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Weird Animals, Sex, and Genome Evolution

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Abstract

Making my career in Australia exposed me to the tyranny of distance, but it gave me opportunities to study our unique native fauna. Distantly related animal species present genetic variation that we can use to explore the most fundamental biological structures and processes. I have compared chromosomes and genomes of kangaroos and platypus, tiger snakes and emus, devils (Tasmanian) and dragons (lizards). I particularly love the challenges posed by sex chromosomes, which, apart from determining sex, provide stunning examples of epigenetic control and break all the evolutionary rules that we currently understand. Here I describe some of those amazing animals and the insights on genome structure, function, and evolution they have afforded us. I also describe my sometimes-random walk in science and the factors and people who influenced my direction. Being a woman in science is still not easy, and I hope others will find encouragement and empathy in my story.

FALLING IN LOVE WITH CHROMOSOMES

I fell in love with kangaroo chromosomes during my undergraduate research project in 1963. They are big and bold; the shortest is longer than human chromosome 1. They are so magnificent that they starred on a 50¢ postage stamp in Australia. Working with stamp designers was interesting: "What color is a chromosome?" "Well, sort of dirty white—but we stain them blue." "Does it matter how many chromosomes there are? We can't fit 16 on a postage stamp." "Okay, well let's do a haploid complement of blue chromosomes." I still smile when I look at the 6-foot poster of that stamp outside the tearoom at La Trobe (**Figure 1*a***).

I always loved the visual part of science; maybe that is what hooked me. Both my parents were academics; my dad was T.J. Marshall of the Marshall equation that describes the movement of fluids through soils. My mum was a lecturer in geography, who waged wars against freeways and overdevelopment in my home town, Adelaide, capital of South Australia, with only a million people (and no urban freeways) and miles of golden beach. But there was no push toward science, and the only subjects for which I won prizes at school were art, creative writing, and geography.

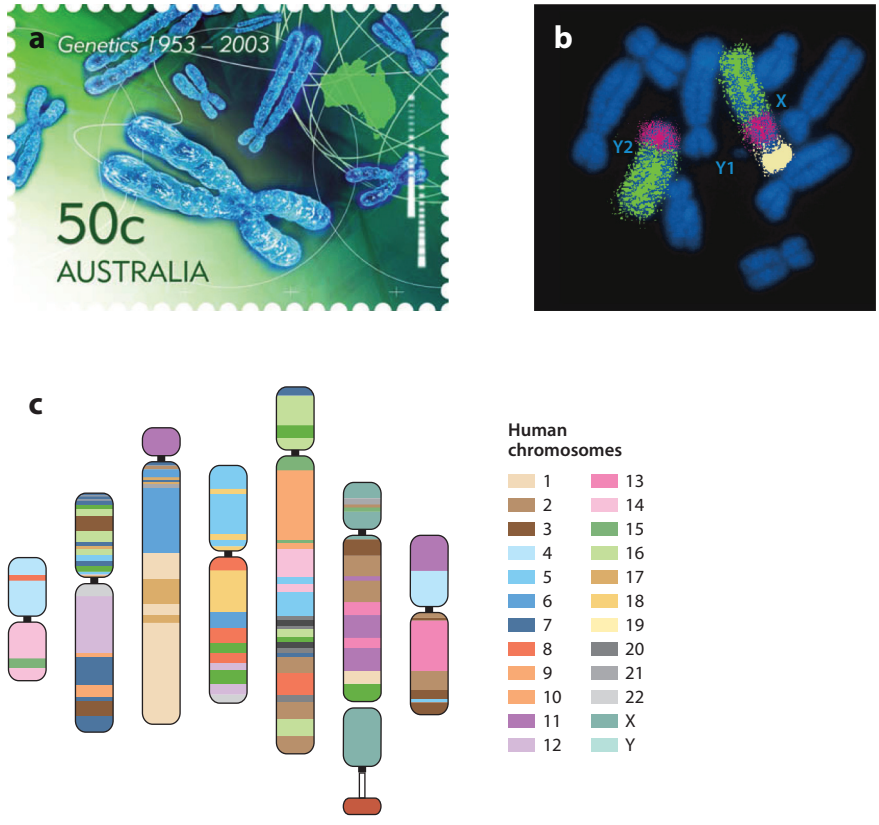

Figure 1

Three images of kangaroo chromosomes. (*a*) Kangaroo chromosome stamp to commemorate the International Congress of Genetics in 2003. (*b*) Comparative painting of swamp wallaby chromosomes with paints from tammar wallaby chromosomes 2 (*green*), 7 (*pink*), and X (*white*). Adapted with permission from Reference 104. (*c*) Kangaroo chromosomes in colors denoting orthology to human chromosomes. Redrawn from "Australia—on the genetic map" tee shirt design by PhD student Jason Lim.

Genetics in Adelaide

In fact, I did not like biology when I finally took a class in my last year of high school—too much stuff and no periodic table to give it form. Then one day our teacher introduced Mendel's laws of inheritance. If you mate a blue budgerigar with a yellow one, all the offspring are green (made sense). But if you intercross the offspring, you got one-quarter blue, one-half green, and one-quarter yellow birds. Wow—there *is* order, I thought, and enrolled for biology the following year (1959) at Adelaide University, in the era when all science students took physics, chem, maths, and "one other."

I was lucky to study at Adelaide. First-year biology, unusually, included a term of genetics, taught by the wonderful Peter Martin, a botanist who moonlighted on marsupial chromosomes. And it boasted a Department of Genetics, staffed largely by Australians trained in R.A. Fisher's department in Cambridge, England. I took a semester of statistics with the great R.A. Fisher, whom I did not warm to (and whose advocacy of cigarette smoking I deplored), but whose extraordinary insights on evolution I later came to revere (1).

I was lucky, too, to spend a semester at the University of California, Berkeley, during my parents' sabbaticals; because I only audited, I could do anything I liked. What I most liked was Dan Mazia's wonderful course on "physicochemical biology," later called cell biology. Mazia (2) was famous for his work on cell division, particularly on how chromosomes were pulled apart at mitosis. When I returned to Adelaide in 1961, I declared I wanted to pursue physicochemical biology, and for my presumption my puzzled advisers steered me into mammoth chemistry and genetics subjects that clashed, were physically at the opposite ends of the campus, and intellectually could have been on different planets. But physical chemistry of macromolecules was strong and included work on thermal denaturation and hybridization of DNA (3) that was to be the lynchpin of molecular biology methods I would use for the rest of my life.

My honours research in 1963 was with David Hayman, a young lecturer in genetics, who worked with Peter Martin on marsupial chromosomes. Mary Lyon in Harwell, England, had just discovered that one chromosome in female mice was genetically inactive (4), and I elected to look for X chromosome inactivation in kangaroo females, using an exotic new technique that Peter had learned at Harwell—tritium autoradiography—to check whether one X replicated late in kangaroos, too. Counting nearly a million silver grains over chromosomes (I dreamed grains) showed that it did (**Figure 2*a***), and I blithely reported that kangaroos are just like mice and humans (5). Wrong—it turns out they are very different, and marsupial X inactivation was to deliver many surprises to our understanding of this model epigenetic silencing system.

Molecular Biology in Berkeley

It is scary to think how casually I made important career decisions. Postgraduate work at Berkeley was one of them. I was offered a PhD scholarship in Adelaide. But I was ready for adventure, so to escape I said the first thing that came into my head: "I want to do a PhD with Dan Mazia at Berkeley." I will swear I had never thought of doing a PhD anywhere, much less at Berkeley, but having said this I had to follow it up with a letter. "Dear Professor Mazia, I am little Jenny Nobody from Adelaide, and I'd like to come and work with you." I could not believe his friendly and helpful response, to the effect that he would love to have me in his lab. But since I had never studied zoology, I should enroll in the sparkly new molecular biology department up the hill.

Berkeley was mopping up after the Free Speech Movement when I arrived in 1965, and succeeding years saw constant antiwar protests; it was impossible not to be involved in daily demonstrations. The other distraction was performing in a musical, *NucleoSide Story*, written by fellow graduate student Phil Carl, the classic tale of star-crossed graduate students Maria and Tony from

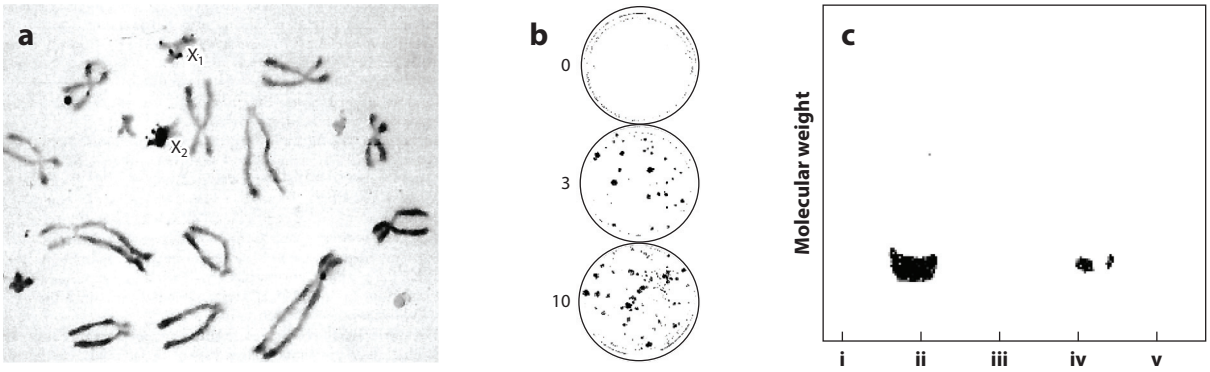

Figure 2

Three views of studying X inactivation. (*a*) Late-replicating kangaroo X. Adapted with permission from figure 2*b* in Reference 5. (*b*) HPRT+ reactivants after treatment of mouse hybrid cells with 0, 3, and 10 μg/ml of the demethylating agent 5-azacytidine. (*c*) Northern blot of RNA from (*i*) HPRT-deficient child, (*ii*) his heterozygous mother, and (*iii–v*) clones of mother's cells with the HPRT+ allele on the active X (*iv*) or inactive X (*iii*, *v*). Adapted from figure 1*a* in Reference 34.

the warring departments of Molecular Biology and Biochemistry. The MoBi graduate class was a bit short of lyric sopranos, so I ended up singing Maria. I married Tony, sung by a tenor called John Graves, who introduced me to the joys of choral singing, which obsesses us both to this day.

