotype.

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Discussion

Vertebrate sex determination is controlled by a limited set of key regulators whose hierarchy has evolved dynamically (11). Most of these genes display pleiotropic effects, controlling regulatory networks in several tissues, often making them indispensable for embryonic viability (22, 23). It is thus plausible that variations in sex determination derive from regulatory changes that alter gene expression patterns but preserve essential functions, as suggested for other evolutionary adaptations (1, 2). These genomic changes appear to be linked to the evolutionary success of moles and demonstrate that regulatory innovation can overcome a priori seemingly incompatible situations such as female fertility in the presence of high androgen levels. But how could an intersex phenotype evolve, and what is its adaptive significance? One potential explanation is the anabolic effects of androgens on muscle mass. Mole ovoestes may have evolved to equalize muscular strength among sexes by increasing androgens in females. Such an advantage, combined with other androgen-derived effects such as aggressive behavior, might have been key for the adaptation to the demanding requirements of a burrowing underground lifestyle (14).

This study also highlights the evolutionary importance of genomic rearrangements and their potential to modulate developmental gene expression. In most cases, genomic rearrangements would have limited effects, as they preserve whole regulatory units and do not disrupt enhancer-promoter interactions (6, 24). However, as shown here, they can also alter the regulatory potential in the local environment of a TAD, as for the CYP17A1 locus, or shuffle the functional content of distant TAD units, exemplified by the FGF9 locus. Similar effects have been observed in human genetic diseases for enhancer duplications (25) and for inversions (8, 9). Therefore, regulation of genes by enhancer elements and their organization in TADs might constitute a mechanism of “evolvability” resulting from a modular system with vast flexibility and enormous evolutionary potential for the origin of novelties. On the basis of this modularity, genomic rearrangements can easily change and reconstitute complex expression patterns, thereby contributing to the saliency nature of phenotypic innovation observed in many phylogenetic lineages. We expect that approaches considering these important aspects will eventually reveal the evolutionary basis of many other traits and substantially enhance the toolbox for unlocking the secrets of phenotypic variation and adaptation across the animal kingdom.

REFERENCES AND NOTES


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Competing interests: The authors declare no competing interests. Data and materials availability: The Whole Genome Shotgun project has been deposited at DDBJ/ENA/GeneBank under the accession number PRJN449424L. All transcription and epigenetic data have been deposited in GEO under accession number GSE25099. Alignments have been deposited at https://www.ncbi.nlm.nih.gov/Genome/PRJ.html. All datasets have been deposited at https://www.ncbi.nlm.nih.gov/Genome/PRJ.html. We thank the members of the Lupiáñez and Mundlos labs for fruitful discussions.

SUPPLEMENTARY MATERIALS

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Materials and Methods

Supplementary Text

Figs. S1 to S20

Tables S1 to S58

References (26–100)

M.D.R. Reproducibility Checklist

Data S1 to S10

View/request a protocol for this paper from Bio-protocol.

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6 of 6
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Intersexuality in female moles
Female moles are intersexual and develop masculinizing ovotestes, a distinctive trait among mammals. Real et al. investigated the origin of this trait by sequencing the Iberian mole genome and applying comparative strategies that integrate transcriptomic, epigenetic, and chromatin interaction data. They identified mole-specific genomic rearrangements that alter the three-dimensional regulatory landscape of the androgen-converting gene CYP17A1 and the pro-testicular factor gene FGF9, both of which show distinct expression patterns in mole gonads. The use of transgenic mice confirms the capability of these factors to increase circulating testosterone levels and to induce gonadal masculinization. This study highlights how integrative approaches can reveal the phenotypic impact of genomic variation. Science, this issue p. 208