The genome of a songbird

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Abstract

The zebra finch is an important model organism in several fields[1,2] with unique relevance to human neuroscience[3,4]. Like other songbirds, the zebra finch communicates through learned vocalizations, an ability otherwise documented only in humans and a few other animals and lacking in the chicken[5]—the only bird with a sequenced genome until now[6]. Here we present a structural, functional and comparative analysis of the genome sequence of the zebra finch (*Taeniopygia guttata*), which is a songbird belonging to the large avian order Passeriformes[7]. We find that the overall structures of the genomes are similar in zebra finch and chicken, but they

differ in many intrachromosomal rearrangements, lineage-specific gene family expansions, the number of long-terminal-repeat-based retrotransposons, and mechanisms of sex chromosome dosage compensation. We show that song behaviour engages gene regulatory networks in the zebra finch brain, altering the expression of long non-coding RNAs, microRNAs, transcription factors and their targets. We also show evidence for rapid molecular evolution in the songbird lineage of genes that are regulated during song experience. These results indicate an active involvement of the genome in neural processes underlying vocal communication and identify potential genetic substrates for the evolution and regulation of this behaviour.

As in all songbirds, singing in the zebra finch is under the control of a discrete neural circuit that includes several dedicated centres in the forebrain termed the 'song control nuclei' (for an extensive series of reviews see [ref. 8]). Neurophysiological studies in these nuclei during singing have yielded some of the most illuminating examples of how vocalizations are encoded in the motor system of a vertebrate brain[9,10]. In the zebra finch, these nuclei develop more fully in the male than in the female (who does not sing), and they change markedly in size and organization during the juvenile period when the male learns to sing[11]. Analysis of the underlying cellular mechanisms of plasticity led to the unexpected discovery of neurogenesis in adult songbirds and life-long replacement of neurons[12]. Sex steroid hormones also contribute to songbird neural plasticity, in part by influencing the survival of new neurons[13]. Some of these effects are probably caused by oestrogen and/or testosterone synthesized within the brain itself rather than just in the gonads[14].

Song perception and memory also involve auditory centres that are present in both sexes, and the mere experience of hearing a song activates gene expression in these auditory centres[15]. The gene response itself changes as a song becomes familiar over the course of a day[16] or as the context of the experience changes[17]. The act of singing induces gene expression in the male song control nuclei, and these patterns of gene activation also vary with the context of the experience[18]. The function of this changing genomic activity is not yet understood, but it may support or suppress learning and help integrate information over periods of hours to days[19].

The chicken genome is the only other bird genome analysed to date[6]. The chicken and zebra finch lineages diverged about 100 million years ago near the base of the avian radiation[7]. By comparing their genomes we can now discern features that are shared (and thus generally characteristic of birds), and features that are most conspicuously different between the two lineages—some of which will be related to the distinctive neural and behavioural traits of songbirds.

We sequenced and assembled a male zebra finch genome using methods described previously[6,20]. A male (the homogametic sex in birds) was chosen to maximize coverage of the Z chromosome. Of the 1.2 gigabase (Gb) draft assembly, 1.0 Gb has been assigned to 33 chromosomes and three linkage groups, by using zebra finch genetic linkage[21] and bacterial artificial chromosome (BAC) fingerprint maps. The genome assembly is of sufficient quality for the analysis presented here (see Supplementary Note 1 and Supplementary Table 1). A total of 17,475 protein-coding genes were predicted from the zebra finch genome assembly using the Ensembl pipeline supplemented by Gpipe gene models (Supplementary Note 1). To extend further the characterization of genes relevant to brain and behaviour, we also sequenced complementary DNAs from the forebrain of zebra finches at 50 (juvenile, during the critical song learning period) and 850 (adult) days post-hatch, mapping these reads (Illumina GA2) to the protein-coding models (Supplementary Note 1). Of the 17,475 protein-coding gene models we find 9,872 (56%) and 10,106 (57%) genes expressed in the forebrain at these two ages (90.7% overlap), respectively. In addition to evidence for

developmental regulation, these reads show further splice forms, new exons and untranslated sequences (Supplementary Figs 1 and 2).

To address issues of large-scale genome structure and evolution, we compared the chromosomes of zebra finch and chicken using both sequence alignment and fluorescent *in situ* hybridization. These analyses showed overall conservation of synteny and karyotype in the two species, although the rate of intrachromosomal rearrangement was high (Supplementary Note 2). We were also surprised to see genes of the major histocompatibility complex (MHC) dispersed across several chromosomes in the zebra finch, in contrast to the syntenic organization of both chicken and human MHCs (Supplementary Note 2).

We assessed specific gene losses and expansions in the zebra finch lineage by constructing phylogenies of genes present in the last common ancestor of birds and mammals (Supplementary Note 2 and Supplementary Fig. 3). Both the zebra finch and the chicken genome assemblies lack genes encoding vomeronasal receptors, casein milk proteins, salivary-associated proteins and enamel proteins—not surprisingly, as birds lack vomeronasal organs, mammary glands and teeth. Unexpectedly, both species lack the gene for the neuronal protein synapsin 1 (*SYN1*); comparative analyses suggest that the loss of *SYN1* and flanking genes probably occurred in an ancestor to modern birds, possibly within the dinosaur lineage (Supplementary Note 2, Supplementary Table 2 and Supplementary Fig. 4). Both zebra finch and chicken have extensive repertoires of olfactory receptor-like sequences (Supplementary Note 2 and Supplementary Fig. 5), proteases (Supplementary Table 3), and a rich repertoire of neuropeptide and pro-hormone genes.

Compared to mammals, zebra finch has duplications of genes encoding several proteins with known neural functions, including growth hormone, (Supplementary Fig. 3), caspase-3 and β-secretase (Supplementary Table 3). Two large expansions of gene families expressed in the brain seem to have occurred in the zebra finch lineage after the split from mammals. One involves a family related to the *PAK3* (p21-activated kinase) gene. Thirty-one uninterrupted *PAK3*-like sequences have been identified in the zebra finch genome, of which 29 are expressed in testis and/or brain (Supplementary Note 2). The second involves the *PHF7* gene, which encodes a zinc-finger-containing transcriptional control protein. Humans only have a single *PHF7* gene, but remarkably the gene has been duplicated independently, many times in both the zebra finch and chicken lineages to form species-specific clades of 17 and 18 genes, respectively (Supplementary Fig. 6). In the zebra finch these genes are expressed in the brain (Supplementary Note 2).

An intriguing puzzle in avian genomics has been the evident lack of a chromosome-wide dosage compensation mechanism to balance the expression of genes on the Z sex chromosome, which is present in two copies in males but only one in females[22,23]. The chicken has been suspected of exerting dosage compensation on a more local level, by the non-coding RNA MHM (male hypermethylated)[24,25], to cause a characteristic variation of gene expression along the Z chromosome. The zebra finch genome assembly, however, lacks an MHM sequence, and genes adjacent to the comparable MHM chromosomal position show no special cluster of dosage compensation (Fig. 1 and Supplementary Note 2). Thus, the putative MHM-mediated mechanism of restricted Z-chromosome dosage compensation is not common to all birds. Chromosomal sex differences in the brain could have a direct role in the sex differences so evident in zebra finch neuroanatomy and singing behaviour.