Molecular biology—what is that? Well, my background in genetics and physical chemistry suddenly made sense, and I lapped up new discoveries about *Escherichia coli* and phage and cracking the genetic code.

But my heart lay in animal cells and chromosomes, so I had to convince Dan Mazia I was worth having in his lab. Dan was on sabbatical when I arrived, but now I must introduce myself. I walked three times around the dark basement corridor of the giant square Life Science Building nerving myself up—and ran straight into the great man, who was guiltily returning from the cigarette machine. "I'm supposed to be giving up," he confessed as he sat me down at his giant old microscope. "What do you see down there?" "A bunch of little dots—sea urchin chromosomes? You should see kangaroo chromosomes . . . ," I replied. Dan Mazia became my lifelong mentor, his ebullience propelling me through mazes of doubt.

It was kangaroo chromosomes that saved me when I took my orals the next year, a terrifying ordeal in which a committee of five could ask you anything they liked. Many years later, one of those committee members, Bill Birky, introduced me at a seminar at the University of Arizona as "Scheherezade," reminding me that I had so distracted the committee with my descriptions of glorious marsupial chromosomes—and my tales of how to catch kangaroos—that they never got around to quizzing me about *Drosophila* genetics or pyrimidine biosynthesis.

Cell Fusion and Cell Biology

I was still transfixed by epigenetic silencing of the mammal X chromosome and designed impossible cell-fusion experiments as my orals proposition, fusing 2X active cells with 1X active cells to see what factors turned silencing off and on. So I rashly decided to put my plan into action as my PhD research project, moving from Mazia's sea urchin lab into Morgan Harris's pristine cell culture lab (a converted shark aquarium in the courtyard of the Life Sciences Building doughnut). I absorbed Morg's kindness and expertise in somatic cell variation (6), which he showed was epigenetic, as well as his meticulous technique to dissect messy cell biology. I learned to grow Sendai virus in

eggs and blundered around fusing mouse, hamster, and human cells—the first fusion experiments in America, I discovered later.

But establishing a source of 2X active cells from early mouse embryos was beyond my capabilities and Morg's patience, so instead I fused cells to dissect control of the cell cycle, discovering cytoplasmic factors (now known to be various kinases) that turn on DNA synthesis and mitosis (7, 8). I observed that chromosomes in cell hybrids were rapidly lost from one parental set but not the other. There seemed to be a pecking order, and human chromosomes were at the bottom.

Why did I never twig that loss of human chromosomes from cell hybrids provided a parasexual cycle that could be harnessed to map human genes? Coincidentally, Frank Ruddle, who was to develop this revolutionary technology for mapping the human genome (9), was Morg's previous student; having completed a dull project on chromosome changes in cultured pig cells, he left the day I arrived, and I did not meet him for another decade.

MAPPING OUT A RESEARCH PATH

In fact, gene mapping was far from my radar when I returned to Australia as a lecturer (a.k.a. assistant professor). This was another complete accident; my sister, a Master's student at the new La Trobe University in Melbourne, encountered an old Adelaide friend at the campus post office and told him that I would return soon when my exchange visitor visa expired (I had a Fulbright). Des Cooper sent me a job advertisement and I applied. Remarkably, they hired me in 1971, with no postdoctoral experience and a single publication.

The professor showed me around my new department. "Your lab," he said grandly, opening the door to a bare room with a sink and a fridge. Hmm, what next? Fortunately, my 17-year-old lab technician knew more than I about ordering equipment and glassware, and between us we laid the foundations of JennyTech Labs.

A Well-Mannered Female Academic Would Resign

Being a new member of staff, and the only woman, was a novel experience. I was all agog at my first staff meeting and eager to help. Until the last item on the agenda—who would organize the Christmas party? All eyes turned to me. I do not remember what I said, but I was never again asked to organize parties. Although my male colleagues treated me cordially, I began to realize there were resources and information I was not privy to. Why did my male colleagues have extra help to wash glassware? There were secret guilty glances when I asked.

How should I spend my research life? Lazily, I puttered along charting DNA synthesis patterns of hamster and mouse chromosomes in hybrid cells, which seemed to stick to their individual agendas. I tested hypotheses to explain why chromosomes were lost from cell hybrids and what lay behind the pecking order of segregation. A series of papers ensued (read by no one much except Hunt Willard, who I would come to know and admire many years later), rejecting one after another hypothesis. Was it differences in growth rate? Spindle constitution? Centromere activity? Only recently have molecular techniques confirmed centromere competition in our old hybrids (10).

No hurry—there was a whole lifetime ahead. John and I were living in a commune in suburban Melbourne. This took a lot of energy, not the least because I had to defend science against charges that it was boring ("just counting things") and downright dangerous. I became pregnant and had to decide whether to resign my lectureship and take up a part-time tutoring position, as was expected of well-mannered women academics. Fortunately, this was not an option because my husband was completing a Master's degree in applied science, having decided that molecular biology would not save the world. There was no maternity leave in those days, so our baby daughter saw a lot of the lab from her basket.

Australian Mammals—Independent Experiments in Mammalian Evolution

My research direction radically changed when Des Cooper persuaded me to use cell fusion technology to map genes in kangaroos. "Why would anyone want to do that?" I asked rudely. Well, marsupials are so distantly related to placental mammals that they constitute an independent experiment in mammalian evolution, he explained patiently.

A case in point was work on marsupial X chromosome inactivation that had followed my demonstration of a late-replicating X in kangaroos. Des, with colleagues at Macquarie University in Sydney, showed that the process was quite different in marsupials and placental mammals. Rather than being random, it was always the paternal X that was inactivated (11, 12)—the first evidence of imprinting in a mammal. Its molecular mechanism, too, is very different (13, 14).

So we needed to know what genes lay on the kangaroo X chromosome; could I make a few mouse-kangaroo cell hybrids and identify marsupial genes that were expressed in hybrids retaining a kangaroo X?

This was easier said than done. I grew virus and fused mouse cells with kangaroo blood cells; nothing survived the selective medium. We tried several marsupial species with no more success. It took years of collaboration with an Adelaide colleague, Rory Hope, to check each step. We recovered our first hybrids from culture vessels that were inadvertently left in the 37°C room for weeks. These struggling colonies had no kangaroo chromosomes but expressed the kangaroo version of the selected marker *HPRT* (15). We nursed along hundreds of these pathetic hybrid clones, finding that they all had fragments of the X chromosome but only six retained recognizable kangaroo chromosomes. Years of somatic cell genetic mapping were done on the few hybrids retaining a marsupial X and revertants that had lost it (16). The fragments gave us information on gene order (17), predicating radiation hybrid mapping (18).

Mapping the X in kangaroos and other marsupials was more interesting than I had anticipated. Family studies had established sex-linked inheritance in kangaroos of genes on the human X that encoded old favorite enzymes G6PD and PGK. We added two more enzyme loci to what was evidently a highly conserved mammal X. It looked as if marsupials, too, obeyed Ohno's law, that X chromosomes had the same gene content in all mammals, which the great Susumo Ohno (19) attributed to protection from translocation, which would disrupt the whole X chromosome–inactivation mechanism.

We enthusiastically adopted the newly developed technique of autoradiographic in situ hybridization (20). Because we had no genomic clones of kangaroo genes [and certainly no bacterial artificial chromosomes (BACs)], we used the weak signal from cDNAs to locate the sequence on a chromosome. We could even use human cDNAs of very conserved genes. Because we did not have to establish electrophoretic differences between allozymes, we could now map anything for which we had cDNA (21).

Breaking Ohno's Law

This enabled us to show that marsupials break Ohno's law. We used in situ hybridization to show that genes on the short arm of the human X are autosomal in kangaroos, mapping together on chromosome 5 (22–24) (**Figure 3*a***).

