major marine taxa was generated using the neighbour-joining method (positions 226–878, *E. coli* numbering) excluding positions with <50% conservation. Uncultured archaeon 'KTK 31A' (GenBank accession number AJ133625) served as the outgroup.

Received 22 June; accepted 1 November 2004; doi:10.1038/nature03170.

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Supplementary Information accompanies the paper on **www.nature.com/nature**.

Acknowledgements This project was funded by the US National Science Foundation. We acknowledge TIGR seqcore, informatics, and IT groups for support. We thank A. Cook for analysis of the taurine pathway and T. M. Davidsen, N. Zafar, L. Zhou, M. Gwinn and T. Creasy for bioinformatics support.

Competing interests statement The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to M.A.M. (mmoran@uga.edu). The complete sequence has been submitted to the GenBank database under accession numbers s_pomeroyi_dss_3_267 CP000031 and s_pomeroyi_dss_3_267 CP000032.

In the platypus a meiotic chain of ten sex chromosomes shares genes with the bird Z and mammal X chromosomes

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Two centuries after the duck-billed platypus was discovered, monotreme chromosome systems remain deeply puzzling. Karyotypes of males[1], or of both sexes[2–4], were claimed to contain several unpaired chromosomes (including the X chromosome) that form a multi-chromosomal chain at meiosis. Such meiotic chains exist in plants[5] and insects[6] but are rare in vertebrates[7]. How the platypus chromosome system works to determine sex and produce balanced gametes has been controversial for decades[1–4]. Here we demonstrate that platypus have five male-specific chromosomes (Y chromosomes) and five chromosomes present in one copy in males and two copies in females (X chromosomes). These ten chromosomes form a multivalent chain at male meiosis, adopting an alternating pattern to segregate into XXXXX-bearing and YYYYY-bearing sperm. Which, if any, of these sex chromosomes bears one or more sex-determining genes remains unknown. The largest X chromosome, with homology to the human X chromosome, lies at one end of the chain, and a chromosome with homology to the bird Z chromosome lies near the other end. This suggests an evolutionary link between mammal and bird sex chromosome systems, which were previously thought to have evolved independently.

Monotremes (mammalian subclass Prototheria) were the earliest offshoot of the mammalian lineage, diverging 210 million years ago from therian mammals (eutherians and marsupials)[8]. Only three monotremes are extant: the platypus (*Ornithorhynchus anatinus*) and two echidna species. Their phylogenetic position, and their mix of mammalian, reptilian and specialized morphological and physiological features, makes monotremes uniquely valuable for comparative genomics and for understanding sex chromosome evolution[9,10].

The correct chromosome number ($2n = 52$) in male and female platypus was established in 1975 (ref. 3). Measurements and banding of mitotic chromosomes revealed several that lacked obvious homologues in males. The largest, defined as the X chromosome because it was present in one copy in males and two in females, shares many genes with the eutherian and marsupial X chromosome[10]. However, the presence of one or more male-specific chromosomes, and of unpaired mitotic chromosomes in females, has long been controversial[1,2,4]. No genes have been mapped to any unpaired chromosomes except for this X chromosome[10], and no male-specific sequences have ever been identified.

At male meiosis in monotremes, several chromosomes assemble in a multivalent chain, analogous to meiotic chains in invertebrates that are the result of translocation heterozygosity. The X chromosome lies at one end, but the other elements are unknown and their numbers in platypus are variously reported as eight[1] or ten[2]. How

the chain elements segregate at meiosis to produce balanced gametes is completely mysterious.

Sex determination in monotremes is another mystery. No platypus homologue of the therian testis-determining gene *SRY* (sex-determining region Y) can be identified (Supplementary Note 1).

Whereas mammals show XY male heterogamety, and testis determination is triggered by the Y-borne *SRY* gene, birds have ZW female heterogamety with no evidence of sex-specific *SRY* sequences[11,12]. The chicken Z chromosome includes the highly conserved *DMRT1* gene (*Drosophila doublesex* and *Caenorhabditis elegans mab-3*-related transcription factor 1)[13]. *DMRT1* maps to the Z, but not the W chromosome in emu[14] as well as chicken[13], making it a candidate for a dosage-sensitive sex-determining gene in birds. Recently a duplicated copy of *DMRT1* was identified on the medaka Y chromosome[15,16]. However, *DMRT1* maps to human chromosome 9, and is autosomal in mammals, although two copies are required for testis differentiation[17].