In mammals, as much as half of their genomes represent interspersed repeats derived from mobile elements, whereas the interspersed repeat content of the chicken genome is only

8.5%. We find that the zebra finch genome also has a low overall interspersed repeat content (7.7%), containing a little over 200,000 mobile elements (Supplementary Tables 4 and 5). The zebra finch, however, has about three times as many retrovirus-derived long terminal repeat (LTR) element copies as the chicken, and a low copy number of short interspersed elements (SINEs), which the chicken lacks altogether. Expressed sequence tag (EST) analysis shows that mobile elements are present in about 4% of the transcripts expressed in the zebra finch brain, and some of these transcripts are regulated by song exposure (next section, Table 1). Figure 2 shows an example of an RNA that was identified in a microarray screening for genes specifically enriched in song control nuclei[26] and now seems to represent a long non-coding RNA (ncRNA) containing a CR1-like mobile element. These results indicate that further experiments investigating a possible role of mobile-element-derived repeated sequences in vocal communication are warranted.

A large portion of the genome is directly engaged by vocal communication. A recent study[27] defined distinct sets of RNAs in the auditory forebrain that respond in different ways to song playbacks during the process of song-specific habituation, a form of learning[16]. We now map each of these song-responsive RNAs to the genome assembly (Table 1 and Supplementary Note 3). Notably, we find evidence that ~40% of transcripts in the unstimulated auditory fore-brain are non-coding and derive from intronic or intergenic loci (Table 1). Among the RNAs that are rapidly suppressed in response to new vocal signals (‘novel down’), two-thirds are ncRNAs.

The robust involvement of ncRNAs in the response to song led us to ask whether song exposure alters the expression of microRNAs—small ncRNAs that regulate gene expression by binding to target messenger RNAs. Indeed we find that miR-124, a conserved microRNA implicated in neurological function in other species[28], is rapidly suppressed in response to song playbacks (Fig. 3). We independently measured this effect by direct Illumina sequencing of small RNAs in the auditory forebrain, and also identified other known and new microRNAs, several of which also change in expression after song stimulation (Supplementary Note 2).

A potential site of action for microRNAs was shown by genomic mapping of transcripts that increase rapidly after new song exposure (Table 1, ‘novel up’). Two of the cDNA clones that measured the most robust increases[27] align to an unusually long (3 kilobases (kb)) 3′ untranslated region (UTR) in the human gene that encodes the NR4A3 transcription factor protein (Fig. 4a). The entire UTR is similar in humans and zebra finches, with several long segments of >80% identity (Fig. 4b). Within these segments we find conserved predicted binding sites for 11 different microRNAs, including five new microRNAs found by direct sequencing of small RNAs from the zebra finch forebrain (Fig. 4b). These findings indicate that this *NR4A3* transcript element may function in both humans and songbirds to integrate many conserved microRNA regulatory pathways.

The act of singing also alters gene expression in song control nuclei[29], and we used the genome assembly to analyse the transcriptional control structure of this response. Using oligonucleotide microarrays, we identified 807 genes in which expression significantly changed as a result of singing. These were grouped by *k*-means clustering into 20 distinct expression profile clusters (Fig. 5a and Supplementary Note 3). Gene regulatory sequences (transcription-factor-binding sites) were predicted across the genome using a new motif-scanning approach (Supplementary Note 1), and we observed significant correlation between changes in expression of transcription factor genes and their predicted targets (Fig. 5b and Supplementary Table 6). Thus, the experience of singing and hearing song engages complex gene regulatory networks in the forebrain, altering the expression of microRNAs,

transcription factor genes, and their targets, as well as of non-coding RNA elements that may integrate transcriptional and post-transcriptional control systems.

Learned vocal communication is crucial to the reproductive success of a songbird, and this behaviour evolved after divergence of the songbird lineage[5]. Thus, it seems likely that genes involved in the neurobiology of vocal communication have been influenced by positive selection in songbirds. With this in mind, we examined the intersection of two sets of genes: (1) those that respond to song exposure in the auditory forebrain as discussed in the previous section; and (2) those that contain residues that seem to have been positively selected in the zebra finch lineage, as determined using phylogenetic analysis by maximum likelihood (PAML) (Supplementary Note 4). There are 214 genes that are common to both lists. Of these, 49 are suppressed by song exposure (Supplementary Table 7), and 6 of these 49 are explicitly annotated for ion channel activity (Table 2). This yields a highly significant statistical enrichment for the term 'ion channel activity' ($P = 0.0016$, false discovery rate (FDR) adjusted Fisher's exact test) and other related terms in this subset of genes (Supplementary Tables 8 and 9). Independent evidence has also demonstrated differential anatomical expression of ion channel genes in song control nuclei[26,30]. Ion channel genes have important roles in many aspects of behaviour, neurological function and disease[31]. This class of genes is highly likely to be linked to song behaviour and should be a major target for future functional studies.

Passerines represent one of the most successful and complex radiations of terrestrial animals[7]. Here we present the first, to our knowledge, analysis of the genome of a passerine bird. The zebra finch was chosen because of its well-developed status as a model organism for a number of fields in biology, including neurobiology, ethology, ecology, biogeography and evolution. In the zebra finch as in the chicken, we see a smaller, tighter genome compared to mammals, with a marked reduction of interspersed repeats. The zebra finch presents a picture of greater genomic plasticity than might have been expected from the chicken and other precedents, with a high degree of intrachromosomal rearrangements between the two avian species, gene copy number variations and transcribed mobile elements. Yet we also see an overall similarity to mammals in protein-coding gene content and core transcriptional control systems.

Our analysis suggests several channels through which evolution may have acted to produce the unique neurobiological properties of songbirds compared to the chicken and other animals. These include the management of sex chromosome gene expression, accelerated evolution of neuronal ion transport genes, gene duplications to produce new variants of *PHF7*, *PAK3* and other neurobiologically important genes, and a new arrangement of MHC genes. Most notably, our analyses suggest a large recruitment of the genome during vocal communication, including the extensive involvement of ncRNAs. It has been proposed that ncRNAs have a contributing role in enabling or driving the evolution of greater complexity in humans and other complex eukaryotes[32]. Seeing that learned vocal communication itself is a phenomenon that has emerged only in some of the most complex organisms, perhaps ncRNAs are a nexus of this phenomenon.

Much work will be needed to establish the actual functional significance of many of these observations and to determine when they arose in avian evolution. This work can now be expedited with the recent development of a method for transgenesis in the zebra finch[33]. An important general lesson, however, is that dynamic and serendipitous aspects of the genome may have unexpected roles in the elaborate vocal communicative capabilities of songbirds.

Methods Summary

Sequence assembly

Sequenced reads were assembled and attempts were made to assign the largest contiguous blocks of sequence to chromosomes using a genetic linkage map[21], fingerprint map and synteny with the chicken genome assembly Gallus_gallus-2.1, a revised version of the original draft[6] (Supplementary Note 1).

Genes

Gene orthology assignment was performed using the EnsemblCompara GeneTrees pipeline and the OPTIC pipeline (Supplementary Note 1). Orthology rate estimation was performed with PAML (pairwise model = 0, Nssites = 0). In all cases, codon frequencies were estimated from the nucleotide composition at each codon position (F3X4 model).

