Methods

Neurophysiology. Experimental animals were collected at Inchanga (KwaZulu/Natal, South Africa) and all field experiments conducted in February 1994–96 and March 1998. Numbers of sensory cells were determined either by backfilling the axons with cobalt lysine and intensifying the staining with silver according to standard methods[23] (for A2–A6), or by counting the number of attachment cells in 5-μm cross-sections of the chordotonal organ stained with methylene blue (for A1). Extracellular recordings of the afferent activity of pleural chordotonal organ receptors were obtained with silver-wire hook electrodes attached to nerve N1 of the respective neuromeres of the metathoracic ganglion or abdominal ganglia. Tuning curves for A2–A6 receptors were obtained by stimulating the preparation with digitized, pure-tone sounds pulses (sampling rate 44 kHz; 50 ms duration) of variable frequency (0.5–8 kHz) and intensity. Tuning curves of A1 receptors were established in a sound-damped chamber in Graz as previously described[24]. To determine how well the time pattern of the male signal is represented in the spike discharge of female chordotonal organs, we stimulated receptors in A2–A6 with digitized models of the male call (sampling rate 44 kHz) at SPLs ranging from 55 to 84 dB, thus simulating different sender–receiver distances.

Behaviour. Playback experiments were conducted as described[12]. Chordotonal organ ablation was done on anaesthetized females by opening the body wall at the attachment site of the hearing organ in A1, removing the organ and sealing the opening with histoacryl. Control animals were similarly sham-treated. Bilaterally operated females were tested the following day using playbacks of the male call at various intensities. Threshold was defined as the SPL of the male call that elicited at least one female chirp in response to the male call in four out of five presentations. In all ablation experiments we subsequently confirmed the absence of neural activity in the afferent nerves of the receptors in A1 with neurophysiological methods. Behavioural tuning experiments were performed in a sound-damped room using digitized, pure-tone sound pulses (sampling rate 22 kHz; 880 ms duration) of 1.7 and 4 kHz, randomly presented at 75 dB SPL at the position of the female and intervals of 40 s.

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A proposed path by which genes common to mammalian X and Y chromosomes evolve to become X inactivated

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Mammalian X and Y chromosomes evolved from an autosomal pair; the X retained and the Y gradually lost most ancestral genes[1,2]. In females, one X chromosome is silenced by X inactivation, a process that is often assumed to have evolved on a broadly regional or chromosomal basis[3]. Here we propose that genes or clusters common to both the X and Y chromosomes (X–Y genes) evolved independently along a multistep path, eventually acquiring dosage compensation on the X chromosome. Three genes studied here, and other extant genes, appear to be intermediates. *ZFX*, *RPS4X* and *SMCX* were monitored for X inactivation in diverse species by assaying CpG-island methylation, which mirrors X inactivation in many eutherians. *ZFX* evidently escaped X inactivation in proto-eutherians, which also possessed a very similar Y-linked gene; both characteristics were retained in most extant orders, but not in myomorph rodents. For *RPS4X*, escape from X inactivation seems unique to primates. *SMCX* escapes inactivation in primates and myomorphs but not in several other lineages. Thus, X inactivation can evolve independently for each of these genes. We propose that it is an adaptation to the decay of a homologous, Y-linked gene.

By studying differences and similarities among homologous genes in extant species, one can draw inferences about ancestral genes and map points of evolutionary divergence. In this manner, one can explore the coevolution of the X and Y chromosomes and the evolution of epigenetic phenomena such as X inactivation. We first examined the inactivation status of individual X-linked genes in a wide range of mammalian species. Specialized reagents that are analogous to those used to assess X inactivation in humans and mice are unavailable for other species[4]. However, methylation of CpG islands, which exist at the 5′ ends of many genes[5], proved to be a widely applicable alternative. For human and murine X-linked genes, a perfect correlation has been observed between 5′ CpG-island methylation and X-inactivation status: transcriptionally silent, X-inactivated alleles are methylated, whereas active alleles are unmethylated[6]. To determine whether this correlation extends to a broad range of eutherians, we examined CpG-island methylation at *ALD*, an X-linked gene known to be X-inactivated in humans[7,8]. Eighteen species representing nine eutherian orders were tested. In all 18 species, methylation was observed in females, where *ALD* is presumably silenced on the inactive X chromosome; no methylation was observed in males, where the single X chromosome is active (Figs 1 and 2). This suggests that CpG-island methylation accompanies X inactivation in a wide range of eutherians.

