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**The Z and W sex chromosomes of birds have evolved independently from the mammalian X and Y chromosomes [1]. Unlike mammals, female birds are heterogametic (ZW), while males are homogametic (ZZ). Therefore male birds, like female mammals, carry a double dose of sex-linked genes relative to the other sex. Other animals with nonhomologous sex chromosomes possess "dosage compensation" systems to equalize the expression of sex-linked genes. Dosage compensation occurs in animals as diverse as mammals, insects, and nematodes, although the mechanisms involved differ profoundly [2]. In birds, however, it is widely accepted that dosage compensation does not occur [3–5], and the differential expression of Z-linked genes has been suggested to underlie the avian sex-determination mechanism [6]. Here we show equivalent expression of at least six of nine Z chromosome genes in male and female chick embryos by using real-time quantitative PCR [7]. Only the Z-linked *ScII* gene, whose ortholog in *Caenorhabditis elegans* plays a crucial role in dosage compensation [8], escapes compensation by this assay. Our results imply that the majority of Z-linked genes in the chicken are dosage compensated.**

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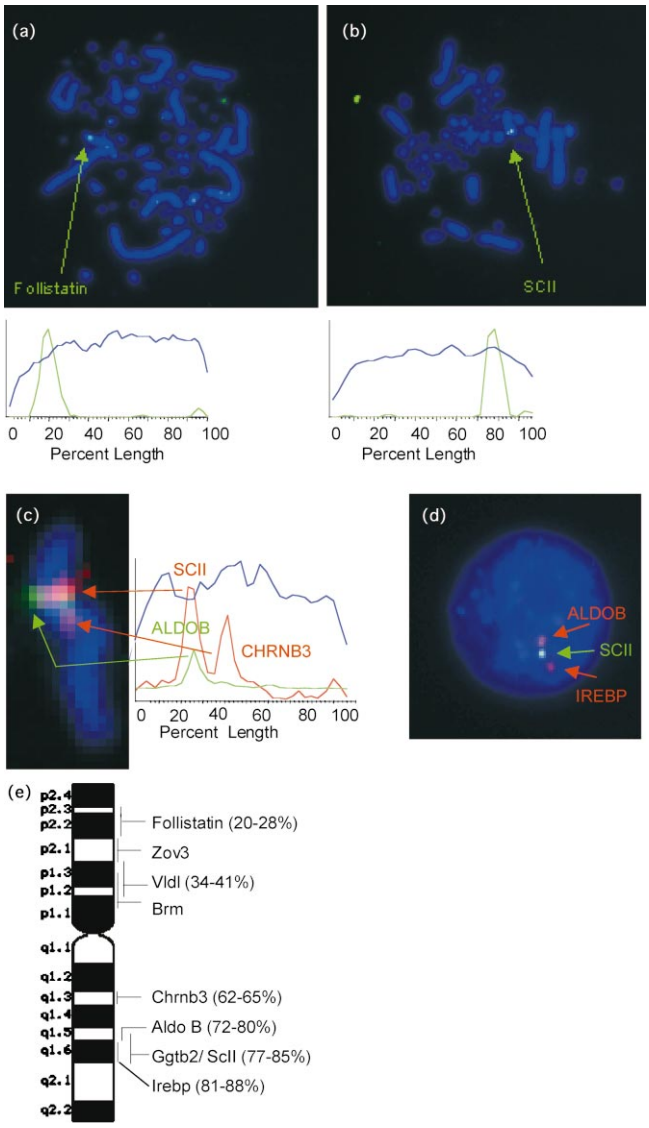
## Results and discussion

The Z-linked enzyme aconitase (also known as iron response element binding protein, IREBP) displays higher activity in the livers of male, as compared to female, domestic fowl, house sparrows, and spotted turtledoves [3]. Although based on a single gene, this represents the main evidence against avian dosage compensation. The lack of sex chromatin [4] or a late-replicating Z chromosome [9] in male avian nuclei also indicates that sex chromosome inactivation, as observed in mammals, is unlikely in birds. Dosage compensation by other methods, however, remains a possibility. In order to clarify whether avian dosage compensation occurs, we aimed to assay gene expression from discrete points along the Z chromosome in male and female chickens. Due to the paucity of physically mapped genes available, we began by constructing our own minimal physical map of the Z chromosome.