Gene expression and evolution

Methods for Illumina read counting, *in situ* hybridization, TaqMan RT–PCR, microarrays, regulatory motif and evolutionary rate analyses are given in Supplementary Notes 1–4.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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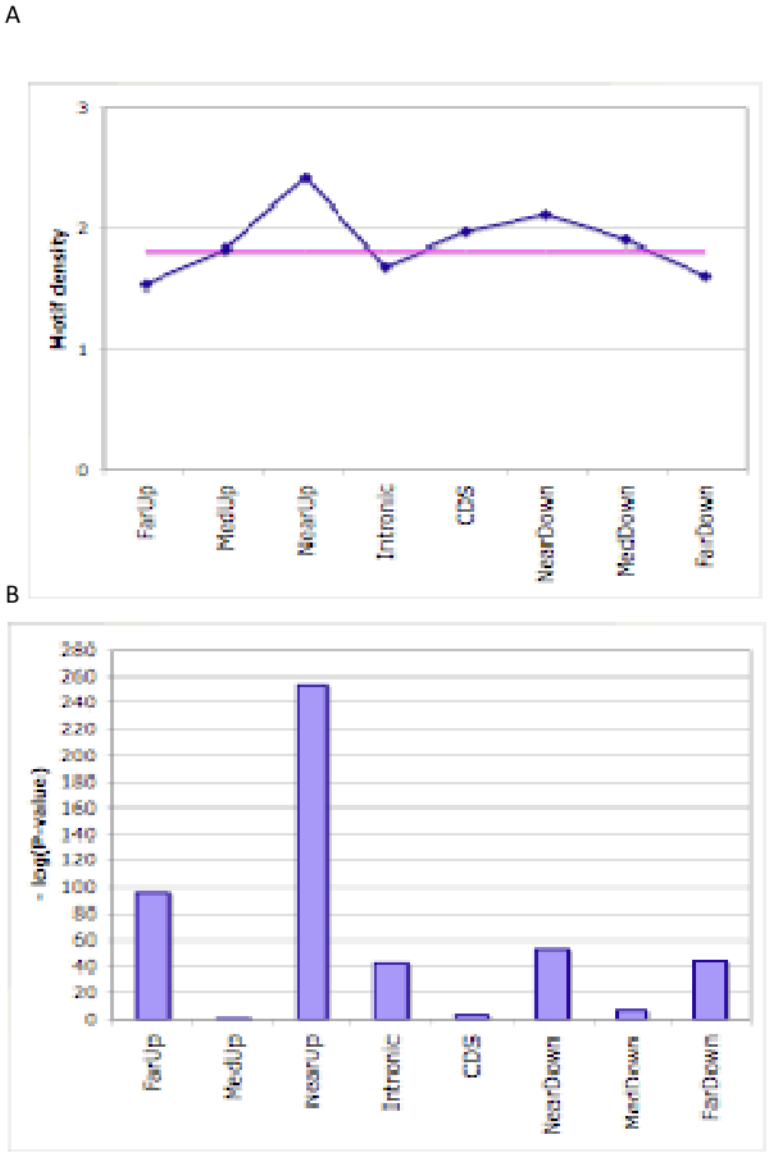
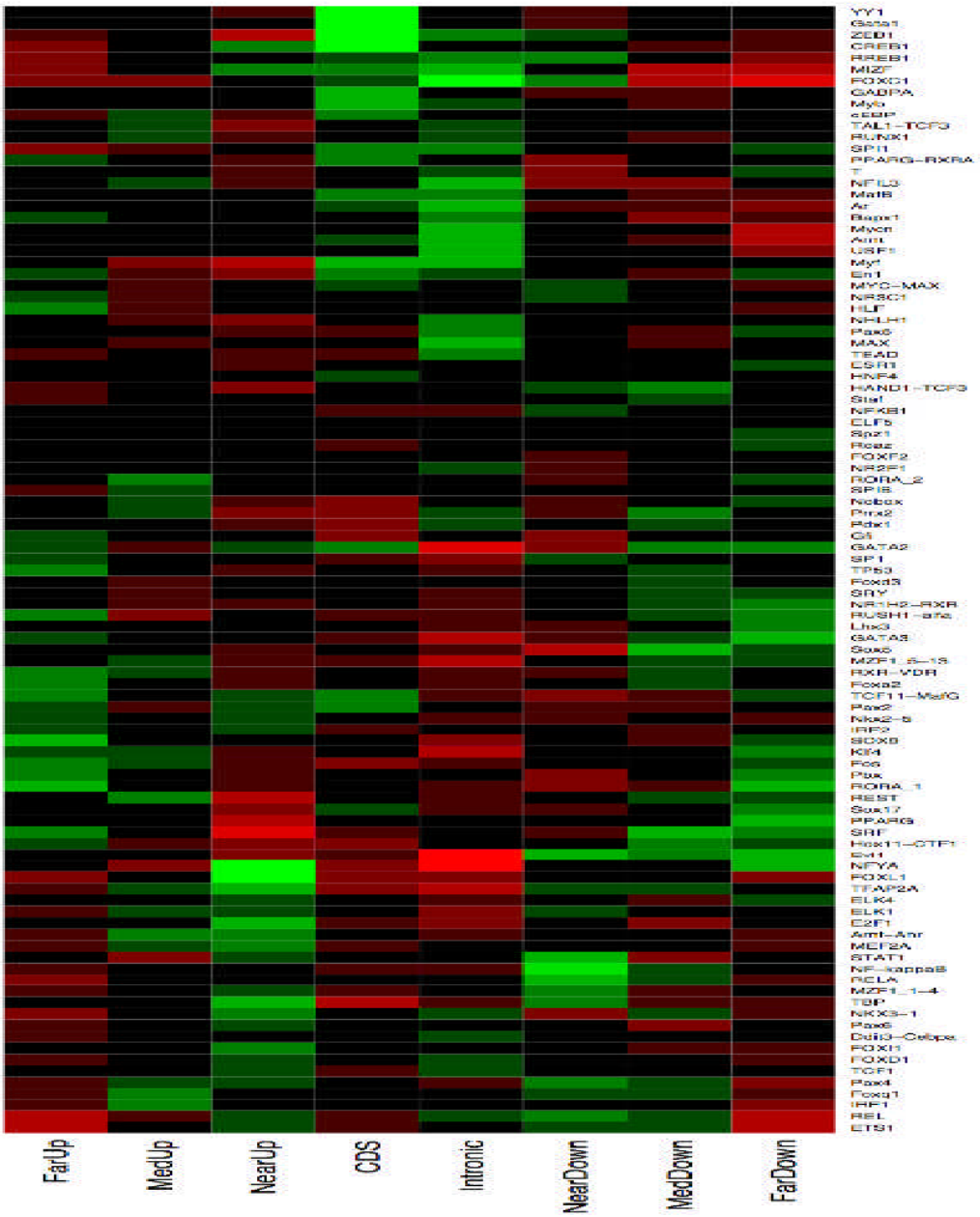
Appendix



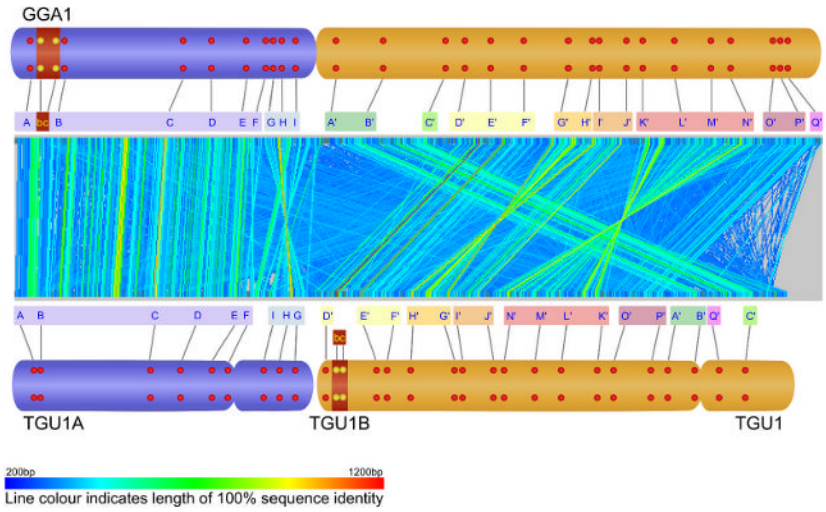
Accessory Figure 1. Predicted motif genome location distribution