We then explored the status, in diverse mammals, of three X-

linked genes that escape X inactivation in humans and that bear CpG islands that are amenable to methylation assays: *ZFX*, *RPS4X* and *SMCX*. We examined the *ZFX* CpG island[9,10] in 19 eutherian species representing eleven orders. In 15 species, neither female nor male DNA exhibited methylation, indicating that *ZFX* escapes X inactivation (Fig. 1). Methylation was observed in females of only four species: mouse (where *Zfx* was previously shown to be X-inactivated[11,12]), rat, hamster and lemming. These species are monophyletic, having evolved from a common ancestor not shared by any of the other 15 eutherian species examined (Fig. 2).

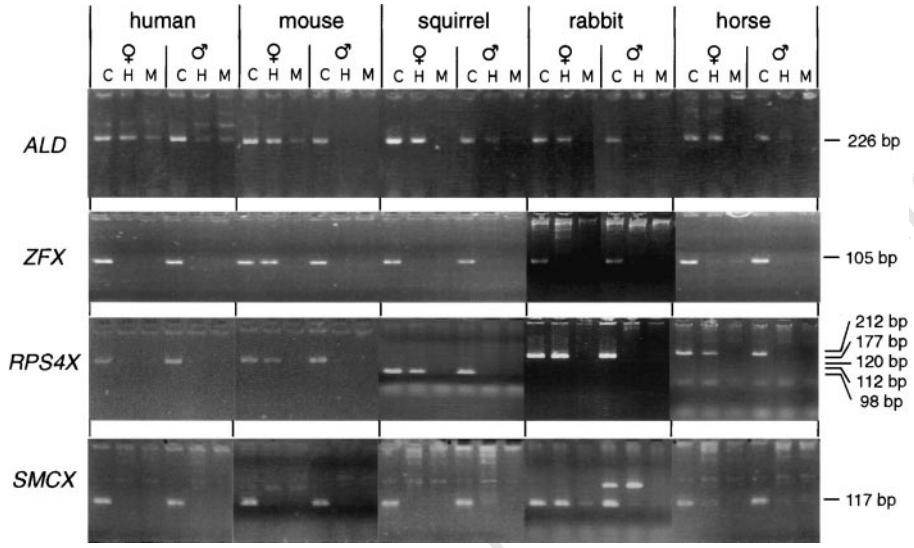

Figure 1 CpG-island methylation studies of four genes in five eutherian species. Female and male genomic DNAs digested with *Hin*dIII (C, positive 'control') or *Hpa*II (H) or *Msp*I (M, negative control) were used as templates in PCR assays. Expected product sizes (in base pairs) are indicated; *RPS4X* product size differs in each species. A strong product in the female H lane (comparable to the female C lane, and stronger than the male H lane) indicates that the gene is methylated and X inactivated, whereas a weak or absent product in the female H lane indicates that the gene is not methylated and escapes X inactivation.