Before we published this discovery, I happened to visit an X-inactivation colleague (Art Riggs) at the City of Hope in Los Angeles. Finding that Susumo Ohno's office was in the same building, I bravely knocked on his door to introduce myself and break the devastating news. Ohno, impeccable in crisp white coat, greeted me politely. When I told him that marsupials had broken his law, he raised his eyebrows; "So?" A long pause. "Come and see what I am doing now." He led me to his

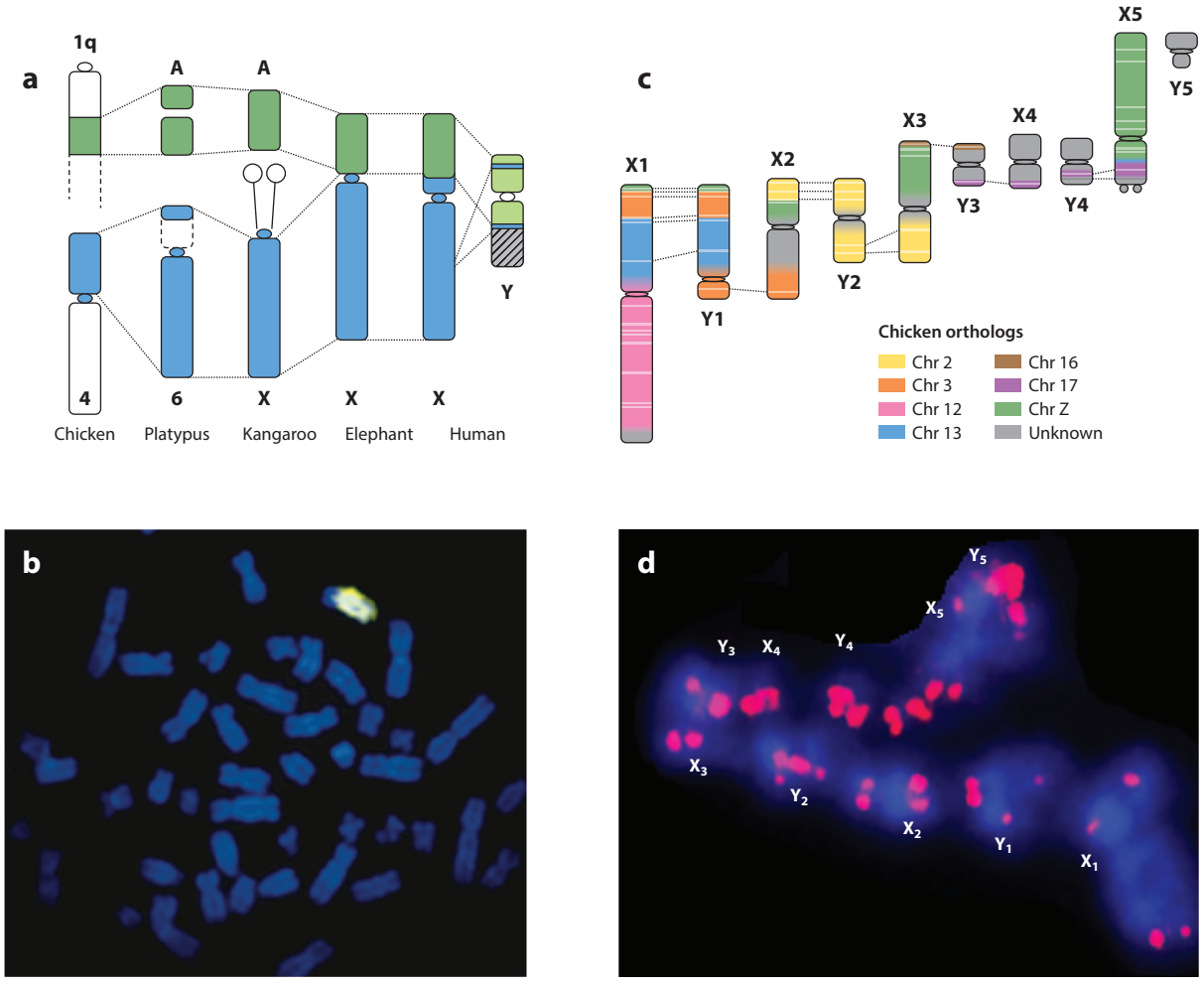

Figure 3

Evolution of mammal sex chromosomes. (*a*) Homologies between human XY, other mammals, and birds. The original X of therian mammals (*blue*) is represented by autosomal regions in birds and monotremes. A separate autosomal region (*green*) underwent centric fusion (as seen in elephant) to the original X in the placental lineage (*green*). The human Y mainly derives from this added region. Adapted with permission from figure 6 in Reference 105. (*b*) Kangaroo X chromosome painted onto human male cell; the human X is labeled over the long arm and part of the short, representing the conserved region of the ancestral therian X (*blue in panel a*). Reprinted with permission from figure 1*c* in Reference 44. (*c*) The 10 sex chromosomes of platypus, colored by homology to chicken chromosomes, form a chain at meiosis held together by 9 pseudoautosomal regions. There is no homology to mammal XY chromosomes but considerable homology to the bird ZW (*green*). Redrawn with permission from figure 2 in Reference 81. (*d*) The 10 platypus sex chromosomes, their ends marked by telomere probes (*pink*), line up in an alternating XY chain at male meiosis. Adapted with permission from figure 3*b* in Reference 80.

computer, on which he was composing DNA music, which I later heard the Stockholm Symphony Orchestra play. A memorable way to learn that it is alright to be wrong.

There were two ways to explain our X mapping results. After marsupials diverged from placental mammals 166 Mya, there had been either an X-autosome fusion in the ancestral placental mammal or a fission of an ancestral marsupial X. I bet on a fusion because the two blocks of genes seemed

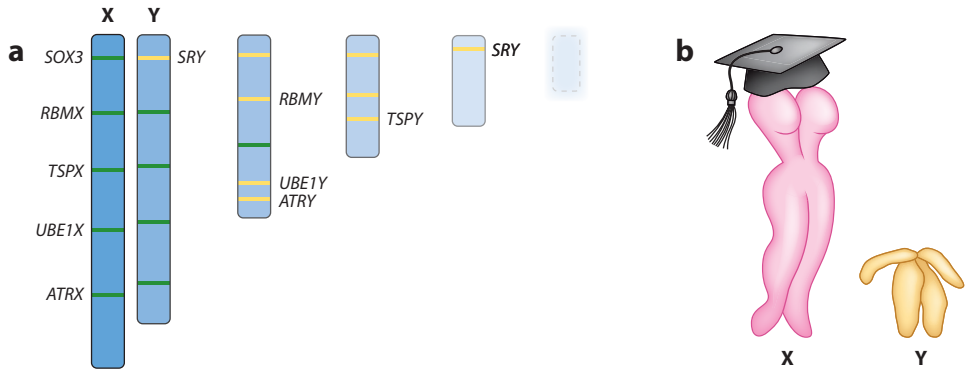

Figure 4

Two views of human sex chromosomes (*a*) Degradation of the mammalian Y. The proto-Y is initially homologous to the proto-X except for the evolution of novel sex-determining gene *SRY* (*left*), but progressively loses active genes (*green bands*). Some genes on the Y acquire a male-specific function (*yellow*), but even these may be pseudogenized and lost as the Y becomes full of repetitive sequences (*lighter blue*). Ultimately even *SRY* is lost and replaced with a novel gene on another chromosome, and the Y becomes redundant and is lost. (*b*) The (smart, sexy) human X chromosome has a disproportionate share of genes involved in intelligence and reproduction. The (wimpy) Y chromosome has few active genes and is a degenerate copy of the X.

to be separate in the third group of mammals, the monotremes (25), and this turned out to be the case also in birds (26). So the marsupial X represented the ancient X, to which a chunk of autosome had been added some time before the eutherian radiation 105 Mya (27). This explained the concentration on the short arm of many genes on the human X that escape inactivation (28): They are not a special class of gene, but were just added recently and not yet incorporated into the inactivation system.

Indeed, the gene content of the X became a research topic on its own, because its accumulation of intelligence and reproduction genes (**Figure 4*b***) is driven by dosage differences (29), the driver also for X chromosome inactivation.

Exploring the Basis of X Inactivation

My first PhD student tried to establish a source of 2X active cells from mouse embryos so we could at last do 2X × 1X active fusion experiments. We grew early mouse embryos on feeder cell layers (the way we nursed reluctant cell clones), observing tight colonies of small cells that would round up and develop all sorts of strange morphologies before floating away. I wish I had shown them to my embryologist friend upstairs—perhaps we could have told the world about embryonic stem cells in 1973.

During the 1980s, I took two sabbaticals in the United States to work on X inactivation in mouse and human. In Gail Martin's lab at the University of California, San Francisco, I fused 2X active teratocarcinoma cells with mouse hybrid cells heterozygous for variants of three X genes. I derived HPRT-deficient variants so I could select for reactivation of the inactive X. I detected reactivation in hybrids, but only locally, around the selected marker (30). My colleague Nobuo Takagi later got full reactivation by fusing 2X active embryonal carcinoma cells with somatic cells from heterozygous mice (31).

I returned to Australia with the HPRT-mouse hybrid cells, which I used to try to reactivate the inactive X with a demethylating agent 5-azacytidine, which Morgan Harris (32) had used to reverse

a thymine-kinase-deficient cell line. It was the most spectacular experiment I ever did; within a week I had reactivant colonies on all my Petri dishes, their frequency dose dependent, and I could show that the selected marker HPRT was expressed from the inactive X (**Figure 2*b***). Although scooped by an independent study showing azacytidine reactivation of the inactive human X in a cell hybrid, I love my little paper proposing that DNA methylation is at the basis of X inactivation (33). Anyway, methylation is only part of the story.

I spent my next sabbatical (1984) in the Seattle laboratory of Stan Gartler, who became a beloved mentor. He was bemused at my determination to test whether transcriptional repression was the molecular basis of X chromosome inactivation. "What else could it be?" he asked (well, we did not know then of the many ways in which genes can be silenced at the RNA and even protein level). Indeed, the assumption that inactivated genes were not transcribed underpinned all theories of how epigenetic silencing worked, although there was no real evidence for transcriptional repression.

Stan had transformed lymphocytes from a woman heterozygous for *HPRT* deficiency, and from her affected son. I cloned sublines with the normal or mutant gene on the active X then showed by Northern analysis that there was no *HPRT* transcript from the cells with a normal allele on the inactive X (**Figure 2*c***). Confident that the first demonstration of transcription repression of the inactive X was of fundamental significance, I sent our paper to *Nature*. But countermanding Mary Lyon's championing, one referee sniffed, "What else could it be?" and it was published in a specialist journal (34). Another beloved but unappreciated paper.