Two new Z chromosome genes, *follistatin* and *ScII*, whose Z-linkage was previously indicated by gene dosage in Southern blots were assigned to distal portions of the p and q arms, respectively (Figure 1). Since human *follistatin* maps to 5p1.4 (OMIM), our results confirm and extend the previously noted conservation of synteny between human 5p1.2–1.4 and this region of Zp [6]. Cytogenetic locations were also assigned to two genetically mapped genes (*CHRNB3* and *aldolase B*) and were confirmed for two previously mapped genes (*VLDLR* and *IREBP*) (Figure 1 and Table 1). W homologs were not detected, by fluorescent in situ hybridization (FISH), for any of the six Z chromosome genes. Incorporating a further three genes with previously known locations, we assembled a set of nine genes to represent both arms of the chicken Z chromosome (Figure 1e).

We next compared gene expression from the nine Z chromosome genes in individual male and female chick embryos by using real-time quantitative PCR. We expected that dosage compensation would result in similar expression levels in male individuals as those seen for females while the lack of dosage compensation would result in an average expression level for ZZ males that was twice that of ZW females. We were surprised to find that relative expression levels for eight of our nine test genes were similar in both sexes, with male:female ratios ranging from 0.8 to 1.4 (Figure 2). $p$ values were consistent with the null hypothesis of a male:female ratio equaling 1.0 ($p > 0.05$) for six of the nine genes (excepting *ScII*, *ZOV3*, and *GGTB2*; Table 1). These results imply dosage compensation for most genes. In contrast, the *ScII* gene gave consistently higher relative expression levels for male embryos at days 3 and 4 of development than for corresponding female embryos (Figure 2). An approximately 2-fold difference ($p = 0.58$) detected for *ScII* expression in males versus females suggests that no dosage compensation occurs for this gene. We calculated male:female ratios for each gene in two data sets corrected for RNA concentrations according to expression from the autosomal genes

**Figure 1**




FISH mapping of Z chromosome genes. **(a–c)** Examples of hybridizations to metaphase chromosomes, with gene names written in the same colors as their hybridized probe signals. In each case a colored plot shows the chromosomal position of the red and green probe signals as a percentage of the entire chromosome, represented in blue. (a) *follistatin* maps to Zp2.2–2.3, and the signal is shown at approximately 20% from p to q. (b) *ScII* maps to Zq1.5–1.6, and the signal is shown at approximately 80% from p to q. (c) *ScII* mapping relative to *aldolase B* (Zq1.5, 75% pq) and *CHRNB3* (Zq1.3, 62% pq). The Z chromosome plot shown is drawn from q to p. **(d)** *ScII* location between *aldolase B* and the previously mapped *IREBP* gene is shown by hybridization to an interphase nucleus. **(e)** Cartoon of the Z chromosome shows physical locations for the nine mapped genes used in the expression analysis. Percentage distances along the chromosome from p to q were derived from multiple experiments.

*GAPDH* and either *growth hormone* or β *actin*. Male:female ratios were largely consistent between these two data sets (Table 1).

Our results indicate that, contrary to current dogma, avian dosage compensation does occur and is demonstrated for at least six of the nine chicken Z chromosome genes analyzed. Compensated genes are found on both arms of the Z chromosome (Table 1) and include the *IREBP* gene, whose sexually dimorphic enzyme activity in avian liver [3] was previously cited as evidence against compensation. This contradiction might arise from the different tissues used for assay. Sex differences in expression levels have been reported previously for liver enzymes [10], while we conducted our experiments on chick embryos prior to formation of the genital ridge specifically to avoid differences due to sexual differentiation.

In addition to the finding that avian dosage compensation exists, we have found one example, the *ScII* gene, that seems to escape such compensation. This is not without precedent since 15% of 224 human X chromosome genes tested have been shown to escape X inactivation [11]. These genes are grouped nonrandomly along the inactive human X chromosome, with one-third of the genes on the short arm escaping inactivation [11]. Athough we have found dosage compensation for two genes located in the same or adjacent cytogenetic band with the *ScII* gene (Table 1), it is still unclear whether *ScII* represents an isolated example or is one of a group of Z chromosome genes that escape dosage compensation. The higher level of *ScII* expression observed in day 3 males continues in the male gonad until at least day 8 of development (data not shown). This sexually dimorphic expression pattern both before and during sex determination suggests a role for *ScII* in gonadal development or in sex-determination itself. Such sexually dimorphic expression resembles that of the *DMRT1* gene demonstrated at day 4.5 [12, 13]. *DMRT1* is thought to represent a conserved component of vertebrate sex-determining pathways [12], and it has been suggested that its sexually dimorphic expression in birds results from the lack of dosage compensation [6]. We were unable to detect *DMRT1* expression in our day 3 and 4 samples, and it is therefore unclear whether the sexually dimorphic expression pattern for *DMRT1* represents escape from dosage compensation or whether it is secondary to other events during the formation of the genital ridge.