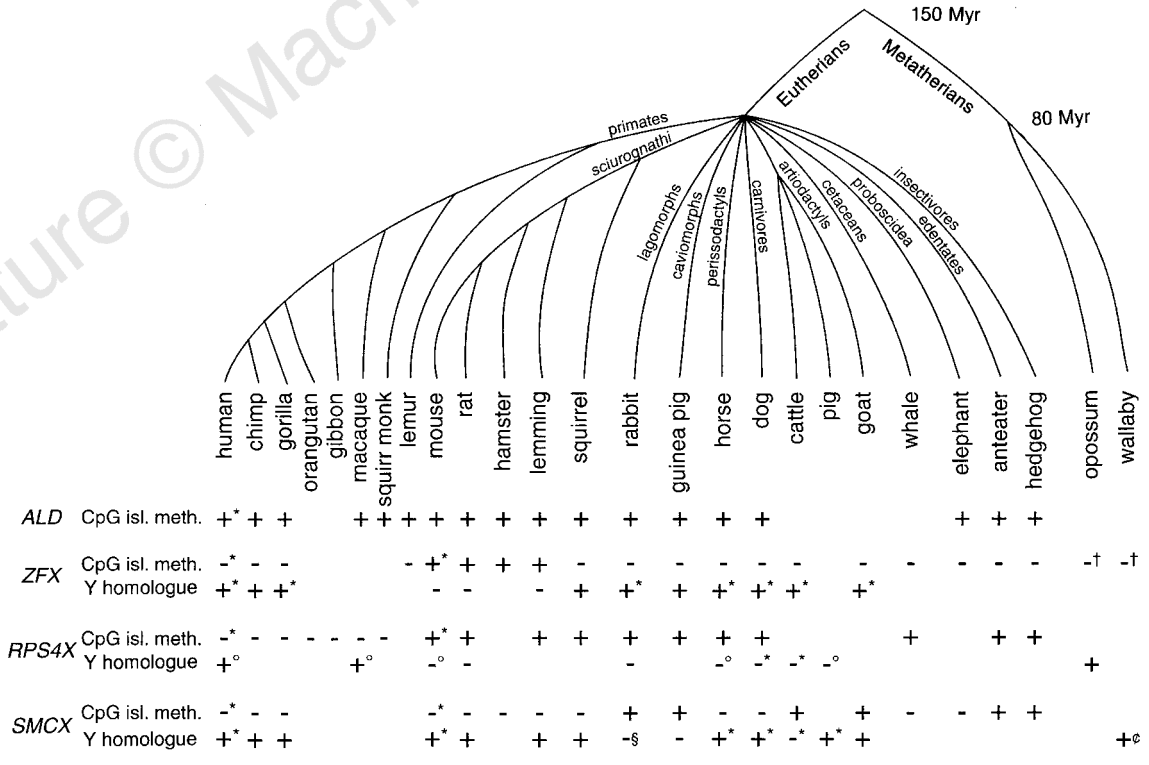

Figure 2 Summary of studies of CpG-island methylation and Y-chromosome homologue in 26 mammalian species, arranged phylogenetically[28]. Genes were scored '+' for CpG-island methylation if methylation was detected in female but not male DNA, and '–' if methylation was detected in neither sex (Fig. 1). For Y homologues, genes were scored '+' if experiments described in the text and Methods yielded reproducible evidence of highly similar, male-specific counterparts. Asterisk, our results confirmed published findings[7,9–17,20,22,26]. Other symbols defined as follows. † CpG island was found to be unmethylated in marsupials, where homologue of human *ZFX* is autosomal[29]. ° Previously published findings[11,13,14,20,22]. § Rabbit *SMCY* was detected by Southern blotting (our studies and ref. 20) but appears not to be expressed (see text) ¢ B. Duffy, J. Graves and collaborators, in preparation. Aguinik and collaborators reported evidence of *SMCY* homologue on kangaroo Y chromosome[20].

We conclude that *ZFX* escaped X inactivation in the common ancestor of all placental mammals, but became subject to X inactivation in myomorph rodents after the divergence of the sciuromorph (squirrel) lineage 40–70 Myr BP.