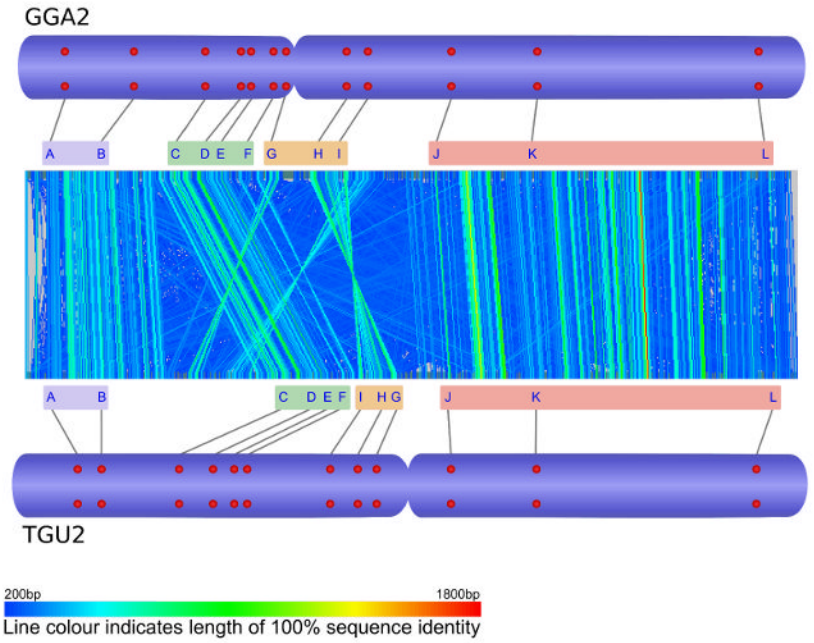
(A) Density of predicted motifs (y axis) for different categories of regions (x axis) in terms of location with respect to their nearest genes, shown in blue. The horizontal pink line is the genome-wide average. See text for definitions of motif density and various region categories. (B) P-values of enrichment or depletion of motif occurrence in each category of regions, using one-tailed Fishers exact tests. Negative logarithms are shown.

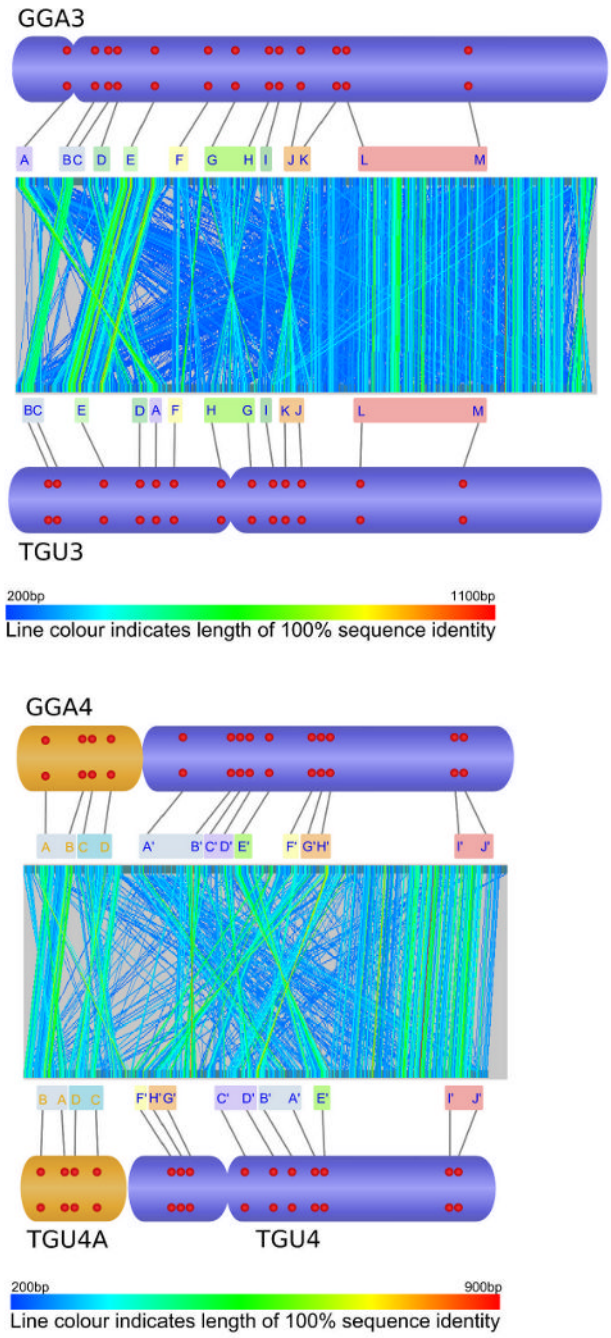


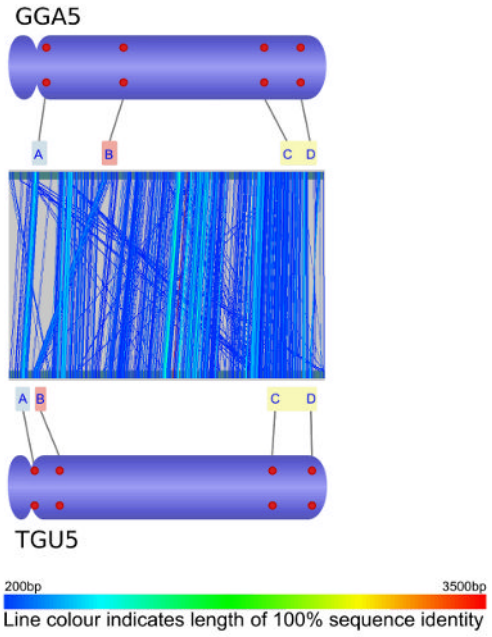
Accessory Figure 2. Motif target counts by defined location

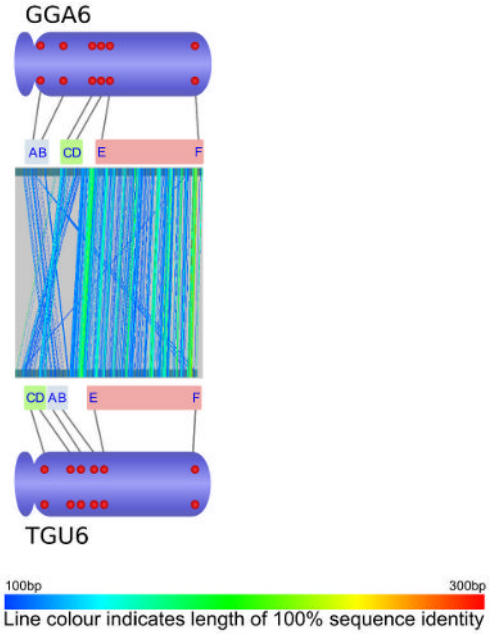
Counts of targets windows of individual motifs in different categories of regions are compared to the respective expected values, with colors shown indicating whether the count is greater or less than expectation. Green cells correspond to counts that are higher than the average and red cells correspond to regions with below-average counts.