We have detected significant within-sex variation in relative expression levels for most genes. We believe that this finding is due to genetic heterogeneity within non-inbred chicken lines. We consider our results to be reliable due to their reproducibility in repeated experiments incorporating two different control genes (Table 1) and two methods of real-time PCR (Figure 2a,b). For the relatively

**Table 1**

**Male:female ratios for expression of chicken Z genes normalized for expression of *GAPDH, growth hormone* gene, or *actin***

| Map location | Z gene | Expression (m:f)* normalized to expression of | | Result | *p* value |
|---|---|---|---|---|---|
| | | *GAPDH* | *GH/actin* | | 1:1 (2:1) |
| Zp2.2–2.3 | *follistatin* | 1.35 (22/16) | nd | Compensated | 0.08 (0.04) |
| Zp2.1 | *ZOV3* | 1.35 (6/6) | 1.47 (7/6) | ? | 0.05 (0.04) |
| Zp1.2–1.3 | *VLDLR* | 0.78 (6/6) | 1.00 (6/6) | Compensated | 0.17 (0.00) |
| Zp1.1–1.2 | *BRM* | 1.23 (6/6) | 1.45 (6/6) | Compensated | 0.26 (0.00) |
| Zq1.3 | *CHRNB3* | 1.33 (6/6) | nd | Compensated | 0.18 (0.05) |
| Zq1.5 | *aldolase B* | 0.94 (6/6) | nd | Compensated | 0.77 (0.01) |
| Zq1.5–1.6 | *ScII* | 2.24 (22/16) | 2.00 (22/16) | Not Compensated | 0.05 (0.58) |
| Zq1.5–1.6 | *GGTB2* | 1.39 (6/6) | 1.07 (6/5) | ? | 0.02 (0.00) |
| Zq1.6 | *IREBP* | 0.83 (6/6) | 1.09 (6/5) | Compensated | 0.30 (0.00) |
| 1q4.1–q4.5 | *GH* | 1.11 (6/6) | 0.70 (2/1) | (Autosomal) | 0.45 (0.02) |

*Relative expression in males:females presented as ratios of average values corrected against either *GAPDH* and *growth hormone* or *GAPDH* and *actin* for the *ScII* and *GH* genes. (n/n) = n males/n females, nd = not done. *p* values indicate degree of consistency with a null hypothesis of a 1:1 ratio of males:females. Bracketed values are for the null hypothesis of a 2:1 ratio of males:females.

small sample size studied, significant individual variation could distort average expression values and result in cases such as those of the *ZOV3* and *GGTB2* genes, for which the expression profiles suggest dosage compensation (Figure 2a) but similarities in male-to-female expression levels are not statistically significant (Table 1). For *ZOV3*, only one male value is outside the range of the female values (Figure 2a). An alternative explanation for the raised level of *ZOV3* transcript in this individual is heterogeneous dosage compensation, in which some but not all individuals are compensated for a particular gene. The *REP1* and *TIMP1* genes are examples of the approximately 5% of human genes that show such heterogeneous inactivation patterns [11, 14].

Dosage compensation is achieved by very different mechanisms in mammals, fruitfies, and nematode worms. All female mammals inactivate one of their two X chromosomes, which results in the activity of only one X. This inactivation thus equalizes female gene dosage with that of males. For the fruitfly *Drosophila*, the single male X chromosome shows increased expression relative to that of females, while in the worm *C. elegans* the hermaphrodite downregulates expression from both X chromosomes [15]. There is no reason to suspect that the mechanism of dosage compensation in birds will be similar to that of mammals, or indeed to any of the three described mechanisms. However, it is interesting to note that the chicken *ScII* gene, which escapes dosage compensation, is a member of the structural-maintenance-of-chromosomes (SMC) family and has a role in mitotic chromosome condensation [16]. *MIX-1* is the *C. elegans* ortholog of *ScII* [17], and in addition to its role in mitotic chromosome condensation, MIX-1 localizes to the X chromosomes of XX hermaphrodites, in conjunction with dosage compensation–specific proteins, and reduces X chromosome gene expression [8]. One speculative possibility is that the sexually dimorphic expression of the *ScII* gene in chickens is related to the establishment or maintenance of dosage compensation.