THE BIG PICTURE

During this time I continued to map marsupial genes—X-borne and autosomal—via in situ hybridization. It was fun—I love jigsaws. Even better was the discovery that marsupial mapping was of interest to the Human Gene Mapping (HGM) consortium, which ran annual international workshops. This body backed up the Human Genome Project, constructing detailed maps of human chromosomes by linkage analysis, somatic cell genetics, and in situ hybridization.

Human Gene Mapping

I joined the HGM consortium when Des Cooper (by then at Macquarie University) invited Steve O'Brien to a meeting in Sydney. Steve seemed more excited than I about our scraps of mapping data and invited me to the HGM workshop in Paris in 1987. Here participants worked in chromosome-based committees, putting mapping data together for each human chromosome. I joined the Comparative Committee, which was dominated by mouse geneticists but also contained Steve and Jim Womack, who mapped cats and cows.

This was the beginning of my lifelong engagement with comparative genomics, although that name had not been invented yet. My role, at many successive meetings in Yale, Baltimore, Oxford, and London, was to scamper between groups, collecting snippets of data on pigs, cows, monkeys, cats, and even fish, sometimes in lists, sometimes on the backs of envelopes. Steve drafted the report while I drew diagrams. Human geneticists initially took little notice of our work, but I made great connections with groups that worked on all manner of interesting problems of wildlife, pest species, and agricultural animals.

Comparative Maps

As I sifted through assignments, I realized with a jolt that most data on agricultural animals were useless to me. Their maps were based on anonymous DNA markers developed to locate traits of

economic importance, and they could not be compared because they were species specific. Please map conserved genes, I would beg, genes with obvious orthology between species. Dave Burt, who was mapping chicken genes, responded with the first useful map of a bird (7), in which I recognized many gene groups from humans, cats, and cows. Steve O'Brien later gave these two types of markers names (Types I and II), and we compiled a how-to report (35) with criteria (including synteny) for recognizing orthology.

The comparative maps expanded as new data started linking synteny groups. Maybe I could get them all onto the same map, I thought; I tried this idea out on Steve, etching them in the sand on the beach during a Lorne Genome conference in Australia in 1990. So in London later that year, with my plastic ruler and pencil, I started drawing genomes, one chromosome below the other, for 35 species.

These maps revealed amazing patterns (36). The genome was far more conserved than we had realized. Whole chromosomes were shared by humans and cats, and great chunks of chromosomes were shared by humans and kangaroos, even birds. The rank outsiders were not kangaroos but mice. "Mice are not mammals at all," I growled. "They are alien beings sent to confuse us." There were highly conserved genomes like humans and cats, and highly fragmented genomes like gibbons and dogs. Gene maps and genomes from other birds and reptiles now show that placental mammals are a glaring exception to a highly conserved arrangement of the vertebrate genome.

This conservation allowed us to spot orthologs by their position in conserved gene blocks and recognize—and date—paralogs with new functions. We showed that comparisons between humans and other species could go two ways, so human genes could be located by the position of their orthologs in other species. Human gene mappers started to take notice. We could also spot conserved genome rearrangements. Some were unique to a species, such as human chromosome 2, present as two smaller chromosomes in other great apes, implying a recent centric fusion in the human lineage. Others were shared between distantly related species, implying an ancient arrangement that was subverted independently in different lineages.

The computer age was upon us, and my PhD student, Matthew Wakefield, worked to get the maps into a computer format. Successions of secretaries and collaborators tried more or less useful programs, while I plodded along with pencil and ruler. Ultimately, the maps were put on a professional footing, taking genome comparisons to new heights (37, 38) and devising tools that permit detailed comparisons between any species (39) and reveal genome evolutionary strategies (40).

Getting Our FISHing License

Counting grains and assessing the significance of peaks, I often dreamed, "If only I could *see* where a gene is." Fluorescent probes made this a reality. I was lucky to recruit Roland Toder from Freiburg, who helped build one of the first UV microscope setups in Australia. Even better, we could use as hybridization probes large insert clones from a dunnart BAC library that we made ourselves; later we requisitioned commercial libraries from our model kangaroo, the tammar wallaby, and the platypus. Fluorescence in situ hybridization (FISH) was a dream come true—at last I could see, in just a single cell, exactly where a gene lived.

This period, too, was the beginning of a long and fruitful collaboration with Malcolm Ferguson-Smith from Cambridge, whose group was perfecting methods for sorting chromosomes by their size and DNA content. Marsupial chromosomes were ideal material for Malcolm's sophisticated setup, and he used them to make chromosome-specific paints, which we could hybridize across species.

Over the next 10 years we collaborated with Willem Rens from Malcolm's group to paint chromosomes from one marsupial species onto chromosomes from closely and distantly related

species, until we had a picture of the genome homologies between all marsupial groups (41) (**Figure 1*b*,*c***). As David Hayman and Peter Martin had predicted decades ago (42, 43), it turned out to be extraordinarily simple; there were essentially only 19 genomic blocks that were arranged and rearranged in different marsupial lineages. We even painted the kangaroo X chromosome onto human chromosomes (44), revealing the two evolutionary blocks that mapping had predicted (**Figure 3*b***).

SEX AND THE Y

My focus on the X chromosome shifted abruptly to the Y in 1988. I was aware—who was not?—of the race to find the gene on the human Y chromosome that triggered testis formation and sex determination in the embryo, and I exulted when a paper appeared that seemed to settle it. A gene, the first cloned from the human Y, was male specific (though curiously it had a partner on the X); it coded for a highly conserved zinc finger protein that was a good candidate for a transcription factor that could control the sex pathway (45). Like many others, I enthusiastically endorsed *ZFY* and hounded my students to read it as a stellar example of positional cloning that would soon revolutionize human genetics.

Search for the Sex-Determining Gene

To my great surprise, I received a phone call that night from the author, David Page, in Boston. Knowing that I mapped genes on marsupial sex chromosomes, he invited me to map *ZFY* in kangaroos. If *ZFY* was the right gene, it should also be on the Y in marsupials as well, which share most of the XY with placental mammals. I gave the job to finishing PhD student Andrew Sinclair and a new student, Jamie Foster, who had elected to study *ZFY* in marsupials. David sent us his *ZFY* clone. Coincidentally, his London rival, Peter Goodfellow, with whom Andrew had already arranged a postdoc, independently sent his version of *ZFY*.

My students mapped both probes in two different marsupial species, using radioactive in situ hybridization. The day came to develop the autorads; they stayed late at the lab counting grains. Andrew called me at 1 AM. "It's on chromosome 5 in kangaroos." A funny place for a sex gene. Both probes mapped to the same spot, to which other human X short arm genes also mapped. Both probes mapped to the orthologous region in the distantly related dunnart. So *ZFY* must have originated on the autosomal region that fused to the X only 105 Mya: It could not be the universal mammalian sex determiner. I called David Page and apologetically told him he had the wrong gene; this conclusion was energetically challenged, but we eventually published a joint paper (46).

Not surprisingly, our conclusion was welcomed in Peter Goodfellow's lab in London, and Andrew became part of a renewed search there for the testis-determining gene. After another year sifting through repetitive sequences using the fragmented clones of the day, he and his colleagues (including Jamie Foster, who extended a European vacation) discovered a small intronless sequence close to the pseudoautosomal region of the Y (47). This *SRY* gene (for Sex Region on the Y) was male specific and conserved in all placental mammals. Jamie returned to clone marsupial *SRY*, which, thankfully, mapped to the kangaroo Y (48).

In his attempt to clone marsupial *SRY*, Jamie detected bands on his low-stringency Southern blots that represented similar genes. One showed dosage differences between sexes and mapped to the X in marsupials, and also in human and mouse. Sequence comparisons suggested it was the ancestor of *SRY* (49), a proposal recently confirmed by the observation of XX male babies and transgenic mice with *SOX3* misexpressed in the gonad (50). Evidently the *SOX3* gene had been truncated by a rearrangement that put it under the control of a gonad-specific promotor. How easy

it is to evolve new sex genes (51)! This gave me the first insight into how sex genes and chromosomes evolve and led to my interest in the changes that are driven by novel sex-determining genes.

The Disappearing Y

By the late 1990s, other genes had been discovered on the human and mouse Y chromosomes. They were hard to find—there are only 27 unique protein coding genes on the male-specific region of the human Y, hidden in masses of repetitive sequence. Page's group proposed that there are two quite different classes of genes on the Y (52). Boring genes like *ZFY* evolved from X copies. Interesting genes with functions in sex and sperm were unique to the Y and originated from autosomes, like *DAZ*, an amplified spermatogenesis Y gene with an autosomal homolog (53).

A hunt for interesting Y genes located *RBMY* in a region of the human Y deleted in azoospermic patients. Margaret Delbridge set out to identify and clone marsupial *RBMY* in my lab, discovering an ortholog on the marsupial Y. *RBMY* must be a very old gene, predating the mammalian divergence 166 Mya (54). But surprisingly, *RBMY*, too, had a partner on the X from which it evolved (55). So did the candidate gonadoblastoma gene, *TSPY* (56), and so do most other Y genes. This suggested that Y genes, even those with a male function, originated from genes on the X.

Unlike the X chromosome, the Y is poorly conserved between species. We identified several genes on the kangaroo Y chromosome with no homolog on the human or mouse Y (57, 58). All had partners on the marsupial (and human) X, verifying that the Y degrades independently in different lineages.