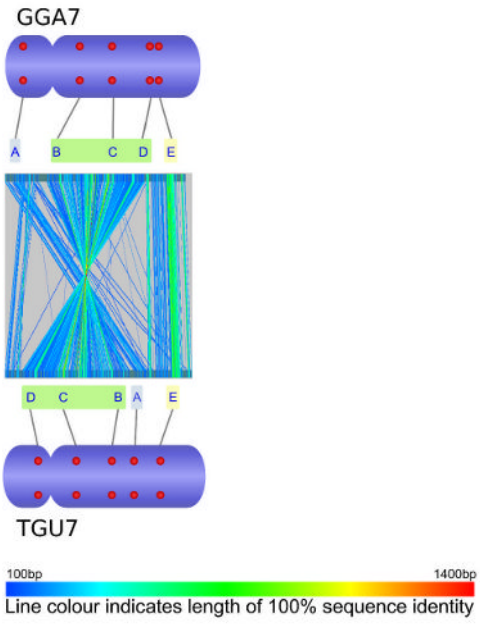


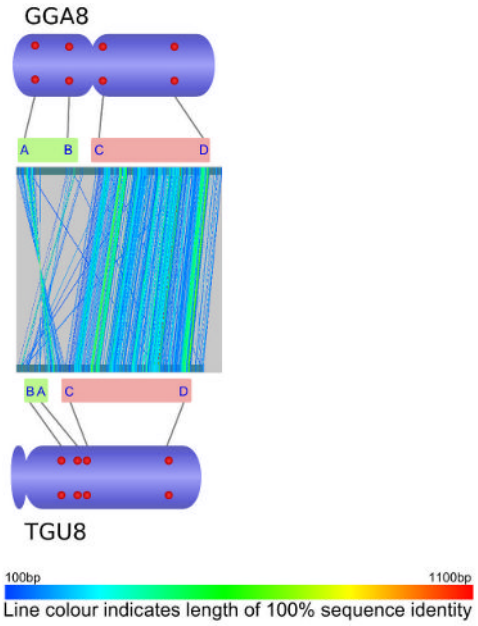


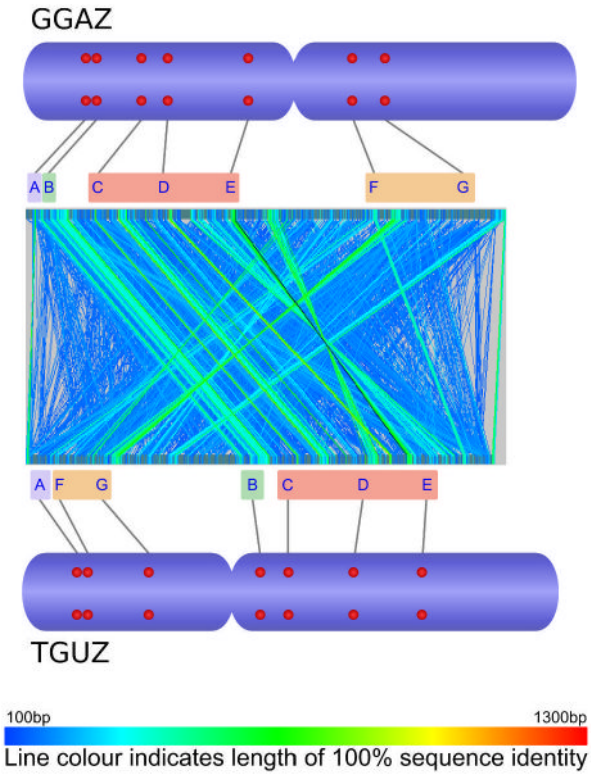






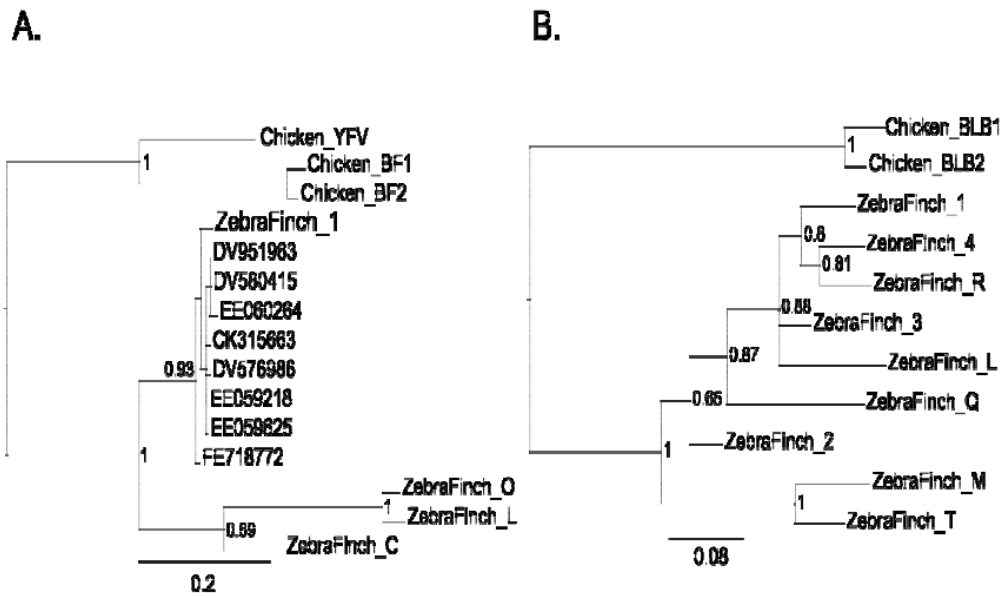






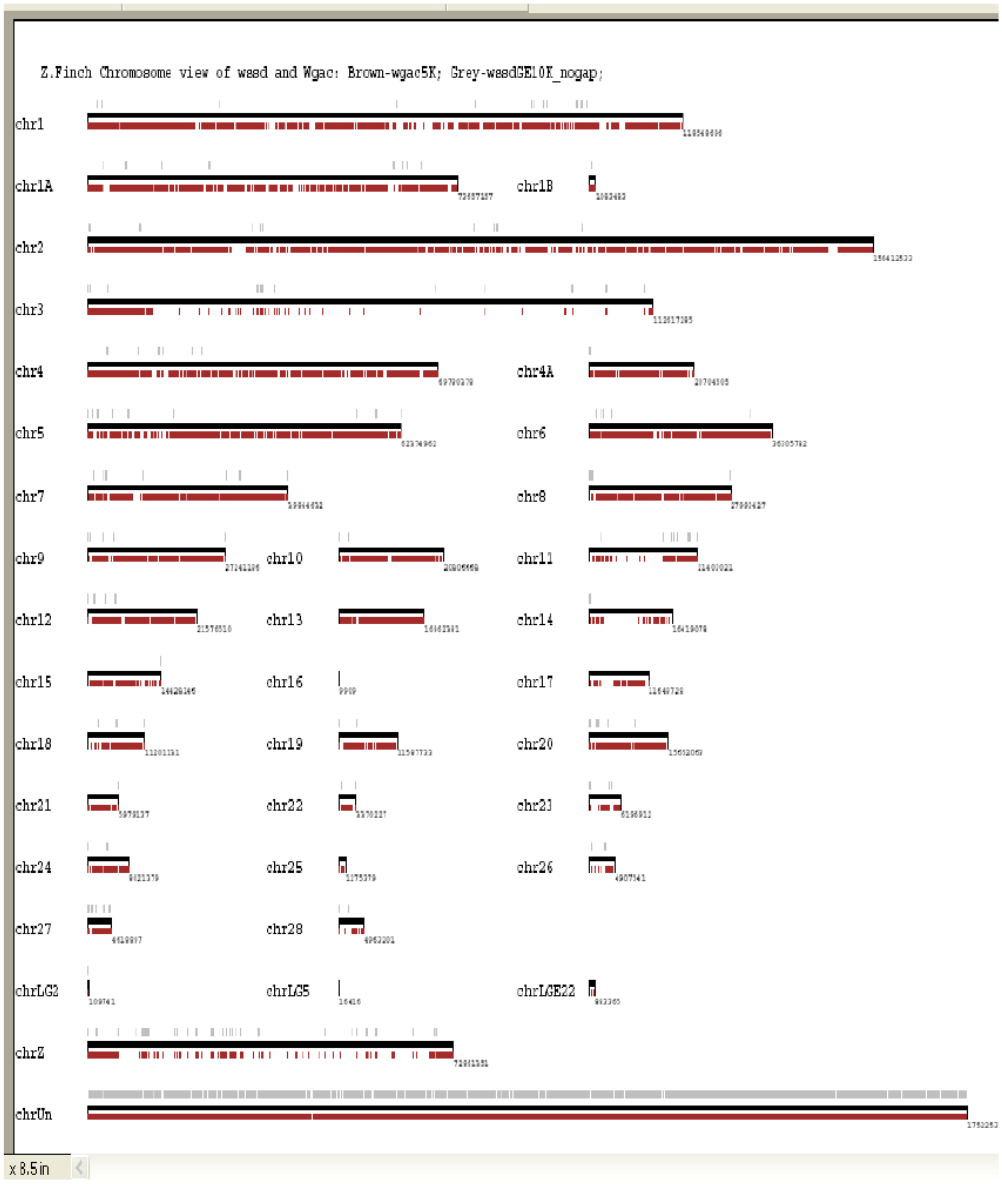
Accessory Figure 3.