Like *ZFX*, *RPS4X* is known to escape X inactivation in humans and to undergo X inactivation in mice[11,13,14]. Given that X inactivation is controlled at the level of the whole chromosome during development, we anticipated that, among diverse mammals, the inactivation status of *RPS4X* might mirror that of *ZFX*. Instead, we found evidence that inactivation of *RPS4X* is much more widespread. Among 11 eutherian orders tested by CpG-island methylation, only primate *RPS4X* (seven species tested) seems to escape X inactivation. In all non-primate species tested (eight eutherian orders), we observed methylation of the *RPS4X* CpG island in female DNA, which probably reflects X inactivation of the gene (Figs 1 and 2).

Finally, we examined *SMCX*, an X-linked gene known to escape inactivation in both mice and humans[15–17]. Because mice appear to inactivate the X chromosome more completely than do other eutherians[11,12,14], we expected that genes escaping inactivation on the mouse X chromosome would do the same in all eutherians. Instead, results from CpG-island methylation studies suggested that *SMCX* is X-inactivated in 5 of 11 eutherian orders (6 of 18 species) tested (Figs 1 and 2).

Our studies yielded several general conclusions. For each of the genes studied—*ZFX*, *RPS4X* and *SMCX*—methylation status was concordant among the four myomorph rodent species tested, and among the seven primate species tested. If CpG-island methylation is a faithful marker of X inactivation, a change in the inactivation behaviour of a given X-linked gene must be only an occasional event during evolution. A single evolutionary transition could account for the X inactivation status of *ZFX* among diverse eutherian species, and no more than a few events could account for the status of either *RPS4X* or *SMCX*. Nonetheless, within a species the methylation status of any one gene—*ZFX*, *RPS4X* or *SMCX*—poorly predicts the other genes' behaviour. Thus, these evolutionary transitions in X inactivation affected individual genes or gene clusters, not whole chromosomes.

Were these occasional changes in X-inactivation status accompanied by the decay of homologous Y-linked genes[18,19]? If so, the Y-linked homologue should exhibit evidence of functional decline in lineages where the X-linked gene has become subject to X inactivation. We found this for all three X-linked genes under study, each known to have a functional homologue on the human Y chromosome[9,13,20]. For *ZFX*, Southern blotting revealed a highly similar, male-specific homologue, *ZFY*, in diverse eutherians where *ZFX* is unmethylated (Figs 2 and 3). Only myomorph rodents, where *ZFX* undergoes X inactivation, showed no evidence of highly conserved *ZFY* genes. Based on previous studies in mice, it seems likely that the tested myomorphs possess *ZFY* genes; their nucleotide sequences may differ substantially from those of non-myomorphs because of circumscribed expression and function, and hence relaxed selective pressures[21]. We infer that: (1) a *ZFY* gene, which closely resembled that in humans, existed in proto-eutherians, where *ZFX* escaped X inactivation; (2) these primitive characteristics were retained in most extant eutherian orders, including sciuromorphs (squirrels; Figs 2 and 3); and (3) myomorph *ZFY* function became restricted and *ZFX* became subject to X inactivation after the divergence of sciuromorphs.

With *RPS4X*, we and other investigators[11,14,22] searched for Y-chromosomal homologues in diverse eutherians by various means. A homologous Y gene, *RPS4Y*, was identified only in primates, the sole eutherian order in which *RPS4X* is known to escape X inactivation (Fig. 2). These findings are of particular interest given present evidence that *RPS4Y* and *RPS4X* genes coexisted in the common ancestor of eutherians and metatherians (marsupials) 150 Myr BP: in opossum, a marsupial, we identified full-length cDNA clones from two different *RPS4* genes, one mapping to the X and the other to the Y chromosome, as in humans (K.J. and D.C.P., unpublished results). Thus it appears that proto-eutherians possessed an *RPS4Y* gene. It has been maintained in modern primates, where *RPS4X* escapes X inactivation. *RPS4Y* was lost in other eutherian lineages, where *RPS4X* is now subject to X inactivation.