Both mammal and bird sex chromosome systems are believed to have evolved from autosomal pairs. An earlier proposition that the mammal XY and the bird ZW systems evolved from the same pair[18] was refuted by the demonstration of homology between the chicken Z chromosome and human chromosome 9. This implied that the two systems evolved independently from different pairs of autosomes[13].

To resolve the longstanding controversy over platypus mitotic and meiotic chromosomes we generated whole chromosome paints by flow sorting chromosomes from cultured male platypus fibroblasts, and identified them by fluorescence *in situ* hybridization (FISH, see Methods) to metaphase chromosomes of males and females[19].

Eight chromosome paints (Fig. 1; Supplementary Table 1) showed different hybridization patterns in females and males, and

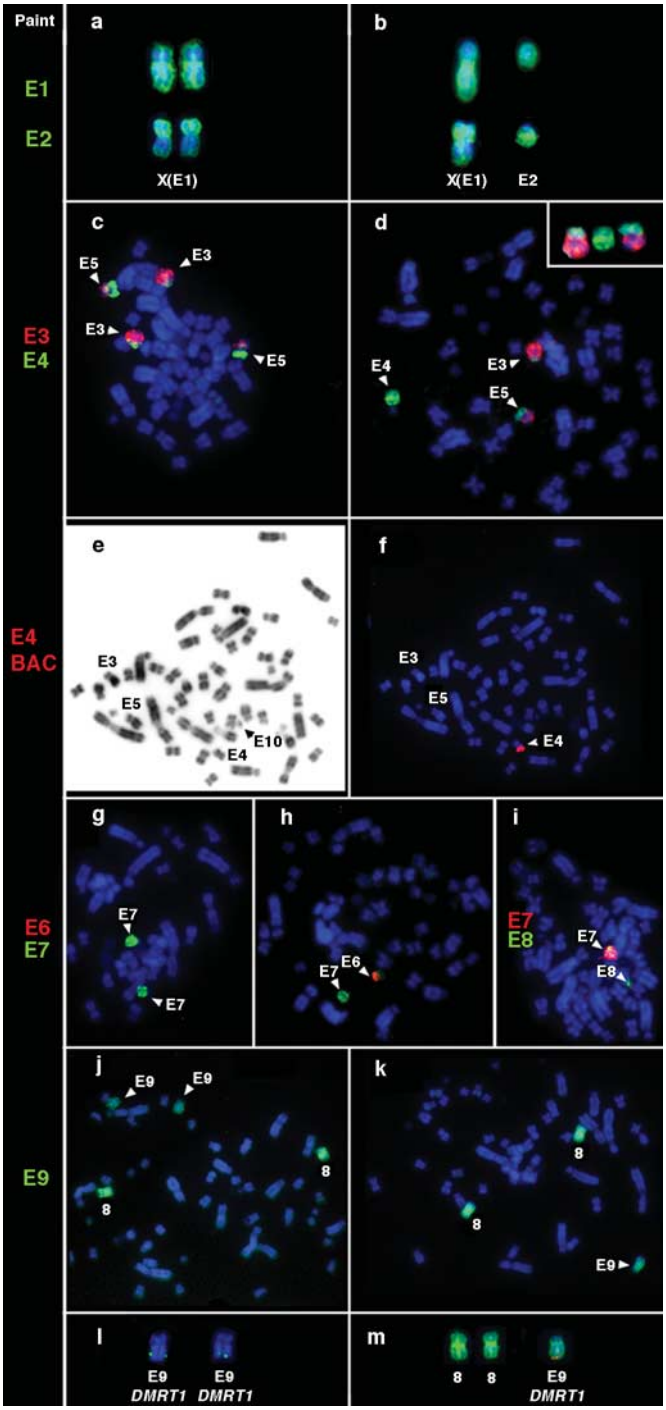

Figure 1 Fluorescence *in situ* hybridization (FISH) of chromosome paints on mitotic chromosomes. The green (FITC-labelled) and red (Cy3-labelled) E symbols indicate which paint was hybridized. **a**, **c**, **g**, **j**, FISH on female metaphase spreads; **b**, **d**, **f**, **h**, **i**, **k**, FISH on male metaphase spread. **e**, Inverted DAPI picture of the male metaphase in **f**. **l**, Localization of the *DMRT1*-containing BAC (green) in female. **m**, Co-localization of the *DMRT1*-BAC (red) and E9 (paint E9, green) in male.