Comparative analysis of marker order on chicken chromosomes 2-8 and Z (GGA2-8, GGAZ) and their zebra finch orthologues (TGU2-8, TGUZ). The central part of each figure was created by aligning whole chromosomal sequences using the program GenAlyzer. Line colour indicates the length of sequences with 100% sequence identity. The tentative chromosomal rearrangements suggested by this analysis were verified using fluorescent in situ hybridization (FISH). Letters indicate the position of chicken and zebra finch BACs with orthologous sequence content in the genome sequences of both species (see accessory file Physical mapping table 2009-09-16.xls for details on the FISH probes used). Red dots on the ideograms illustrate the physical chromosomal position as determined by FISH.



Accessory Figure 4. Bayesian phylogenies of zebra finch MHC genes

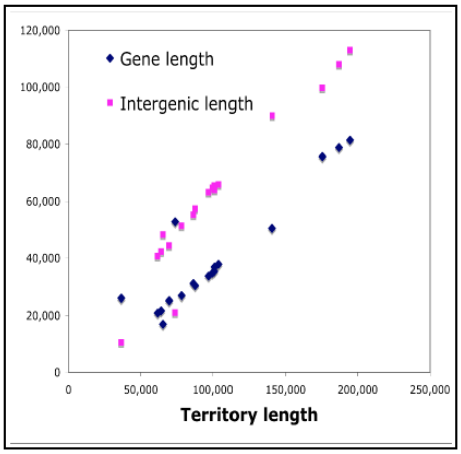
(A) Class I genes and (B) Class IIB genes were compared to sequences from the chicken MHC-B complex. Putatively functional zebra finch genes with open reading frames are given numerical suffixes and putative pseudogenes are given lettered suffixes. For Class I we also include a chicken sequence from the MHC-Y region (YFV). For zebra finch class I, we show the placement of eight brain ESTs (indicated by their GenBank accession numbers) supporting the expression of MHC Class I genes in the brain. Posterior probabilities are given for well-supported nodes in the tree.



Accessory Figure 5.
General view showing WGAC (>5kb) and WSSD on all chromosomes. Grey above lines is WSSD and red below lines is WGAC. ChrUn was treated as a “distinct” chromosome.

Gene set	size	terr_len	gene_len	intergenic_len	p-value
All genes	8436	87,768	30,355	57,413	-
Group 1	82	61,667	20,973	40,694	6.9E-3
Group 2	48	66,301	18,967	48,334	4.0E-1
Group 3	6	73,868	52,931	21,037	9.3E-2
Group 4	210	194,474	81,456	113,018	1.0E-22
Group 5	95	175,420	75,882	99,738	1.5E-8
Group 6	2	36,521	26,028	10,493	7.3E-1
Group 7	1100	100,885	35,416	65,470	2.0E-10
Group 8	1049	78,327	26,893	51,433	2.1E-8
Group 9	390	69,660	25,217	44,443	8.3E-7
Group 10	378	140,510	50,507	90,003	2.0E-17
down_only (4,5,6)	1312	101,048	36,972	64,076	9.7E-1
fast_down (4,5,6)	301	166,840	78,631	108,009	1.7E-28
fast_up (1,2,3)	136	64,057	21,715	42,343	3.9E-3
slow_down (3,5,6)	1136	86,430	31,167	55,264	1.4E-4
slow_up (2,6,7)	1138	99,855	34,799	64,856	9.3E-10
switch (3,6,9,10)	768	103,752	37,886	65,886	3.3E-2
up_only (1,2,7)	1212	96,810	33,772	63,138	2.4E-7

A)



B)

Accessory Figure 6.

(A) Characteristics of co-expressed gene sets from Dong et al. [58] (See S3 supplementary notes). “Gene set”: name of the gene set, as in the original paper. “All genes” refers to the genes on the array in Dong et al. Numbers in parentheses indicate component subsets of a set. “Size”: number of genes in set. “terr_len”: average gene territory length of a gene set. “gene_len”: average coding sequence length. “intergenic_length”: average of (territory length – gene length). “p-value”: statistical significance of enrichment for short (pink cells) or long (green cells) territories, as measured by 2-tailed Wilcoxon Rank Sum tests. (B) Average gene length and intergenic length of a gene set (y axis) versus average gene territory length. Each point corresponds to a gene set.

Accessory Table 1
Zebra finch chromosome nomenclature and length

Chicken (GGA) and finch (TGU) chromosome names suggested by Itoh et al., 2005 (Supplementary Notes 1).

		Ordered			Random	
TGU	**GGA**	**Itoh et al., 2005**	**TGU length**	**TGU %GC**	**TGU length**	**TGU %GC**
Tgu1	1	3	118548696	0.39	1193292	0.4
Tgu1A	1	4	73657157	0.39	689636	0.46
Tgu1B	1	NA	1083483	0.5	142794	0.45
Tgu2	2	1	156412533	0.39	1793874	0.39
Tgu3	3	2	112617285	0.39	1378982	0.46
Tgu4	4	5	69780378	0.39	5148506	0.4
Tgu4A	4	micro	20704505	0.43	258280	0.44
Tgu5	5	6	62374962	0.41	2517995	0.42
Tgu6	6	7	36305782	0.41	2096744	0.43
Tgu7	7	8	39844632	0.41	603983	0.44
Tgu8	8	9	27993427	0.41	5113623	0.46
Tgu9	9	10	27241186	0.43	369730	0.42

TGU	GGA	Ordered			Random	
		Itoh et al., 2005	TGU length	TGU %GC	TGU length	TGU %GC
Tgu10	10	NA	20806668	0.43	559132	0.46
Tgu11	11	NA	21403021	0.42	295904	0.44
Tgu12	12	NA	21576510	0.43	345412	0.43
Tgu13	13	NA	16962381	0.44	2653678	0.47
Tgu14	14	NA	16419078	0.45	252098	0.5
Tgu15	15	NA	14428146	0.46	359589	0.46
Tgu16	16	NA	9909	0.48	187953	0.49
Tgu17	17	NA	11648728	0.48	210589	0.48
Tgu18	18	NA	11201131	0.46	474824	0.48
Tgu19	19	NA	11587733	0.46	200344	0.46
Tgu20	20	NA	15652063	0.46	300503	0.47
Tgu21	21	NA	5979137	0.46	1862743	0.49
Tgu22	22	NA	3370227	0.48	803674	0.5
Tgu23	23	NA	6196912	0.49	548500	0.48
Tgu24	24	NA	8021379	0.48	186162	0.49
Tgu25	25	NA	1275379	0.52	472805	0.48
Tgu26	26	NA	4907541	0.5	1627540	0.49
Tgu27	27	NA	4618897	0.49	208747	0.49
Tgu28	28	NA	4963201	0.5	199714	0.51
TguLGE22	LGE22C19W28_E50C23	NA	883365	0.51	454016	0.5
TguLGE22A	LGE22C19W28_E50C23	NA				
Tgun2	NA	NA	109741	0.49		
Tgun5	NA	NA	16416	0.42		
TguZ	Z	Z	72861351	0.39	2969867	0.41
TguUn	Un	Un			175225315	0.42

Abbreviations are as follows TGU – zebra finch, GGU – chicken, ordered presents linkage or other ordering information that allowed unambiguous contig or supercontig chromosomal placement, random – represents partial ordering information that only allows chromosome placement but not order on the chromosome. GC% is the percentage of G and C bases per total bases counted.