## Materials and methods

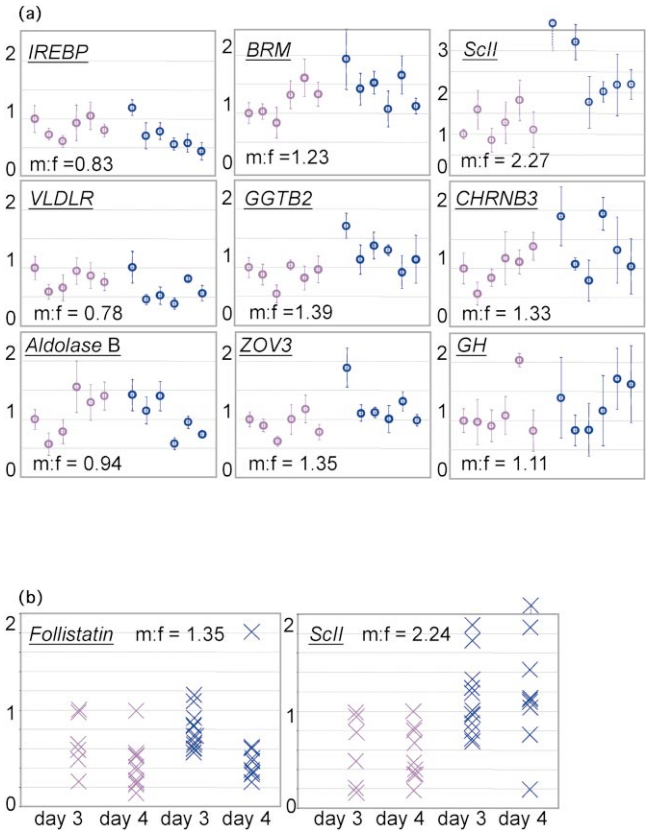
*Physical mapping of the Z chromosome*

All genes were mapped by the three-color fluorescent in situ hybridization (FISH) of BAC and YAC clones to metaphase and interphase chicken chromosomes. We obtained the FISH probes by screening YAC and BAC libraries [18, 19] or by hybridizing HGMP-distributed spotted filters (produced by Richard Crooijman). Alternatively, Richard Crooijman carried out two-dimensional PCR screening. The library identification numbers and known insert sizes of Z chromosome gene clones used were as follows: *follistatin,* YAC987 (900 Kb); *VLDLR*, YAC1117 (600 Kb) BAC35o07; *CHRNB3*, BAC51e3 (120 Kb); *aldolase B*, BAC98c17 (130 Kb); *ScII*, BAC76g06 (190 Kb); *IREBP*, BAC11k15. BAC labeling and FISH procedures were as previously described [20]. Recombinant YACs were gel purified, and fluorescent nucleotides were incorporated by degenerate oligonucleotide PCR [21]. Cytogenetic bands were assigned by reverse DAPI banding, and percentage distances from p to q were also assigned to each probe.