So the Y is essentially a degraded copy of the X (**Figure 4*a***). I presented this idea in 1998 at an international meeting; my title ended with "... a Feminist view," which I thought summed it up (59). This was rendered as "a *Feminist's* view," perhaps accounting for some of the hostility that this idea engendered for decades. My infamy was compounded in 2000 when I cowrote a comment in *Nature*, including a back-of-the-envelope calculation that if the Y continues to degrade at the same rate as in the past, it will disappear in a few million years, perhaps leading to new humanoid species with incompatible sex-determining systems (60).

This was not entirely fanciful. I was fascinated by a group of rodents in Eastern Europe that had, indeed, lost their Y chromosome (61, 62) and invented new sex-determining genes and chromosomes. I follow the mole voles eagerly, as well as a second rodent group (the spiny rats of Japan) that has also lost its Y and *SRY* (63) and appears to have made a master switch out of a gene upstream in the sex-determining network (64).

BECOMING A LADY

The 1990s began badly for me, with a near-fatal cerebral hemorrhage, year-long convalescence, and years of disability. But in 1999 I was elected to the Australian Academy of Science, which had the amazing effect that everything I said was suddenly worth listening to. I was reminded of Eliza Doolittle's line in George Bernard Shaw's *Pygmalion* (a.k.a. *My Fair Lady*): "The difference between a lady and a flower girl is not how she behaves, but how she is treated." Executive positions in the Academy gave me chances to influence science policy and education and, with the award of the international L'Oreal Prize in 2006, emboldened me to speak out about women in science.

I received many invitations to apply for deanships and associate-prodeputy-vice-chancellorships; universities were desperate for senior women. This was flattering, but a moment's thought reminded me that the things senior administrators do all day were precisely the parts of my job that I disliked: juggling inadequate budgets and resolving disputes. I did apply for the chair of my department and was knocked back. "You came across as a bit aggressive," the chair of the

selection committee told me. There was an enquiry, the upshot of which was a personal chair, an ideal situation for which I was later to offer heartfelt thanks to my detractors on the selection committee.

Australian National University and Canberra

The invitation I did accept was for a research-only position at the well-resourced Australian National University (ANU) in Canberra in 2001. Since John (and his vineyard), our two daughters, and my 95-year-old father decried moving to Canberra, I spent the next 10 years doing weekly 500-km commutes. I relished my time aloft—to think and plan on Tuesday as I flew to Canberra, and to integrate and dream going home on Friday.

I set up a new research unit. "What are you going to call it?" asked the director. Off the top of my head I said "comparative genomics," a term I had never heard used in 2000. Now I could legitimately compare anything with anything, and I did.

We started by expanding comparative maps of marsupials. Postdoc Janine Deakin developed efficient screening for kangaroo BACs that contained conserved genes. She streamlined FISH that, while hardly high throughput, increased our rate of mapping to 50/week, up from my original 1/year. Her dense physical gene maps complemented painting homologies in comparisons between marsupial groups (65), and with outgroups (**Figure 1*b*,*c***). This resolved longstanding disputes about the evolution of the marsupial karyotype, confirming that ancestral marsupials had 7 long chromosomes that underwent fission in different lineages, rather than the reverse (66).

What Can Genetics Do for Marsupials?

Australian mammals were turning out to be a goldmine of new genetic information. "But what is genetics doing for marsupials?" I was often asked accusingly.

In fact, I briefly worked on conservation of Australian animals in the 1980s, collaborating with colleagues Neil Murray and Bill Sherwin to study the endangered eastern barred bandicoot (67). And I worked on population and family studies of platypus in 1989, using crude molecular markers (remember RFLPs?) and D-loop variants (68). I decided that conservation genetics is hard—you never get enough samples, particularly of endangered species.

With encouragement (bordering on compulsion) from Steve O'Brien and participation from his collaborators at the Smithsonian, I agreed to a field trip to study koala populations in 1986—my first and last field trip. I broke my leg in a rabbit hole, looking upward to spot koalas high in the trees. Encased in plaster, I was threatened with jail for breaching permit conditions because I unwisely provided an aliquot of blood from each animal for hormone tests as well as DNA preparation. "The koalas didn't care," I protested later to a stern department official. Even though we produced the first population studies of Victorian koala populations (69), as well as analysis of sperm (70), it confirmed to me that fieldwork is hard, and permitology is tricky.

But our ability to map genes efficiently stood us in good stead to help solve a mysterious wildlife disease. The iconic Tasmanian devil—the largest extant marsupial carnivore—was in trouble. Animals with horrible facial tumors had been found in the mid-1990s, and these tumors seemed to be spreading across the island state. Was it a virus or a pollutant?

Cytologist Anne-Maree Pearse, who had volunteered to look at tumor chromosomes, brought preparations to show me in Canberra. Her photos showed me that tumors from several different animals all had a very abnormal karyotype with missing and rearranged chromosomes. But, remarkably, these abnormal karyotypes were all the same. This suggested that the pathogen was a clone of a tumor cell that arose in a single animal and was transmitted by biting (71). Genetic

analysis supported this radical xenograft theory (72, 73). Later Janine supervised chromosome painting and mapping to detail the chromosome changes, which pointed to a chromothrypsis event that left two chromosomes in smithereens in a female founder (74). We also accidentally discovered an extraordinary parent-specific imprinted telomere maintenance mechanism in this marsupial family (75).

During a postdoc in my lab, Liz Murchison (a Tasmanian who trained at Cold Spring Harbor) and a student examined transcriptomes from normal devil cells and tumors. Finding that many overexpressed tumor genes were typical of Schwann cells (76), they concluded that the founder had a Schwannoma, cells of which were transmitted to other animals by biting. Tumor cells were then passed to other animals in an expanding wave that has now engulfed most of the island and killed 90% of animals (77). Much ongoing genetic research aims to establish and reintroduce genetically robust animals from disease-free captive-bred populations.

"Platypus is Furry Bird"

My new freedom at ANU allowed me to launch an ambitious project on platypus. This amazing mammal is one of the basal monotremes, which diverged from other (therian) mammals 190 Mya. They are famous for their mixture of reptile-like and mammal-like characteristics, the most extraordinary of which is laying eggs. This was accepted only when a female was observed in the act: "Platypus oviparous, ovum meroblastic," read a famous four-word telegram.

We did some rudimentary gene mapping at La Trobe. Platypus chromosomes are challenging; many are tiny, almost microchromosomes (78). The sex chromosomes are quite bizarre; comparing karyotypes between male and female platypuses reveals not one pair but many that do not match in males (79). A new postdoc in my lab, Frank Grützner, also from Freiburg, worked with Willem Rens on platypus chromosome paints, finding that males had five male-specific Ys, and five Xs that were present in two copies in females (80). At male meiosis, these ten lined up in alternate order; then the Xs all segregated to one pole and the Ys to the other to fertilize female and male embryos (**Figure 3*d***).

Mapping platypus sex chromosomes now became possible using paints or anchor BACs to identify chromosomes and colocate labeled BACs bearing conserved genes. Postdocs Paul Waters and Fred Veyrunes, from Montpelier, found, surprisingly, that none of the X chromosomes shared any homology with the human (or kangaroo) X. Instead, all of the genes on the ancient therian X lay together on platypus chromosome 6, present in two copies in both sexes (81). Instead, the five platypus X chromosomes were made up of large regions shared by human autosomes. Extraordinarily, the largest X shared almost complete homology with the chicken Z chromosome, and bits of Z were scattered among the other Xs (**Figure 3*c***). "Platypus is furry bird," was my entry in a challenge to compose a modern equivalent of that famous four-word telegram.

This observation set a start date for mammal sex chromosome differentiation at 190 Mya. So the Y chromosome is disappearing even faster than I had calculated from a start date of 310 Mya provided by the divergence of mammals and reptiles that I had used in my previous calculation (82).

Weird Animal Genomes

Chromosomes and gene maps were our entrée to genomics in the 2000s. This was early days; the human genome had been completed, at astronomical cost, and mouse was nearly done. Spending $80 million to sequence a kangaroo did not appeal to our Australian politicians (maybe a sheep or cow?), and I had got nowhere pleading for funding. I was overjoyed to be approached by the US National Health Genome Research Institute (NHGRI) to develop a proposal to sequence a marsupial.

Of course, it must be a kangaroo. I rounded up the world experts on kangaroo genetics, all three of us. With Des Cooper, Marilyn Renfree, and younger colleagues I wrote a white paper to sequence the tammar wallaby. Disappointingly, NHGRI responded that, although we had made an excellent case for sequencing a marsupial, they had decided it should be an American marsupial. The Brazilian short-tailed gray opossum *Monodelphis domestica* was duly sequenced at the Broad Institute (83). The best I could do was to ensure that Australian scientists were involved in the analysis, and I was gratified that 25 authors of the *Nature* cover story were Aussies.

In the meantime, we put together an application for a Centre of Excellence in Kangaroo Genomics, along with the director of a sequencing facility, Sue Forrest, and bioinformaticist Terry Speed. We did not get it (I was told later that we wanted too much money for sequencing, which they did not regard as "research"). But they cobbled together funds from various grants into what I called a "Centre of VeryGoodness," which at least gave us some security. We struggled to sequence the tammar wallaby, finally publishing it in 2011 (84). Now several marsupials are being sequenced, some (like koala) to a very high level of assembly.

A year later, I was approached again by NHGRI, this time to sequence platypus. I orchestrated a white paper with platypus researchers. This time I was confident; platypus is unique, iconic, an outgroup to all other mammals. And there are no American monotremes. The platypus genome was sequenced at Washington University in St. Louis in 2008, again with many Australian coauthors (85).