Accessory Table 2

Summary of gene orthologs defined from sequence homology, gene trees and conservation of synteny.

Species Comparison	No Homologs	1:1 Orthologs	1:M Orthologs	M:M Orthologs	Total Orthologs	Total Genes
Zebra finch/Chicken	3,863	10,161	3,563	135	13,859	17,722
Zebra finch/Human	4,695	10,861	2,028	140	13,029	17,724
Chicken/Human	5,035	11,429	1,141	119	12,689	17,724

Accessory Table 3

All (motif, region category) pairs with z-scores greater than 3 or less than -3 are shown.

Factor	Region	Z-Score	Factor	Region	Z-Score	Factor	Region	Z-Score
FOXL1	NearUp	8.33	Arnt	Intronic	3.58	MIZF	FarDown	-3.09
CREB1	CDS	7.75	NFYA	FarDown	3.58	Klf4	Intronic	-3.14
FOXC1	Intronic	7.25	STAT1	NearDown	3.58	Myf	NearUp	-3.27
YY1	CDS	6.83	Evi1	NearDown	3.45	Sox5	NearDown	-3.35
Gata1	CDS	6.76	GATA3	FarDown	3.42	REL	FarDown	-3.36
NFYA	NearUp	6.03	USF1	Intronic	3.38	ETS1	FarUp	-3.39
ZEB1	CDS	5.07	MIZF	Intronic	3.36	PPARG	NearUp	-3.41
NF-kappaB	NearDown	4.06	PPARG	FarDown	3.32	Arnt	FarDown	-3.45
E2F1	NearUp	3.93	MAX	Intronic	3.23	ZEB1	NearUp	-3.47
RORA_1	FarUp	3.92	Mycn	Intronic	3.23	ETS1	FarDown	-3.49
NFIL3	Intronic	3.87	GABPA	CDS	3.22	TFAP2A	Intronic	-3.5
Sox5	MedDown	3.82	SRF	MedDown	3.17	Mycn	FarDown	-3.59
Myf	Intronic	3.8	Myb	CDS	3.09	REST	NearUp	-3.88
Evi1	FarDown	3.78	TFAP2A	NearUp	3.08	MZF1_5-13	Intronic	-3.91
Ar	Intronic	3.76	Myf	CDS	3.01	SRF	NearUp	-4.02
SOX9	FarUp	3.75	FOXC1	MedDown	-3.04	GATA2	Intronic	-4.09
RELA	NearDown	3.71	TBP	CDS	-3.04	FOXC1	FarDown	-4.23
RORA_1	FarDown	3.71	REL	FarUp	-3.05	NFYA	Intronic	-5.18
TBP	NearUp	3.65	MIZF	MedDown	-3.09	Evi1	Intronic	-5.56

Accessory Table 4
Comparison of motif – region preferences

Comparisons were made to human (see S1 Supplementary notes; Blanchette et al. 2006). Shown are the z-scores for motifs that were common to the two studies. We note that Blanchette et al. studied only one “FOX” motif, one “GATA” motif and one “RORA” motif, whereas our compendium includes multiple versions (marked in gray, column 1). Columns 2 – 6 are z-scores from this study, columns 7 – 9 indicate overrepresentation (“u”, pink) or depletion (“d”, green) of a motif in a particular region category as reported by Blanchette et al. Cells with bold outlines in columns 7 – 9 are cases where our findings agree with those of Blanchette et al (our z-score is > 2 or < -2). Cells with bold outlines in columns 2 – 6 are cases where our findings disagree with those of Blanchette et al. In all other cases, either of the two studies reports a non-significant enrichment or depletion.

	Songbird (this study)					Human (Blanchette et al.)		
factor	FarUp	MedUp	NearUp	CDS	Intronic	farup	nearup	intron
CREB1	-2.72	-0.98	2.31	7.75	-0.39		u	
E2F1	0.2	0.52	3.93	-1.09	-2.16	d	u	u
ELK1	-1.02	1.01	1.82	-0.6	-2.49		u	
Foxa2	2.4	0.27	-1.18	-0.67	-1.29	d	u	
FOXC1	-2.36	-2.15	0.17	1.15	7.25	d	u	

	Songbird (this study)					Human (Blanchette et al.)		
factor	**FarUp**	**MedUp**	**NearUp**	**CDS**	**Intronic**	**farup**	**nearup**	**intron**
FOXD1	-1.11	-0.9	1.74	-0.9	1.23	d	u	
Foxd3	0.5	-1.13	0.03	-0.79	-0.88	d	u	
FOXF2	0.43	0.21	0.07	-0.55	0.19	d	u	
FOXI1	-0.55	-0.59	2.11	-0.09	0.9	d	u	
FOXL1	-2.71	-0.23	8.33	-2.58	-2.87	d	u	
Foxq1	-1.44	2.23	-0.8	-0.9	-0.2	d	u	
GABPA	0.4	0.9	0.67	3.22	0.29		u	
Gata1	-0.67	-0.7	-0.86	6.76	0.4	u	d	
GATA2	1.12	-1.24	1.41	2.15	-4.09	u	d	
GATA3	1.7	-0.4	-0.03	-1.09	-3	u	d	
MEF2A	-1.56	1.63	2.66	-1.13	-0.16	d	u	
NFYA	0.65	-2.9	6.03	-2.84	-5.18		u	d
NR2F1	-0.41	0.11	0.19	-0.29	1.23		u	
Pbx	2.6	-0.13	-1.25	0.46	-0.83	u		
RORA_1	3.92	-0.85	-1.68	0.09	-1.66	u	d	
RORA_2	0.03	2.17	-0.91	-0.46	-0.95	u	d	
Spz1	-0.3	0.62	-0.71	-0.87	-0.6	d		u
SRY	0.67	-1.86	-0.53	0.01	-1.06	d	u	
TAL1-TCF3	0.48	1.04	-2.7	0.96	1.18	u	d	
TBP	-0.88	-0.55	3.65	-3.04	-1.23	d	u	

Accessory Table 5
Orthologs predicted with the OPTIC pipeline

Orphaned genes have no ortholog predicted in any of the other species. These often represent rapidly evolving genes or those in large families whole phylogeny may not have been inferred accurately.

Species	Genes	Transcripts	Genes with orthologs		Orphaned genes
H. sapiens	20,907	46,259	18,485	88%	2,422
M. musculus	22,848	40,052	19,067	83%	3,781
C. familiaris	19,292	25,546	17,436	90%	1,856
M. domestica	19,458	32,544	17,025	87%	2,433
O. anatinus	17,936	26,821	14,879	83%	3,057
G. gallus	16,723	22,181	14,465	86%	2,258
T. guttata	17,475	18,191	15,820	91%	1,655
T. nigroviridis	19,581	23,097	15,371	78%	4,210

Accessory Table 6
Strict 1:1 orthologs across eight species

Each set contains exactly one gene of each species.