Like *RPS4X*, homologues of *SMCX* are found on human and marsupial Y (and X) chromosomes, implying that proto-eutherians possessed an *SMCY* gene (ref. 20 and B. Duffy, J. Graves and collaborators, in preparation). Previous and present Southern blotting studies suggest that *SMCY* has been lost, or diverged greatly, in cattle and guinea pigs—both species in which *SMCX* seems to be subject to X inactivation. However, in rabbits and goats, male-specific homologues are detectable by Southern blotting, although *SMCX* is apparently subject to X inactivation (Fig. 2; see also ref. 20). In rabbits, we used screening of cDNA libraries to recover 13 *SMCX* but no *SMCY* cDNA clones. This contrasts with the balanced expression of *SMCX* and *SMCY* in humans and mice[15,20]. Thus, at least in rabbits, *SMCX* is X inactivated and little or no SMCY is expressed.

Our findings based on three X–Y gene pairs suggest that differentiation of X and Y chromosomes occurred on a gene-by-gene or cluster-by-cluster basis, and has been an ongoing process in the eutherian lineages that radiated some 80 Myr BP. Extant eutherian sex chromosomes are only partly differentiated; a subset of genes has retained primitive or intermediate characteristics. We speculate that many extant genes represent intermediates on a general pathway by which eutherian X–Y genes or clusters evolved from autosomal genes (Fig. 4). Autosomal genes were funnelled into the pathway in two ways: by their mere presence on emergent sex chromosomes or by additions to those sex chromosomes through translocation of autosomal material[2]. This was followed by suppression of X–Y recombination. These steps occurred at the chromosomal or regional level, and gave rise to functionally equivalent X-linked (but not X-inactivated) and Y-linked genes. We postulate that subsequent steps occurred on a gene-by-gene or cluster-by-cluster basis. Three processes interacted, perhaps in coupled increments, resulting in an X-linked, X-inactivated gene with complete loss of the Y gene. This

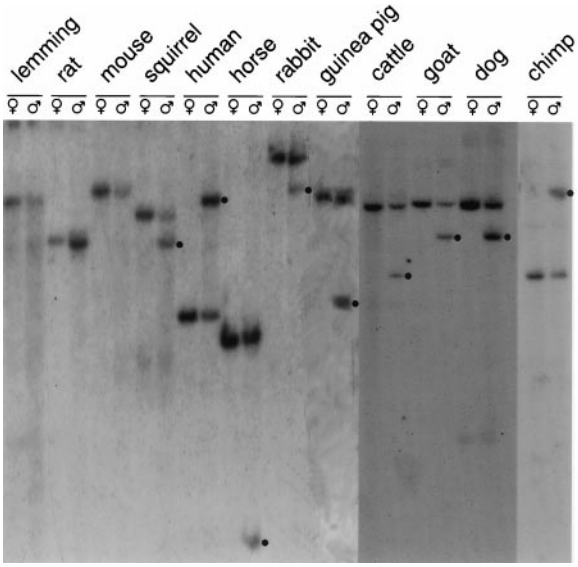

Figure 3 Southern blotting reveals closely related, Y-chromosomal homologue of *ZFX* in many eutherians, but not myomorph rodents. A 395-bp *Bss*HII fragment from human *ZFY* CpG island[10] was hybridized to *Eco*RI-digested genomic DNAs (5 μg per lane; female rat lane is underloaded). A black dot is placed immediately to right of each male-specific fragment observed.