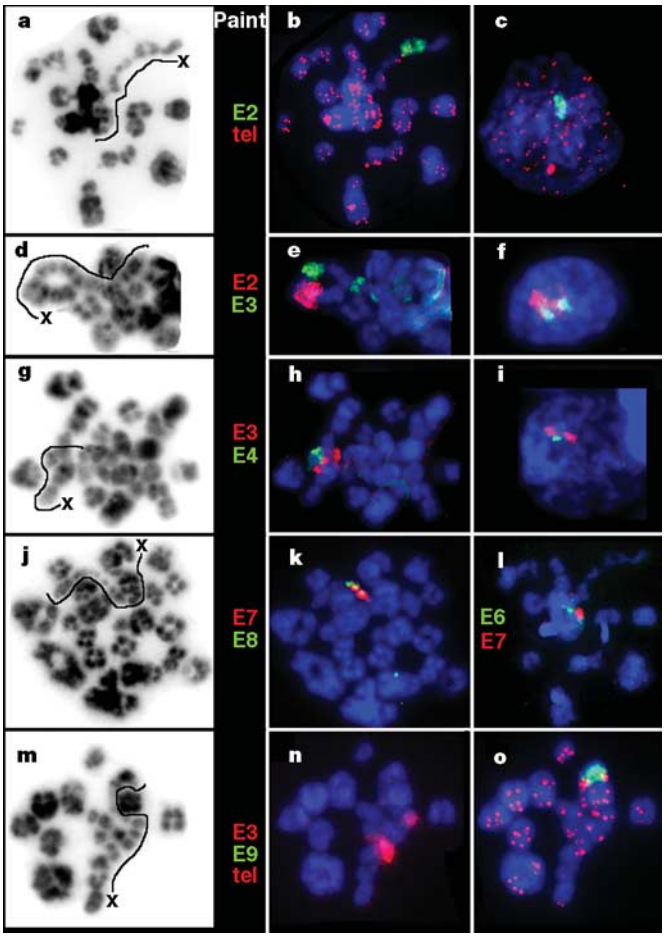

Figure 2 Order of chromosomes in the meiotic chain. Left column (**a**, **d**, **g**, **j**, **m**) shows the inverted DAPI picture of the meiotic metaphase I cells in the middle column (**b**, **e**, **h**, **k**, **n**). The chain is outlined by a black line. The start is indicated with an X. The green (FITC) and red (Cy3) labels indicate which paint was hybridized. **c**, Pachytene cell; Xp and E2 are paired. **f**, Pachytene cell; proximity of X/E2 with the unpaired E3 and E5. **i**, Pachytene cell. **l**, Metaphase I cell. **o**, Metaphase I cell; FISH with E9 and a telomeric repeat (tel).

hybridized to the meiotic chain (Fig. 2). To avoid pre-judging the identity of the chromosomes in the chain, we designated them elements E1 (the X), E2, E3 and so on. Five of these paints detected homology with other chain elements; for instance, the E2-derived paint hybridized also to E1p. The painting patterns at mitosis (Fig. 1), and their positions at meiosis (Fig. 2) identified nine unpaired chromosomes (E1–E9) in males that form the meiotic translocation chain. A tiny tenth element E10, present as the smallest chromosome in the male karyotype but absent from females, is clearly attached to E9 at the end of the chain (Fig. 1e, Fig. 3a, b and Supplementary Fig. 2). Thus males have a single copy of five X chromosomes that are paired in females, and five male-specific Y chromosomes. Females contain no unpaired mitotic chromosomes. Technically, therefore, the platypus has an $X_1X_1X_2X_2X_3X_3X_4X_4X_5X_5$ female: $X_1Y_1X_2Y_2X_3Y_3X_4Y_4X_5Y_5$ male sex chromosome system.