Comparative Genomics

Sequence from marsupials and monotremes expanded our horizons. Now we could investigate genes and gene families and figure out how they evolved. An example was the α and ß globin gene clusters, which were thought to have arisen by segmental duplication and differential decay, we could now trace to insertion of the ß cluster into another chromosome (86). We also put together the opossum major histocompatibility complex region, finding it arranged more like frog than human, implying that the familiar arrangement in placental mammals is a new construct (87).

Another big surprise came when we searched for the Prader-Willi-Angelman imprinted region in marsupials and monotremes. It was simply not there; it had been cobbled together recently by a translocation and insertion of many elements (88). Indeed, we spent a lot of time searching for missing sequences—another example was the *XIST* gene that controls X chromosome inactivation in placental mammals but not marsupials (89).

We accidentally stumbled upon several novel human genes. Because we had to work at such low stringency, we often saw bands on Southerns that represented paralogs. Looking for the monotreme prion protein gene turned up its ancient and conserved ancestor, *Shadoo* (90). As well as *SOX3*, *TSPX*, and *RBMX* on the X (discovered looking for Y genes), we unearthed a whole family of autosomal paralogs (91). Surely each new human gene would attract medical grants; however, I soon found that although grants are bestowed on diseases searching for a gene, referees have little time for genes searching for a disease.

SLITHERING DOWN THE EVOLUTIONARY TREE

The name I had chosen so casually—comparative genomics—allowed me to stray far from mammals. We had already worked on birds, showing by chromosome painting that the Z chromosome of the flightless emu is homologous to the chicken Z. But unlike the tiny degraded chicken W, the emu W was virtually homologous to the Z, illustrating an early stage in W degeneration (92). Painting also revealed that in marine turtles with no sex chromosomes (they determine sex by

temperature), the chicken Z is homologous to chromosome 6, illustrating the autosomal origin of the ZW pair.

Why not snakes, then? Snake sex chromosomes became famous in the 1960s, when Ohno (19) proposed that three snake families represent stages in the progressive degeneration of the W chromosome. I bet that the snake and bird Z were homologous; I was wrong. Gene mapping later revealed a completely different origin (93), although, remarkably, they do share repetitive sequence (94).

With a new student (ex-curator of reptiles at the Australian Museum) we forged a strong relationship with reptile ecologists at the Institute of Applied Ecology (IAE) at the University of Canberra near ANU. They worked on sex determination in the central bearded dragon, a loveably spiky lizard. It evidently had genetic sex determination (GSD) because the sex ratio was 50:50 over a range of temperatures. But closely related species had temperature sex determination (TSD). Could we cross them to map genes involved in GSD and TSD?

Sex in Dragons

I talked my IAE colleagues, Arthur Georges and Steve Sarre, into working with my group, proposing that we do fancy cytogenetics to identify cryptic sex chromosomes, microdissect the ZW or XY, make DNA, and identify conserved genes that would refer us immediately to an orthologous region on the chicken—or human—genome. Comparative genomics in action! It was a rash proposal, but the granting agency loved it, and our project, "Sex in Dragons," was born.

I had written it around a postdoc in my lab and was dismayed when she elected to move to Yale. But that day, I received an email from a young Bangladeshi scientist, newly arrived in Australia from a PhD in Scotland, who had worked on fish sex chromosomes and, unusually, had excellent skills in cytology as well as molecular biology. I recruited him on the spot, and Tariq Ezaz (now a professor at IAE) became an essential part of the Dragon project.

Lizard chromosomes are hard to characterize; like those of snakes and birds, they have six macrochromosomes and a flotilla of tiny microchromosomes. Tariq compared male and female karyotypes using every kind of banding we could think of, as well as comparative genome hybridization. He identified a female-specific microchromosome, distinguished by a chunk of heterochromatin from its slightly smaller partner—a classic W and Z (95). He microdissected them, then used the DNA to identify orthologs of genes on chicken chromosome 23, a region not previously implicated in vertebrate sex determination (96).

This provided candidate sex-determining genes. Great excitement ensued when *RSPO1* (sex-reversing in humans) was identified in the chicken synteny group; however, this gene was on another evolutionary block and autosomal in dragons (97). A new candidate gene is currently being investigated.

Temperature-Induced Sex Reversal and GSD-TSD Turnover

I had long been interested in TSD, cosupervising (with Andrew Sinclair in the 1980s) a project to detect differential gene activity in alligator eggs incubated at male- and female-determining temperatures (98–100).

An IAE student isolated a female-specific repetitive sequence, which enabled us to discover that incubation at high temperatures reversed sex. At 36°C, all the hatchlings were female; half were ZW, but the other half were sex-reversed ZZ. Evidently the ZW system determined sex within the physiological temperature range but was overridden by high temperatures (101). This implied that GSD and TSD were not, as was believed, controlled by different molecular mechanisms.

Rather, temperature interacted epigenetically with a common sex pathway. Other reptiles also show temperature sex reversal (102), and it may be quite common.

This ability to determine sex in two ways, as well as the phylogeny that implied at least four switches between GSD and TSD, suggested that sex in dragons was quite labile. IAE postdoc Clare Holleley found, surprisingly, that sex-reversed ZZ females were fully fertile—in fact, they laid more eggs and had a better hatch rate than their normal ZW sisters. She could therefore mate sex-reversed ZZ females to normal ZZ males, completely eliminating the W. All the offspring were ZZ—and their sex was determined entirely by incubation temperature (103). Clare had flipped the sex-determining system from GSD to TSD in a single generation.

Was this occurring in the wild? Field observations over eight years show an increase of sex-reversed ZZ females from 6.7% to 22.2% as the ambient temperature rose gradually in its home range. Global warming has obvious implications for TSD species; GSD reptiles were thought to be robust to climate change, but in fact they may be susceptible to extreme temperatures.

At Last, the Mechanism of TSD?

Dragons have now afforded a breakthrough in our understanding of TSD, a hoary old problem investigated by endocrinologists and ecologists for 50 years.

Transcriptome analysis by PhD student Ira Deveson, working with Clare Holleley, compared sex-reversed ZZ female dragons to normal ZW females and ZZ males. They discovered a transcript that is specific for sex reversal, not shared by normal males or females. Two *Jumonji* genes, with links into the sex-determining pathway, retain an intron loaded with stop codons in sex-reversed females, so that the protein product, part of the polycomb response complex, would be truncated (103). This intron retention also distinguishes male and female alligators and turtles with TSD, making it a strong candidate for a switch between testis/ovary development. Sex-reversed dragons also have a high activity of genes associated with a stress response. Thus, extreme temperature may trigger a stress response that feeds directly into epigenetic control of the sex-determining pathway via alternative *Jumonji* transcripts.

So sex can ultimately be epigenetic. It is a wonderful system for exploring how the environment interacts with the genome.

CONCLUSIONS

Fields I tried to sidestep—gene mapping and evolution—turned out to be central to my career. And topics I swore I never would pursue—like human genetics, cancer, conservation genetics, and global warming—I have made surprising inroads into. I even published on *Drosophila* epigenetics and pyrimidine biosynthesis! Everything is related to everything, and it is very satisfying to me that the two strands of my career, epigenetics and sex chromosomes, seem to have coalesced in our understanding of Sex in Dragons.

I returned to Melbourne in 2011, to a position at La Trobe University. My job description is merely to "be distinguished." I miss the excitement of a lab full of enthusiastic young people, but I do not miss endless grant writing or midnight messages that the freezer has died. I love being engaged in continuing projects on vertebrate genomics and sex determination, many with my ex-students and postdocs.

I have been lucky to have had so much freedom to follow my nose, and I wish I thought that such freedom was still a hallmark of academia in Australia and the world. It has been exciting to work on such a variety of animals: 26 vertebrates in all. You never know what secrets lurk in the genomes of weird animals, or when your little discovery will transform fundamental knowledge.

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LITERATURE CITED

1. Fisher RA. 1930. *The Genetical Theory of Natural Selection*. Oxford: Clarendon
2. Mazia D. 1961. How cells divide. *Sci. Am.* 205:100–20
3. Gulland JM, Jordan DO. 1947. The macro-molecular behaviour of nucleic acids. *Symp. Soc. Exp. Biol.* 1947(1):56–65
4. Lyon MF. 1961. Gene action in the X-chromosome of the mouse (*Mus musculus* L.). *Nature* 190:372–73
5. Graves JAM. 1967. DNA synthesis in chromosomes of cultured leucocytes from two marsupial species. *Exp. Cell Res.* 46:37–57
6. Harris M. 1964. *Cell Culture and Somatic Variation*. New York: Holt, Rinehart and Winston
7. Graves JAM. 1972. Cell cycles and chromosome replication patterns in interspecific somatic hybrids. *Exp. Cell Res.* 73:81–94
8. Graves JAM. 1972. DNA synthesis in heterokaryons formed by fusion of mammalian cells from different species. *Exp. Cell Res.* 72:393–403
9. Ruddle FH. 1973. Linkage analysis in man by somatic cell genetics. *Nature* 242:165–69
10. Brown JD, Carone DM, Flynn BL, Finn CE, Mlynarski EE, O'Neill RJ. 2011. Centromere conversion and retention in somatic cell hybrids. *Cytogenet. Genome Res.* 134:182–90
11. Sharman GB. 1971. Late DNA replication in the paternally derived X chromosome of female kangaroos. *Nature* 230:231–32
12. Cooper DW. 1971. Directed genetic change model for X chromosome inactivation in eutherian mammals. *Nature* 230:292–94
13. Cooper DW, Johnston PG, Graves JAM, Watson JM. 1993. X-inactivation in marsupials and monotremes. *Semin. Dev. Biol.* 4:117–28
14. Graves JAM. 2016. Evolution of vertebrate sex chromosomes and dosage compensation. *Nat. Rev. Genet.* 17:33–46
15. Graves JA, Chew GK, Cooper DW, Johnston PG. 1979. Marsupial–mouse cell hybrids containing fragments of the marsupial X chromosome. *Somat. Cell Genet.* 5:481–89
16. Dawson GW, Graves JAM. 1984. Gene mapping in marsupials and monotremes. I. The chromosomes of rodent-marsupial (*Macropus*) cell hybrids, and gene assignments to the X chromosome of the grey kangaroo. *Chromosoma* 91:20–27
17. Dobrovic A, Graves JAM. 1986. Gene mapping in marsupials and monotremes. II. Assignments to the X chromosome of dasyurid marsupials. *Cytogenet. Cell Genet.* 41:9–13
18. Cox DR, Burmeister M, Price ER, Kim S, Myers RM. 1990. Radiation hybrid mapping: a somatic cell genetic method for constructing high-resolution maps of mammalian chromosomes. *Science* 250:245–50
19. Ohno S. 1967. *Sex Chromosomes and Sex-Linked Genes*. Berlin: Springer-Verlag
20. Gall JG, Pardue ML. 1969. Formation and detection of RNA-DNA hybrid molecules in cytological preparations. *PNAS* 63:378–83
21. Spencer JA, Watson JM, Graves JAM. 1991. The X chromosome of marsupials shares a highly conserved region with eutherians. *Genomics* 9:598–604