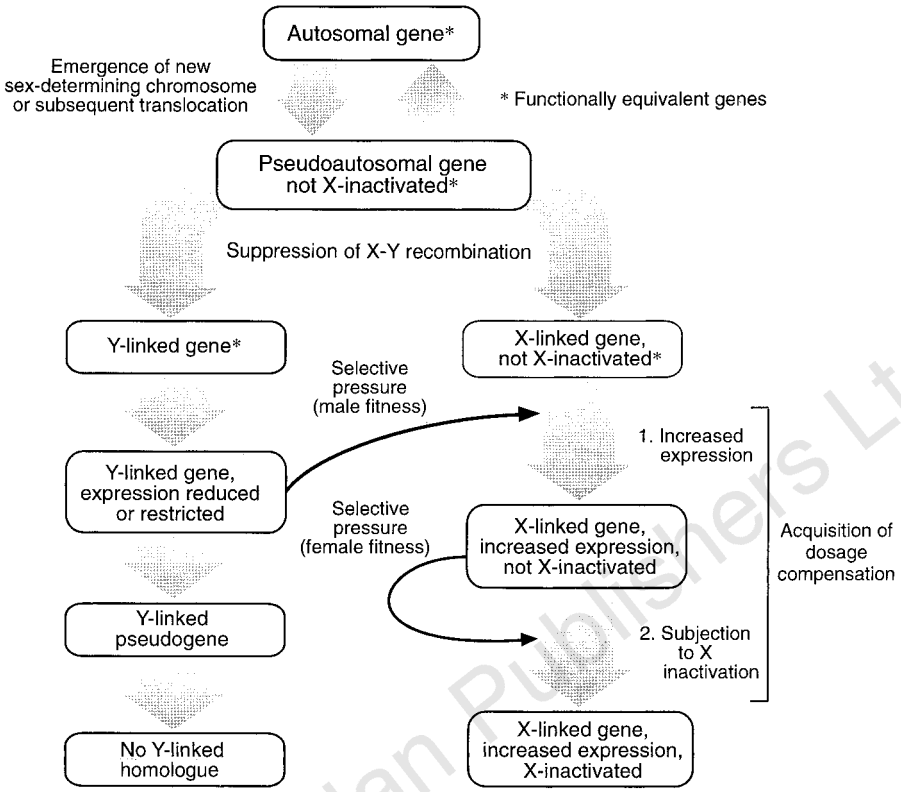

Figure 4 A proposed pathway for X–Y gene evolution in mammals. This could account for most X-linked and X–Y homologous genes in extant mammals, many of these genes existing at intermediate steps in the pathway. We are aware of one gene that cannot be accounted for by this pathway: the human pseudoautosomal gene *SYBL1*, which is X inactivated and is transcriptionally silenced on the Y chromosome[30].

began with Y-gene decay, followed by upregulation of X-linked gene expression, and concluded with X inactivation. Y-gene decay initially may have taken the form of reduced or restricted expression, as in the case of mouse *Zfy* or rabbit *SMCY*. Expression of the X-linked gene or cluster increased as an adaptation to reduced or restricted expression of its Y-linked homologue. This compensated for loss of Y function and restored optimal expression levels in males[18,23,24]. According to our model, X inactivation was a counter-response, restoring optimal expression levels in females.

Our model provides a more comprehensive explanation than the traditional view, which emphasized that X inactivation was rapidly implemented during evolution on a broadly regional or chromosome-wide basis[1,3]. Our model conflicts with the hypothesis that rapid evolutionary spreading of X inactivation preceded Y-gene decay and drove its initial steps[2]. This 'X-driven' hypothesis predicts that inactivated X-linked genes with functionally comparable Y-linked homologues should exist as evolutionary intermediates; to our knowledge, such genes have not been found in any mammal. To the contrary, recent studies suggest that numerous human X-linked genes escape X inactivation but have no detectable Y counterparts[8]. The 'Y-driven' pathway of X–Y gene evolution that we propose readily accounts for such genes as intermediates. Our model is not at odds with the view that an X-inactivation signal spread rapidly through large chromosomal regions. However, we speculate that this signal initially had no effect on the expression of many X-linked genes or clusters, perhaps because the required gene- or domain-specific regulatory elements[25] did not exist. Local and longer-range regulatory elements subsequently evolved, bringing with them X inactivation on a gene-by-gene or cluster-by-cluster basis. However, our comparative X–Y data suggest that the evolution of X-linked gene expression was more than the random playing out of such regulatory influences. The path was evolutionarily driven and constrained, through natural selection, by coupled changes on the Y chromosome. □