To discover whether the male-specific elements (Ys) harbour male-specific DNA sequences, despite their partial homology with X chromosomes, we used paint E4 to probe a platypus bacterial artificial chromosome (BAC) library. Several positive clones hybridized specifically to E4 but not E3 or E5 in males, and produced no signal at all in females, implying that E4 contains male-specific DNA (Fig. 1e, f).

To enumerate the elements of the chain, telomere repeats were hybridized to male meioses to detect chromosome ends. Chains clearly contained ten elements (Fig. 3a, b).

To determine the order of elements in the chain, paints E1–E9 were hybridized onto male meiotic metaphase I cells. This confirmed that the previously identified X chromosome lies at one end of the chain (Fig. 2a, b), its short arm paired with the male-specific E2 (Fig. 2c). Chromosome E3 lay close to E2 (Fig. 2f). These elements show no detectable homology, so they must pair over a very restricted region or form some other kind of association such as that between the X and autosomal meiotic chains in artificially constructed translocation heterozygous mice[20,21]. Cross-hybridization between paint E3 and element E5q demonstrated homology between these elements, but chromosome order in the middle of the chain was clearly E3–E4–E5 (Fig. 2e, g–i).

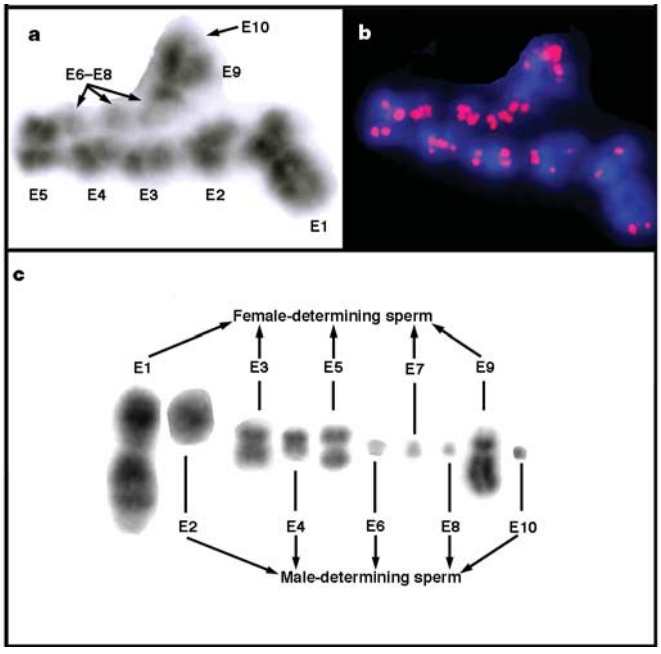

Figure 3 The elements of the chain. **a**, Inverted DAPI picture of a meiotic chain in male platypus. **b**, The same meiotic chain as in **a**, hybridized with a telomeric repeat in red (TAACCCx7). **c**, Order and segregation of the members of the chain (mitotic chromosomes).

E5 is followed in the chain by the male-specific E6, which shares some homology with the next element E7 (Figs 1h and 2l). E7 is followed by the small male-specific E8 (Fig. 2k). The chain ends with the large unpaired element E9 (Fig. 2o) to which is attached the tiny male-specific terminal element E10 (Figs 2o and 3a, b). We