22. Sinclair AH, Wrigley JM, Graves JAM. 1987. Autosomal assignment of OTC in marsupials and monotremes: implications for the evolution of sex chromosomes. *Genet. Res.* 50:131–36
23. Wilcox SA, Watson JM, Spencer JA, Graves JAM. 1996. Comparative mapping identifies the fusion point of an ancient mammalian X-autosomal rearrangement. *Genomics* 35:66–70
24. Spencer JA, Sinclair AH, Watson JM, Graves JAM. 1991. Genes on the short arm of the human X chromosome are not shared with the marsupial X. *Genomics* 11:339–45
25. Watson JM, Spencer JA, Riggs AD, Graves JAM. 1991. Sex chromosome evolution: Platypus gene mapping suggests that part of the human X chromosome was originally autosomal. *PNAS* 88:11256–60
26. Burt DW, Bumstead N, Bitgood JJ, Ponce de Leon FA, Crittenden LB. 1995. Chicken genome mapping: a new era in avian genetics. *Trends Genet*. 11:190–94
27. Graves JAM. 1995. The origin and function of the mammalian Y chromosome and Y-borne genes—an evolving understanding. *Bioessays* 17:311–20
28. Brown CJ, Carrel L, Willard HF. 1997. Expression of genes from the human active and inactive X chromosomes. *Am. J. Hum. Genet.* 60:1333–43
29. Kohn M, Kehrer-Sawatzki H, Vogel W, Graves JA, Hameister H. 2004. Wide genome comparisons reveal the origins of the human X chromosome. *Trends Genet*. 20:598–603
30. Graves JAM, Young GJ. 1982. X-chromosome activity in heterokaryons and hybrids between mouse fibroblasts and teratocarcinoma stem cells. *Exp. Cell Res.* 141:87–97
31. Takagi N, Yoshida MA, Sugawara O, Sasaki M. 1983. Reversal of X-inactivation in female mouse somatic cells hybridized with murine teratocarcinoma stem cells in vitro. *Cell* 34:1053–62
32. Harris M. 1982. Induction of thymidine kinase in enzyme-deficient Chinese hamster cells. *Cell* 29:483–92
33. Graves JAM. 1982. 5-Azacytidine-induced re-expression of alleles on the inactive X chromosome in a hybrid mouse cell line. *Exp. Cell Res.* 141:99–105
34. Graves JAM, Gartler SM. 1986. Mammalian X chromosome inactivation: testing the hypothesis of transcriptional control. *Somat. Cell Mol. Genet.* 12:275–80
35. O'Brien SJ, Graves JAM. 1991. Report of the committee on comparative gene mapping. *Cytogenet. Cell Genet.* 58:1124–51
36. O'Brien SJ, Eisenberg JF, Miyamoto M, Hedges SB, Kumar S, et al. 1999. Genome maps 10. Comparative genomics. Mammalian radiations. Wall chart. *Science* 286:463–78
37. Murphy WJ, Larkin DM, Everts-van der Wind A, Bourque G, Tesler G, et al. 2005. Dynamics of mammalian chromosome evolution inferred from multispecies comparative maps. *Science* 309:613–17
38. Larkin DM, Pape G, Donthu R, Auvil L, Welge M, Lewin HA. 2009. Breakpoint regions and homologous synteny blocks in chromosomes have different evolutionary histories. *Genome Res.* 19:770–77
39. Donthu R, Lewin HA, Larkin DM. 2009. SyntenyTracker: a tool for defining homologous synteny blocks using radiation hybrid maps and whole-genome sequence. *BMC Res. Notes* 2:148
40. Larkin DM. 2010. Role of chromosomal rearrangement and conservative chromosome sites in amniote evolution. *Mol. Gen. Microbiol. Virol.* 25:1–7
41. Rens W, O'Brien PC, Fairclough H, Harman L, Graves JAM, Ferguson-Smith MA. 2003. Reversal and convergence in marsupial chromosome evolution. *Cytogenet. Genome Res.* 102:282–90
42. Rofe R, Hayman D. 1985. G-banding evidence for a conserved complement in the Marsupialia. *Cytogenet. Cell Genet.* 39:40–50
43. Hayman DL, Martin PG. 1969. Cytogenetics of marsupials. In *Comparative Mammalian Cytogenetics*, ed. K Benirschke, pp. 191–217. Berlin: Springer
44. Glas R, Graves JAM, Toder R, Ferguson-Smith M, O'Brien PC. 1999. Cross-species chromosome painting between human and marsupial directly demonstrates the ancient region of the mammalian X. *Mamm. Genome* 10:1115–16
45. Page DC, Mosher R, Simpson EM, Fisher EM, Mardon G, et al. 1987. The sex-determining region of the human Y chromosome encodes a finger protein. *Cell* 51:1091–104
46. Sinclair AH, Foster JW, Spencer JA, Page DC, Palmer M, et al. 1988. Sequences homologous to *ZFY*, a candidate human sex-determining gene, are autosomal in marsupials. *Nature* 336:780–83
47. Sinclair AH, Berta P, Palmer MS, Hawkins JR, Griffiths BL, et al. 1990. A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. *Nature* 346:240–44

48. Foster JW, Brennan FE, Hampikian GK, Goodfellow PN, Sinclair AH, et al. 1992. Evolution of sex determination and the Y chromosome: SRY-related sequences in marsupials. *Nature* 359:531–33
49. Foster JW, Graves JAM. 1994. An SRY-related sequence on the marsupial X chromosome: implications for the evolution of the mammalian testis-determining gene. *PNAS* 91:1927–31
50. Sutton E, Hughes J, White S, Sekido R, Tan J, et al. 2011. Identification of *SOX3* as an XX male sex reversal gene in mice and humans. *J. Clin. Investig.* 121:328–41
51. Graves JAM. 2013. How to evolve new vertebrate sex determining genes. *Dev. Dyn.* 242:354–59
52. Lahn BT, Page DC. 1997. Functional coherence of the human Y chromosome. *Science* 278:675–80
53. Saxena R, Brown LG, Hawkins T, Alagappan RK, Skaletsky H, et al. 1996. The *DAZ* gene cluster on the human Y chromosome arose from an autosomal gene that was transposed, repeatedly amplified and pruned. *Nat. Genet.* 14:292–99
54. Delbridge ML, Harry JL, Toder R, O'Neill RJ, Ma K, et al. 1997. A human candidate spermatogenesis gene, *RBM1*, is conserved and amplified on the marsupial Y chromosome. *Nat. Genet.* 15:131–36
55. Delbridge ML, Lingenfelter PA, Disteche CM, Graves JAM. 1999. The candidate spermatogenesis gene *RBMY* has a homologue on the human X chromosome. *Nat. Genet.* 22:223–24
56. Delbridge ML, Longepied G, Depetris D, Mattei MG, Disteche CM, et al. 2004. TSPY, the candidate gonadoblastoma gene on the human Y chromosome, has a widely expressed homologue on the X—implications for Y chromosome evolution. *Chromosome Res.* 12:345–56
57. Pask A, Renfree MB, Graves JAM. 2000. The human sex-reversing ATRX gene has a homologue on the marsupial Y chromosome, ATRY: implications for the evolution of mammalian sex determination. *PNAS* 97:13198–202
58. Murtagh VJ, O'Meally D, Sankovic N, Delbridge ML, Kuroki Y, et al. 2012. Evolutionary history of novel genes on the tammar wallaby Y chromosome: implications for sex chromosome evolution. *Genome Res.* 22:498–507
59. Graves JAM. 2000. Human Y chromosome, sex determination, and spermatogenesis—a feminist view. *Biol. Reprod.* 63:667–76
60. Aitken RJ, Graves JAM. 2002. The future of sex. *Nature* 415:963
61. Just W, Baumstark A, Suss A, Graphodatsky A, Rens W, et al. 2007. *Ellobius lutescens*: sex determination and sex chromosome. *Sex. Dev.* 1:211–21
62. Just W, Rau W, Vogel W, Akhverdian M, Fredga K, et al. 1995. Absence of *Sry* in species of the vole *Ellobius*. *Nat. Genet.* 11:117–18
63. Sutou S, Mitsui Y, Tsuchiya K. 2001. Sex determination without the Y chromosome in two Japanese rodents *Tokudaia osimensis* and *Tokudaia osimensis* spp. *Mamm. Genome* 12:17–21
64. Kuroiwa A, Handa S, Nishiyama C, Chiba E, Yamada F, et al. 2011. Additional copies of CBX2 in the genomes of males of mammals lacking *SRY*, the Amami spiny rat (*Tokudaia osimensis*) and the Tokunoshima spiny rat (*Tokudaia tokunoshimensis*). *Chromosome Res.* 19:635–44
65. Deakin JE, Graves JAM, Rens W. 2012. The evolution of marsupial and monotreme chromosomes. *Cytogenet. Genome Res.* 137:113–29
66. Deakin JE, Delbridge ML, Koina E, Harley N, Alsop AE, et al. 2013. Reconstruction of the ancestral marsupial karyotype from comparative gene maps. *BMC Evol. Biol.* 13:258
67. Sherwin WB, Murray ND, Graves JAM, Brown PR. 1991. Measurement of genetic variation in endangered populations: bandicoots (Marsupialia: Peramelidae) as an example. *Conserv. Biol.* 5:103–8
68. Gemmell NJ, Western PS, Watson JM, Graves JAM. 1996. Evolution of the mammalian mitochondrial control region—comparisons of control region sequences between monotreme and therian mammals. *Mol. Biol. Evol.* 13:798–808
69. Taylor AC, Graves JAM, Murray ND, Sherwin WB. 1991. Conservation genetics of the koala (*Phascolarctos cinereus*). II. Limited variability in minisatellite DNA sequences. *Biochem. Genet.* 29:355–63
70. Wildt DE, Bush M, O'Brien SJ, Murray ND, Taylor A, Graves JAM. 1991. Semen characteristics in free-living koalas (*Phascolarctos cinereus*). *J. Reprod. Fertil.* 92:99–107
71. Pearse AM, Swift K. 2006. Allograft theory: transmission of devil facial-tumour disease. *Nature* 439:549
72. Deakin JE, Belov K. 2012. A comparative genomics approach to understanding transmissible cancer in Tasmanian devils. *Annu. Rev. Genom. Hum. Genet.* 13:207–22