Methods

CpG island methylation. Methylation was assayed by polymerase chain reaction (PCR) using *Hin*dIII, *Hpa*II- or *Msp*I-digested genomic DNAs as templates. DNA was prepared from human lymphoblastoid cell lines; BALB/c mouse spleen or liver; liver from Sprague–Dawley rat, Syrian gold hamster, lemming (*Myopus schisticolor*), pilot whale, giant anteater, hedgehog (*Erinaceus europaeus*), wallaby (*Macropus eugenii*) and opossum (*Monodelphis domestica*); squirrel (*Sciurus carolinensis*) kidney; Hartley guinea pig kidney or liver; and blood of all other species tested. For PCR, 50 ng template DNA in 20 μl of 2.5 mM $MgCl_2$, 5 mM NH_4Cl, 12.5 mM NaCl, 50 mM KCl, 12.5 mM Tris pH 8.2 and 1 μM of each primer, was heated to 100 °C for 5 min before adding dNTPs (to 125 μM each) and 2 U Taq polymerase. Thirty cycles of 1 min at 94 °C, 1 min at 62 °C, and 1 min at 72 °C were followed by extension for 2 min at 72 °C, unless otherwise specified. PCR products were subjected to electrophoresis in 4% NuSieve agarose (FMC Corp.), 90 mM Tris-borate pH 8.3, 2 mM EDTA, 0.5 mg l^{-1} ethidium bromide and visualized with UV light. All assays were repeated multiple times, in most cases on multiple male and female samples from the same species, with concordant results. Some X-linked genes may escape X inactivation in a subset of cells or developmental stages[4,8], a subtlety that these studies of adult tissue methylation would not detect.

For *ALD*, PCR primers were chosen from CpG-island sequences found to be conserved between human and mouse: GTGACATGCCGGTGCTCTCCA and CGCTGCAGGAATACCCGGTTCAT amplify a 226 base pair (bp) product that includes six CCGG (*Hpa*II/*Msp*I) sites in humans and three CCGG sites in mice. The PCR annealing temperature was 60 °C, and 0.5 μl DMSO was added to each reaction. For *ZFX*, we previously described PCR primers that span a highly conserved 105-bp region containing two CCGG sites[10]. For *RPS4X*, a unique set of primers was required for nearly every species. First, CpG-island sequences for PCR-amplified from genomic DNAs using primers CCTA(G/A)CGCAGCCATGGTAAG (overlapping the exon 1/intron 1 boundary) and TCTTGGGACCACGAGCCT (in exon 2). (For dog, the 5′ primer was GCCTCGCGCAGCCATGGT.) Sequencing of these PCR products confirmed the presence of a CpG island in intron 1 in each species and enabled us to select

primers with which to assay *RPS4X* methylation in particular species; primer and underlying sequences have been deposited at GenBank: human, accession no. G36429; chimp and gorilla, G36430 and G36431; rabbit, G36432; guinea pig, G36433; mouse, G36434; rat, G36435; lemming, G36436; squirrel, G36437; dog, G36438; anteater, G36439; hedgehog, G36440; whale, G36441; and horse, G36442. For *SMCX*, PCR primers were chosen from CpG-island sequences found to be conserved between human and mouse: CCTCGGGCCCACCATGGAG and CTGATTTTCGCGATGTAGCC amplify a 117-bp product that includes three CCGG sites in humans and two in mice. We selected these conserved *SMCX* primers after sequencing 5′ portions of the mouse transcript (GenBank AF0398940; obtained by 5′ RACE cloning) and comparing these with previously published 5′ human *SMCX* sequences[20].