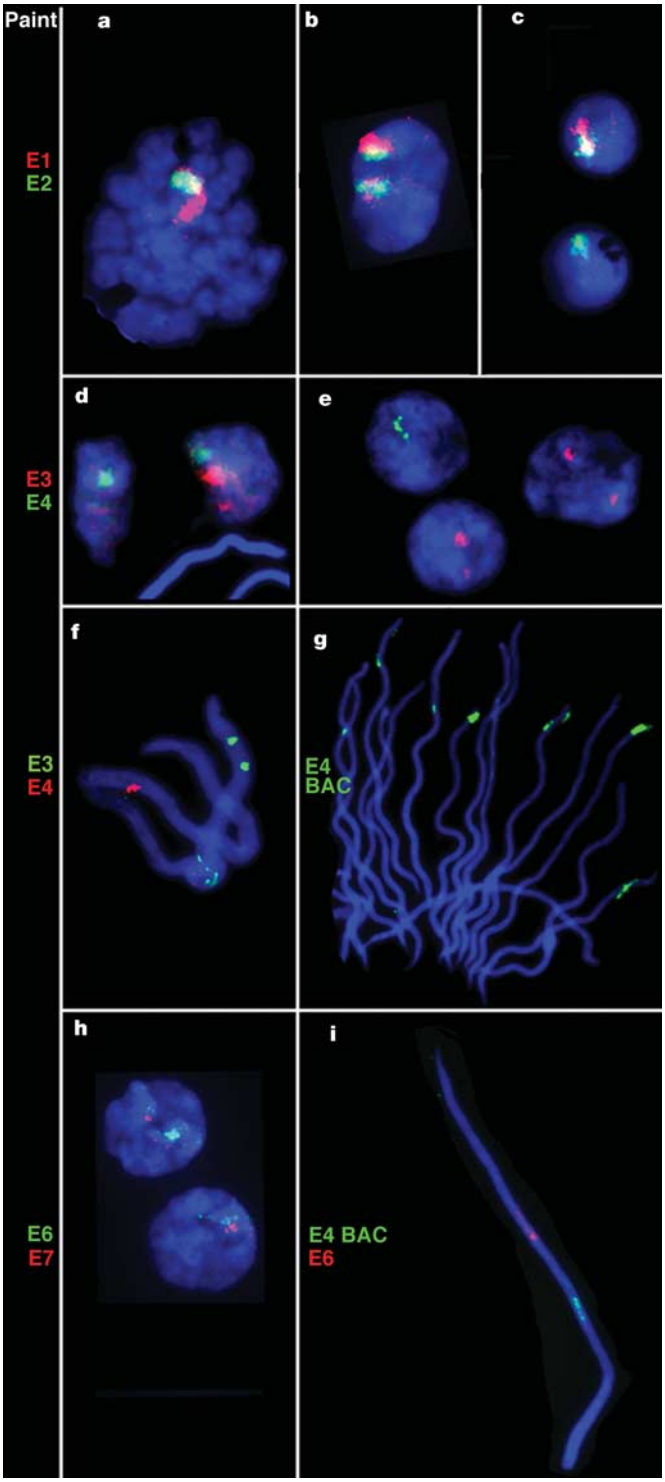

Figure 4 Alternate segregation of the chain. The column on the left indicates which paints were used, in green (FITC) or red (Cy3). **a**, Metaphase I cell. E1 and E2 are paired. **b**, Telophase I cell showing segregation of E1 and E2. **c**, Round spermatids. **d**, Meiotic telophase I cell showing segregation of E3/E5 and E4. **e**, Round spermatids. E3/E5 or E4 are present. **f**, Elongated sperm with E3/E5, or E4. **g**, Bundle of sperm showing an alternating pattern of male- and female-determining (labelled and unlabelled, respectively) sperm. **h**, Round spermatid with either E6 or E7. **i**, Elongated sperm with E4 and E6.

conclude that adjacent elements share homology, and the chain represents a complex system of sex-linked translocation heterozygosity.

Segregation of partially homologous adjacent elements would lead to unbalanced sperm. It was therefore proposed that the elements of a translocation chain undergo alternate segregation to form only two different types of spermatids containing normal or translocated elements[1,9]. The alternating pattern of E-odd (E1, 3, 5, 7, 9) and E-even (E2, 4, 6, 8, 10) elements that we observed in the platypus chain, equivalent to alternating X and Y chromosomes, is consistent with this proposal. To demonstrate alternate segregation directly, we co-hybridized several combinations of E-even and E-odd paints onto telophase 1 cells and spermatids. E-odd and E-even paint(s) never co-located (Fig. 4 and Supplementary Table 2), implying that highly efficient alternating segregation occurs in platypus.