73. Bender HS, Graves JAM, Deakin JE. 2014. Pathogenesis and molecular biology of a transmissible tumor in the Tasmanian devil. *Annu. Rev. Anim. Biosci.* 2:165–87
74. Deakin JE, Bender HS, Pearse AM, Rens W, O'Brien PC, et al. 2012. Genomic restructuring in the Tasmanian devil facial tumour: Chromosome painting and gene mapping provide clues to evolution of a transmissible tumour. *PLOS Genet*. 8:e1002483
75. Bender HS, Murchison EP, Pickett HA, Deakin JE, Strong MA, et al. 2012. Extreme telomere length dimorphism in the Tasmanian devil and related marsupials suggests parental control of telomere length. *PLOS ONE* 7:e46195
76. Murchison EP, Tovar C, Hsu A, Bender HS, Kheradpour P, et al. 2010. The Tasmanian devil transcriptome reveals Schwann cell origins of a clonally transmissible cancer. *Science* 327:84–87
77. Jones ME, Cockburn A, Hamede R, Hawkins C, Hesterman H, et al. 2008. Life-history change in disease-ravaged Tasmanian devil populations. *PNAS* 105:10023–27
78. Wrigley JM, Graves JAM. 1988. Karyotypic conservation in the mammalian order monotremata (subclass Prototheria). *Chromosoma* 96:231–47
79. Murtagh CEA. 1977. A unique cytogenetic system in monotremes. *Chromosoma* 65:37–57
80. Grutzner F, Rens W, Tsend-Ayush E, El-Mogharbel N, O'Brien PC, et al. 2004. In the platypus a meiotic chain of ten sex chromosomes shares genes with the bird Z and mammal X chromosomes. *Nature* 432:913–17
81. Veyrunes F, Waters PD, Miethke P, Rens W, McMillan D, et al. 2008. Bird-like sex chromosomes of platypus imply recent origin of mammal sex chromosomes. *Genome Res*. 18:965–73
82. Graves JAM. 2006. Sex chromosome specialization and degeneration in mammals. *Cell* 124:901–14
83. Mikkelsen TS, Wakefield MJ, Aken B, Amemiya CT, Chang JL, et al. 2007. Genome of the marsupial *Monodelphis domestica* reveals innovation in non-coding sequences. *Nature* 447:167–77
84. Renfree MB, Papenfuss AT, Deakin JE, Lindsay J, Heider T, et al. 2011. Genome sequence of an Australian kangaroo, *Macropus eugenii*, provides insight into the evolution of mammalian reproduction and development. *Genome Biol*. 12:R81
85. Warren WC, Hillier LW, Graves JAM, Birney E, Ponting CP, et al. 2008. Genome analysis of the platypus reveals unique signatures of evolution. *Nature* 453:175–83
86. Patel VS, Cooper SJ, Deakin JE, Fulton B, Graves T, et al. 2008. Platypus globin genes and flanking loci suggest a new insertional model for beta-globin evolution in birds and mammals. *BMC Biol*. 6:34
87. Belov K, Deakin JE, Papenfuss AT, Baker ML, Melman SD, et al. 2006. Reconstructing an ancestral mammalian immune supercomplex from a marsupial major histocompatibility complex. *PLOS Biol*. 4:e46
88. Rapkins RW, Hore T, Smithwick M, Ager E, Pask AJ, et al. 2006. Recent assembly of an imprinted domain from non-imprinted components. *PLOS Genet*. 2:e182
89. Hore TA, Koina E, Wakefield MJ, Graves JAM. 2007. The region homologous to the X-chromosome inactivation centre has been disrupted in marsupial and monotreme mammals. *Chromosome Res*. 15:147–61
90. Premzl M, Sangiorgio L, Strumbo B, Graves JAM, Simonic T, Gready JE. 2003. Shadoo, a new protein highly conserved from fish to mammals and with similarity to prion protein. *Gene* 314:89–102
91. Lingenfelter PA, Delbridge ML, Thomas S, Hoekstra HE, Mitchell MJ, et al. 2001. Expression and conservation of processed copies of the RBMX gene. *Mamm. Genome* 12:538–45
92. Shetty S, Griffin DK, Graves JAM. 1999. Comparative painting reveals strong chromosome homology over 80 million years of bird evolution. *Chromosome Res*. 7:289–95
93. Nanda I, Schlegelmilch K, Haaf T, Schartl M, Schmid M. 2008. Synteny conservation of the Z chromosome in 14 avian species (11 families) supports a role for Z dosage in avian sex determination. *Cytogenet. Genome Res.* 122:150–56
94. O'Meally D, Patel HR, Stiglec R, Sarre SD, Georges A, et al. 2010. Non-homologous sex chromosomes of birds and snakes share repetitive sequences. *Chromosome Res*. 18:787–800
95. Ezaz T, Quinn AE, Miura I, Sarre SD, Georges A, Graves JAM. 2005. The dragon lizard *Pogona vitticeps* has ZZ/ZW micro-sex chromosomes. *Chromosome Res*. 13:763–76
96. Ezaz T, Moritz B, Waters P, Graves JAM, Georges A, Sarre SD. 2009. The ZW sex microchromosomes of an Australian dragon lizard share no homology with those of other reptiles or birds. *Chromosome Res*. 17:965–73

97. Ezaz T, Azad B, O'Meally D, Young MJ, Matsubara K, et al. 2013. Sequence and gene content of a large fragment of a lizard sex chromosome and evaluation of candidate sex differentiating gene R-spondin 1. *BMC Genom*. 14:899
98. Western PS, Harry JL, Graves JAM, Sinclair AH. 1999. Temperature-dependent sex determination in the American alligator: *AMH* precedes *SOX9* expression. *Dev. Dyn.* 216:411–19
99. Western PS, Harry JL, Graves JAM, Sinclair AH. 1999. Temperature-dependent sex determination: upregulation of *SOX9* expression after commitment to male development. *Dev. Dyn.* 214:171–77
100. Western PS, Harry JL, Graves JAM, Sinclair AH. 2000. Temperature-dependent sex determination in the American alligator: expression of *SF1*, *WT1* and *DAX1* during gonadogenesis. *Gene* 241:223–32
101. Quinn AE, Georges A, Sarre SD, Guarino F, Ezaz T, Graves JAM. 2007. Temperature sex reversal implies sex gene dosage in a reptile. *Science* 316:411
102. Radder RS, Quinn AE, Georges A, Sarre SD, Shine R. 2008. Genetic evidence for co-occurrence of chromosomal and thermal sex-determining systems in a lizard. *Biol. Lett.* 4:176–78
103. Holleley CE, O'Meally D, Sarre SD, Graves JAM, Ezaz T, et al. 2015. Sex reversal triggers the rapid transition from genetic to temperature-dependent sex. *Nature* 523:79–82
104. Serov O, Chowdhary B, Womack JE, Graves JAM. 2005. Comparative gene mapping and chromosome painting in mammals and the reconstruction of the ancestral mammal karyotype. In *Mammalian Genomics*, ed. A Ruvinsky, JAM Graves, pp. 349–89. Wallingford, UK: CABI
105. Graves JAM, Renfree MB. 2013. Marsupials in the age of genomics. *Annu. Rev. Genom. Hum. Genet.* 14:393–420