*Real-time quantitative PCR*

Embryos were dissected and staged at days 3 (stage 20/21) and 4 (stage 23/24) of development and were stored frozen while DNA extracted from extraembryonic tissues was used to sex individual embryos by established procedures [22]. RNA was extracted from whole chick embryos with RNAzol B (AMS Biotechnology), and cDNA was produced by oligo dT priming. RT-PCR primers were as follows: *follistatin* (X87609), 5′-TGTGCTCCGGATTGCTCTAA-3′ and 5′-CTTCAAGTT CGGGCTGTTCTTT-3′; *ZOV3* (D16151), 5′-ATCGCCATCAATGAC TCCC-3′ and 5′-GCTTCCCTTTACTCTTGCC-3′; *VLDLR* (X80207), 5′-CCTCAGTCAACCCAGTGTATC-3′ and 5′-ACTCCAACTCATCAC TACCATC-3′; *BRM* (X91638), 5′-TGTTGCTGTGTCATAATGCTC-3′ and 5′-ACTCCTCTTCATCATCCTCATC-3′; *CHRNB3* (X83739), 5′-TCC TCAGCAACTTACCACC-3′ and 5′-GCAATCACGACTTTCTCTCC-3′; *aldolase B* (M10946), 5′-GCAAGAAACAACTAACGCTG-3′ and 5′-ACT GCAAAATGTGGTAATGGG-3′; *GGTB2* (U19890), 5′-CCAATGGAT GACAGGAACAC-3′ and 5′-GCAGACACACCTCCAAAATAC-3′; *ScII* (X80792), 5′-CTGGCATCGTATCTGGAGTATCA-3′ and 5′-ACGAA CATACTGAAAAGCCACATAGA-3′; *IREBP* (D16150), 5′-AATACCA GTAACCCCTCAGTC-3′ and 5′-CATAACCCACTACATCAAACCC-3′; *GH* (D90458), 5′-TACGACAAGTTCGACATCCACC-3′ and 5′-TCAT

**Figure 2**




Expression analysis for nine Z chromosome genes and one autosomal gene on individual male and female embryos. **(a)** Relative expression levels for eight Z chromosome genes and *growth hormone* gene, corrected for *GAPDH* expression, in the same six female and six male day 4 embryos by SYBR Green real-time quantitative PCR analysis. Females (left) are shown in pink, and males (right) are shown in blue. Expression levels in each case are relative to the first female, whose value was scaled to 1.0. Male:female (m:f) values are given. The first male value for the *ScII* plot was 6.0 and could not therefore be plotted within the chart. Standard deviations of the mean values are shown. **(b)** Relative expression levels for 22 male and 16 female day 3 and 4 samples for *follistatin* and *ScII* genes. The expression levels are corrected for *GAPDH* expression by TaqMan analysis. Male:female (m:f) values for combined results from days 3 and 4 are given. One male day 4 *ScII* value was 3.9 and could not be plotted on the chart.

CACCTTCAGGTAGGTCTC-3′; *GAPDH* (M11213), 5′-ATCTTTAAC CACTGCTCCTTG-3′ and 5′-CATGCTGAGCCTATTCACTG-3′; and β-actin (L08165), 5′-TATTGCTGCGCTCGTTGTTG-3′ and 5′-GGG CGACCCACGATAGATG-3′. Probes for TaqMan assays were labeled with either FAM and TAMRA or VIC and TAMRA and were as follows: *ScII*, CGCCTTAAATTCTCCACTTCTCGTGTCATTT; *follistatin*, TTCCT GTAGGTTTTCCCATCTAAGCCACACA; and *actin,* CGAAACCGGC CTTGCACATACCG. RT-PCR was carried out with the SYBR Green or TaqMan reagents and procedures (Applied Biosystems), and amplifications were detected with the ABI PRISM 7700 Sequence Detection System. We used RNA from the same embryos to analyze the expression of all nine genes. All results are averages from triplicate or quadruplicate PCR reactions. Relative expression levels were calculated after correction for expression of *GAPDH*, *beta actin*, or *growth hormone* gene, none of which are Z linked and which were assayed in triplicate or quadruplicate in parallel with the test gene. Since assays of all cDNAs with any one pair of a test gene and a control gene were carried out simultaneously, we were able to assign an expression value of 1.0 to the first female in the data set and calculate relative expression levels for all other samples. We assayed all genes except *follistatin* by using SYBR Green analysis, which depends upon nonspecific binding of dye to DNA. The specificity of the reaction was therefore tested for each primer pair. *follistatin* and *ScII* were both tested with the TaqMan assay, whose specificity derives from fluorescence energy transfer (FRET) probes [7]. *ScII* was assayed by both TaqMan and SYBR Green methods and gave a similar spread of relative expression levels and male:female ratios in both experiments (Figure 2).

Statistical significance was assessed in an unrelated samples 2-tailed *t* test in which equal variance was not assumed. *p* values indicate the degree of consistency with a null hypothesis of no difference in the means or of a 2-fold difference, and *p* values $>0.05$ were taken to be significant.

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