Fertilization of eggs (all E-odd: $X_1X_2X_3X_4X_5$) by E-odd sperm would result in an $X_1X_1X_2X_2X_3X_3X_4X_4X_5X_5$ female zygote homozygous for all X chromosomes. Fertilization by E-even ($Y_1Y_2Y_3Y_4Y_5$) sperm results in $X_1Y_1X_2Y_2X_3Y_3X_4Y_4X_5Y_5$ male zygotes with five X and five Y chromosomes (Fig. 3c).

Multiple sex chromosome systems deriving from X-autosome or Y-autosome translocations usually impose sterility in male carriers. Heterozygosity for sex chromosome–autosome translocations in mouse and humans leads to meiotic arrest or predominantly aneuploid sperm[20,21], although fertile $X_1Y_1X_2Y_2$ sex chromosome systems occur in a few mammalian taxa[7,22,23]. The efficient 5X:5Y alternate segregation in platypus is therefore remarkable.

The evolution of the 5X and 5Y chromosomes of the platypus translocation chain is of considerable general interest. As for translocation complexes in invertebrates, this chain is likely to have evolved step by step, starting from a translocation between a heteromorphic sex pair and an autosome. Additional chromosome pairs became involved subsequently by serial sex chromosome–autosome translocations.

The original sex pair is therefore likely to be represented by an X and Y chromosome at one or the other end of the chain. Which end is ancestral can be assessed from the degree of homology between terminal X and Y elements. X_1 (with homology to the human X) and Y_1, at one end of the chain, are completely homologous over the whole short arm of the X chromosome (Fig. 1a, b). This large pseudoautosomal region suggests that Y_1 is at an early stage of Y chromosome differentiation. In contrast, the tiny Y_5 at the other end of the chain is almost entirely degenerated, and its homology to X_5 is too small to be detected by our methods (Fig. 1j, k). This implies that Y_5 has undergone almost complete degeneration, and is near the endpoint proposed for heteromorphic sex chromosome systems[10,24]. Indeed, this tiny male-specific element appears to be absent from the mitotic karyotype of the related echidna, and from its 9-membered chain[25]. Thus we propose that the chain was initiated from translocation of an original X_5Y_5 pair with an autosome.

In the absence of any significant autosomal mapping, the identity of the original X_5Y_5 pair cannot be immediately ascertained. Speculating that the platypus sex chromosome system might retain some features of the bird system, we used *DMRT1* as a marker to determine whether X_5 and/or Y_5 has homology to the bird Z chromosome. We therefore cloned the presumed *DMRT1* gene from a platypus BAC library and sequenced two exons and three conserved intron sequences (N.E.-M. *et al.*, manuscript in preparation; Supplementary Fig. 3). We mapped *DMRT1* to male and female platypus chromosomes, and detected unambiguous signals on X_5 (Fig. 1l, m). No signal was present on Y_5. This implies that X_5 has at least some homology to the bird Z chromosome.

We therefore conclude that the platypus sex chromosome chain was initiated from an original sex chromosome pair with homology to the bird ZW system. During early mammalian evolution, sequential translocation recruited four other autosome pairs, most recently the pair representing the mammalian XY system.

Our observation that the two ends of the chain share homology to the mammal XY and the bird ZW systems provides the first link between these sex chromosome systems and challenges the accepted view that mammal and bird sex chromosomes evolved independently.

In the absence of *SRY*, it is unclear how monotreme sex is determined. The degenerate Y_5 is unlikely to bear a male-dominant gene because it is small and heterochromatic, and has been lost in the related echidna. E2 at the other end is also unlikely because of its complete homology and meiotic pairing with Xp. One or more male-determining genes could be present on any of the other male-specific (Y) chromosomes. The *DMRT1* gene on chromosome X_5, being present in two doses in females and one in males (that is, the opposite situation from birds) is unlikely to play a similar role. However, the unprecedented localization of *DMRT1* on the sex chromosomes in a mammal raises the possibility that a *DMRT1*-based sex-determining system was ancestral to all mammals. *SRY* could either have been lost in monotremes, or have evolved on the proto-Y chromosome of therian mammals after their divergence from monotremes 210 million years ago. □