Y-chromosome homologues. We searched for Y-specific homologues of *ZFX/ZFY* and *SMCX/SMCY* in mammalian species by Southern blotting of *Eco*RI-digested male and female genomic DNAs. For *ZFY*, we used two hybridization probes in separate experiments, with entirely concordant results (Figs 2 and 3): (1) a 395-bp genomic *Bss*HII fragment[10] from the 5′ CpG island of human *ZFY*, and (2) pDP1007, a 1.3-kb genomic fragment containing the zinc-finger exon[26] of human *ZFY*. Probes were labelled with ^{32}P by random-primer synthesis and hybridized overnight to Southern blots at 67 °C (65 °C for pDP1007) in 1 mM EDTA, 0.5 M $NaPO_4$ pH 7.2 and 7% sodium dodecyl sulphate (SDS). Blots were then washed three times for 20 min each at 62 °C in 0.1 × SSC (1 × SSC = 0.15 M NaCl, 15 mM Na citrate pH 7.4), 0.1% SDS and exposed at −80 °C with X-ray film backed with an intensifying screen for one day. The *SMCY* hybridization probe (pCM4) and conditions were described previously[20].

We also searched for Y-chromosome homologues of *RPS4X/RPS4Y* and *SMCX/SMCY* in particular species by cDNA selection, or by screening cDNA libraries. In cDNA selection[27], human *RPS4Y* coding sequence (as selector) was hybridized at 55 °C to cDNA libraries (Clontech) prepared from adult male rat, rabbit, dog or cattle liver. Selection products were cloned into plasmid vectors and sequenced. cDNA libraries prepared from adult male dog liver (Clontech) and adult male opossum spleen (Stratagene) were screened with the entire human *RPS4Y* coding sequence as probe, at low stringency (overnight hybridization at 58 °C in 1 mM EDTA, 0.5 M $NaPO_4$ pH 7.2, 7% SDS; subsequent washing three times for 20 min each at 50 °C in 1 × SSC, 0.1% SDS). Once dog and opossum *RPS4X* clones were identified, by sequencing, these were used as probes for high-stringency rescreening of their respective libraries (hybridization at 65 °C and washes at 65°C in 0.1 × SSC, 0.1% SDS). We anticipated that *RPS4Y* clones, if present, would be detected in the low-stringency screen but not in the high-stringency screen. In this manner, we identified opossum *RPS4Y* (and *RPS4X*) cDNA clones, confirmed by sequencing (GenBank AF051137 and AF051136, respectively) and mapping studies (K.J. and D.C.P., unpublished results; complete description will be published elsewhere), but in dog we detected only *RPS4X* clones. Similarly, we screened a cDNA library (Clontech) prepared from adult male rabbit liver at low stringency using a 370-bp mouse *Smcx* cDNA fragment (prepared from clone pCM4 (ref. 20) by PCR using primers CCTTCCAAGTTCAACAGTTATGG and CATACGTATGACTCAATAAACTGGG), identifying 13 *SMCX* but no *SMCY* clones.

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Signal-dependent noise determines motor planning

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When we make saccadic eye movements or goal-directed arm movements, there is an infinite number of possible trajectories that the eye or arm could take to reach the target[1,2]. However, humans show highly stereotyped trajectories in which velocity profiles of both the eye and hand are smooth and symmetric for brief movements[3,4]. Here we present a unifying theory of eye and arm movements based on the single physiological assumption that the neural control signals are corrupted by noise whose variance increases with the size of the control signal. We propose that in the presence of such signal-dependent noise, the shape of a trajectory is selected to minimize the variance of the final eye or arm position. This minimum-variance theory accurately predicts the trajectories of both saccades and arm movements and the speed–accuracy trade-off described by Fitt's law[5]. These profiles are robust to changes in the dynamics of the eye or arm, as found empirically[6,7]. Moreover, the relation between path curvature and hand velocity during drawing movements reproduces the empirical 'two-thirds power law'[8,9]. This theory provides a simple and powerful unifying perspective for both eye and arm movement control.

The trajectories of eye and arm movements (that is, the change in position and velocity over time) are not inevitable consequences of