Methods

Isolation of chromosome-specific painting probes

Cell culture, flow sorting, chromosome paint production and FISH were performed according to the protocol described previously[26]. In short, chromosomes were stained with 40 μg ml^{-1} chromomycin A3 (Sigma), 2 mmol l^{-1} $MgSO_4$ and 2 μg ml^{-1} of Hoechst 33258 (Sigma) and incubated for at least 2 h. Ten minutes before flow analysis, sodium sulphate and sodium citrate were added to a final concentration of 10 and 25 mmol l^{-1}, respectively. The stained chromosomes were sorted on a FACStar Plus flow sorter (Becton Dickinson). Five-hundred of each chromosome type were sorted directly into separate 500-μl polymerase chain reaction (PCR) tubes containing 30 μl sterile distilled water. Flow-sorted chromosomes were used as templates for amplification or labelling by degenerate oligonucleotide-primed PCR (DOP-PCR[27]). In the labelling reaction 50 μM dATP, dGTP and dCTP; 20 μM dTTP; and 50 μM of either biotin-16-dUTP or digoxigenin-11-dUTP were added. Chromosome paints were assigned to chromosomes by fluorescence microscopy (Leica DMRXA).

Preparation of chromosomes, meiotic cells and sperm

Mitotic metaphase chromosomes were prepared from exponentially growing platypus fibroblast cell lines from different individuals. Primary cultures were set up from toe web from previously trapped animals[28] as well as from four male and one female cell lines derived from animals captured at the upper Barnard river, New South Wales, Australia during breeding season (AEEC permit R.CG.07.03 (F.G.), Environment ACT permit LI 2002 270 (J.A.M.G.), NPWS permit A193 (R.C.J.)). The captured animals were killed with an intraperitoneal injection of pentobarbitone sodium (Nembutal) at a dose of 0.1 mg g^{-1} body weight. Meiotic cells and sperm were obtained by crushing the testis. The material was either directly fixed in methanol/acetic acid (3:1) or incubated in 0.075 M KCl at 37 °C as hypotonic treatment and then fixed.

Chromosome painting

Chromosome paints were hybridized to chromosomes of different individuals to exclude cell culture artefacts. For FISH, the slides were treated with 100 μg ml^{-1} RNase A/2 × SSC at 37 °C for 30 min and with 0.01% pepsin in 10 mM HCl at 37 °C for 10 min. After re-fixing for 10 min in 1 × PBS, 50 mM $MgCl_2$, 1% formaldehyde, the preparations were dehydrated in an ethanol series. Slides were denatured for 2.5 min at 75 °C in 70% formamide, 2 × SSC, pH 7.0, and again dehydrated. For hybridization of one half-slide, 10 μl of biotinylated and/or digoxigenated probe DNA was coprecipitated with 10–20 μg of boiled genomic platypus DNA (as competitor), and 50 μg salmon sperm DNA (as carrier), and redissolved in 50% formamide, 10% dextran sulphate, 2 × SSC. The hybridization mixture was denatured for 10 min at 80 °C. Preannealing of repetitive DNA sequences was carried out for 30 min at 37 °C. The slides were hybridized overnight in a moist chamber at 37 °C. The slides were then washed three times for 5 min in 50% formamide, 2 × SSC at 42 °C and once for 5 min in 0.1 × SSC, pH 7.0 at 60 °C and blocked with 4 × SSC, 3% BSA, 0.1% Tween 20 at 37 °C for 30 min. Probes were detected with fluorescein isothiocyanate (FITC)-conjugated avidin and Cy3-conjugated anti-digoxigenin antibody. Chromosomes and cell nuclei were counterstained with 1 μg ml^{-1} 4,6-diamidino-2-phenylindole (DAPI) in 2 × SSC for 1 min and mounted in 90% glycerol, 0.1 M Tris-HCl, pH 8.0 and 2.3% DABCO. Images were taken with a Zeiss Axioplan epifluorescence microscope equipped with a CCD (charge-coupled device) camera (RT-Spot, Jackson Instruments), which was controlled by an Apple Macintosh computer. IPlab imaging software was used to capture grey scale images and to superimpose the source images into a colour image.

Isolation of platypus specific BAC clones and platypus *DMRT1*

A commercially available platypus BAC library (OA__Bb, Clemson University Genomics Institute, USA) was used to screen for male-specific BAC clones as well as for the platypus

DMRT1 gene. As a probe, 10 μl of DOP-PCR amplification of paint E4 were used. For *DMRT1* a 287-base-pair (bp) *Pst*I digest of chicken *DMRT1* complementary DNA, containing the DM (Doublesex and MAB-3) domain was hybridized. For both experiments the probe DNA was labelled radioactively with [^{32}P]-dATP and the BAC library screened according to the manufacturer's instructions (http://www.genome.clemson.edu/protocols/hyb_filter.html). Positive clones were ordered from the Clemson University Genomics Institute (CUGI). For *DMRT1*, a final positive clone was verified by PCR using primers from conserved *DMRT1* specific regions[29]. This clone was then sequenced after subcloning into a TOPO Shotgun subcloning kit (Invitrogen) according to the manufacturer's instructions. Sequences were deposited at EMBL. The *DMRT1* BAC as well as the E4 specific BAC clones were labelled by standard nick translation with biotin or digoxigenin and 400–600 ng of labelled DNA were used to map the clones by FISH, as described above.

Received 17 June; accepted 8 September 2004; doi:10.1038/nature03021.
Published online 24 October 2004.

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Supplementary Information accompanies the paper on **www.nature.com/nature**.

Acknowledgements We thank P. Miethke and D. McMillan for technical assistance, D. Zarkower for providing the chicken *DMRT1* cDNA and A. Pask for information on *SOX* genes and *SRY* in monotremes, and provision of Supplementary Fig. 1. This work was supported by the Australian Research Council (F.G. and J.A.M.G.). W.R. and M.A.F.-S. are supported by a Wellcome Trust grant to the Cambridge Resource Centre for Comparative Genomics. Facilities were provided by Macquarie Generation and Glenrock Station, NSW. Approval to collect animals was granted by the New South Wales National Parks and Wildlife Services, New South Wales Fisheries, Environment Australian Capital Territory and the Animal Experimentation Ethics Committee, Australian National University.

Competing interests statement The authors declare that they have no competing financial interests.

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Lipocalin 2 mediates an innate immune response to bacterial infection by sequestrating iron

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Although iron is required to sustain life, its free concentration and metabolism have to be tightly regulated[1]. This is achieved through a variety of iron-binding proteins including transferrin and ferritin[2]. During infection, bacteria acquire much of their iron from the host by synthesizing siderophores that scavenge iron and transport it into the pathogen[3,4]. We recently demonstrated that enterochelin, a bacterial catecholate siderophore,

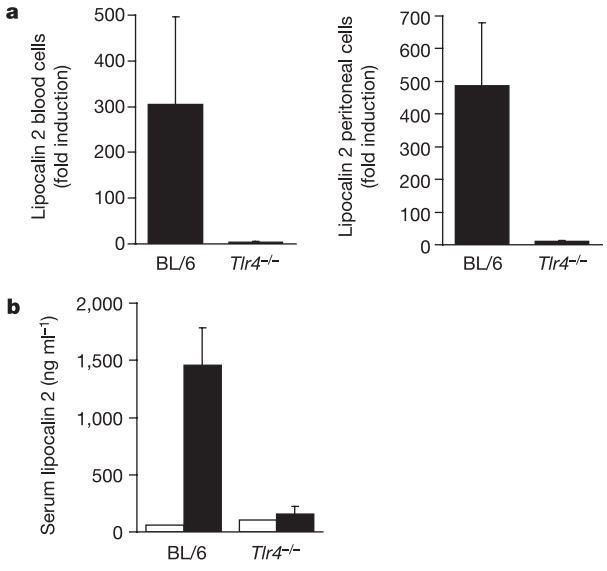

Figure 1 Lipocalin 2 production is induced through TLRs. Lipocalin 2 mRNA and protein measured 4 h after i.p. injection of C57BL/6 wild-type and TLR4-deficient mice with 10 μg LPS ($n = 3$ mice). **a**, Lipocalin 2 mRNA from blood cells and peritoneal cells is shown as fold induction values relative to the mRNA from PBS-injected mice. **b**, Serum levels of lipocalin 2 protein after LPS injection (filled bars) are compared to the levels in PBS-injected mice (open bars). Errors bars show the s.d. for each experiment.