Species	(1:1)n ortholog sets		Species	(1:1)n ortholog sets	
Human	20,907	100%	Zebrafinch	17,475	100%
+Mouse	15,272	73%	+Chicken	10,868	62%
+Dog	14,229	68%	+Platypus	8,024	46%
+Opossum	12,411	59%	+Opossum	7,238	41%
+Platypus	9,508	45%	+Dog	6,850	39%
+Chicken	7,884	38%	+Mouse	6,615	38%
+Zebrafinch	5,262	25%	+Human	6,474	37%
+Tetraodon	4,344	21%	+Tetraodon	4,344	25%

Accessory Table 7
Location of novel transcript models with respect to ENSEMBL gene models

Associated transcript models are within 1kb of an ENSEMBL exon, while intergenic sequences are more than 1kb distant from the closest ENSEMBL exon. Note that the numbers do not sum to 100% as some transcript models contain both intronic and intergenic

Set	total	Intronic		Associated		Intergenic	
array	8,409	915	11%	2,275	27%	5,019	60%
chicken	24,249	7,844	32%	1,970	8%	14,020	58%
estima	13,036	1,778	14%	3,230	25%	7,651	59%
Embryo	110,634	28,349	26%	13,324	12%	68,464	62%
Liver	176,210	49,068	28%	8,971	5%	117,604	67%
Muscle	128,814	34,783	27%	6,704	5%	86,892	67%
Skin	107,841	26,999	25%	11,035	10%	69,348	64%
Spleen	108,492	28,411	26%	9,922	9%	69,673	64%
Testes	63,418	12,223	19%	10,486	17%	40,307	64%

Accessory Table 8

Annotation of transcribed loci (TL). Based on the overlap with ENSEMBL exons TL are annotated first into “known”, “novel”, and “ambiguous” sets. This first level annotation is followed by a second level annotation. Known TL are annotated as protein coding (“pc”), pseudogene (“pseudo”), non-protein coding (“npc”) or UTR (“utr”) depending on the kind or part of an ENSEMBL gene with which they overlap. Novel TL are classified by their location as gene-associated (“assoc”), “intronic” or “intergenic” TL. The category “assoc” contains TL that extend at least partially within 1kb of either terminal exon of an ENSEMBL gene.

set	total	known	novel	ambiguous	pc	pseudo	npc	utr	intronic	associated	intergenic
all	15009	3301	8409	3299	3253	5	3	40	915	2275	5019

set	total	known	novel	ambiguous	pc	pseudo	npc	utr	intronic	associated	intergenic
unchanged	12248	2565	7060	2623	2525	4	3	33	741	1902	4258
FastDown	435	34	343	58	34	0	0	0	90	46	194
FastUp	125	44	34	47	42	0	0	2	1	16	15
SlowDown	1112	371	442	299	367	1	0	3	70	120	239
SlowUp	1217	318	630	269	314	0	0	4	36	205	372

Accessory Table 9

Novel TL are statistically significantly depleted in intronic sequences. The only exception is the set FastDown.

set	depletion	P-Value
Fastdown	8.00%	0.1300
FastUp	82.00%	0.0001
SlowDown	42.00%	0.0010
SlowUp	79.00%	0.0001
unchanged	64.00%	0.0001

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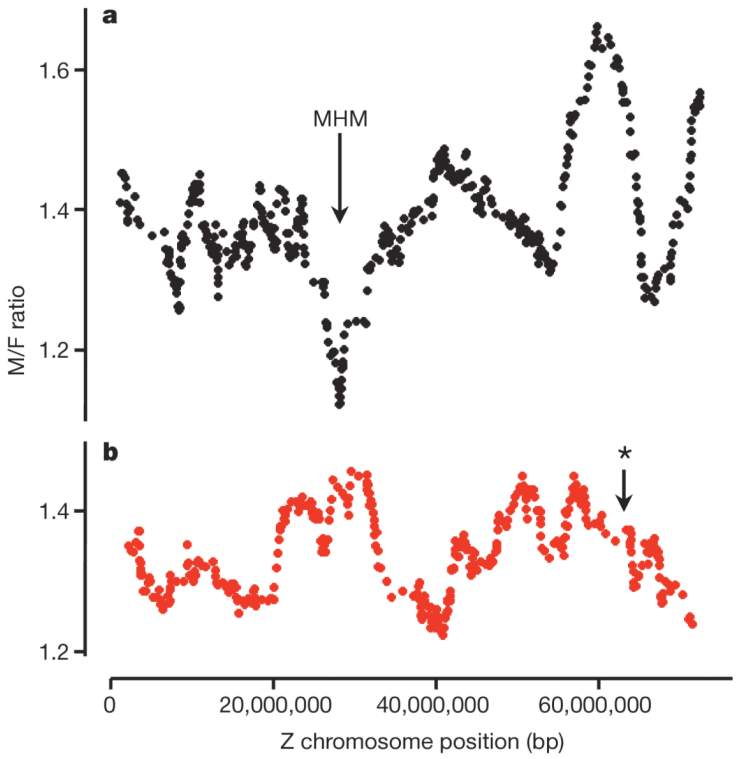

Figure 1. Divergent patterns of dosage compensation in birds

a, **b**, The male to female (M/F) ratio of gene expression, measured by species-specific microarrays, is plotted along the Z chromosome of chicken (**a**) and zebra finch (**b**). Each point represents the average M/F ratio of a sliding window of 30 genes plotted at the median gene position and stepping one gene at a time along the chromosome. Note region of lower M/F ratios in chicken surrounding the locus of the MHM (male hypermethylated) ncRNA. In zebra finch, genes adjacent to the comparable MHM position (asterisk) show no special cluster of dosage compensation (low M/F ratios), and no MHM sequence appears in the genome assembly. bp, base pairs.

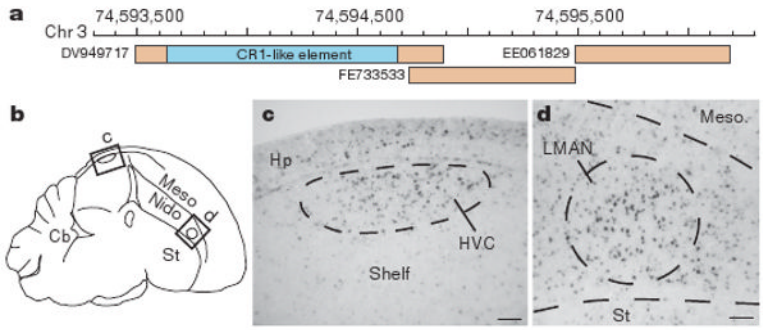

Figure 2. Enriched expression of a CR1-like element in the zebra finch song system

a, Genomic alignment of an RNA containing a CR1-like retrotransposon element (in blue) and adjacent ESTs, with respective GenBank accession numbers. **b**–**d**, DV949717 is expressed in the brain of adult males with enrichment in song nuclei HVC (letter-based name) and LMAN (lateral magnocellular nucleus of the anterior nidopallium), as revealed by *in situ* hybridization. The diagram in **b** indicates areas shown in photomicrographs in **c** and **d**. Cb, cerebellum; Hp, hippocampus; Meso, mesopallium; Nido, nidopallium; Shelf, nidopallial shelf region; St, striatum. Scale bars, 0.1 mm.

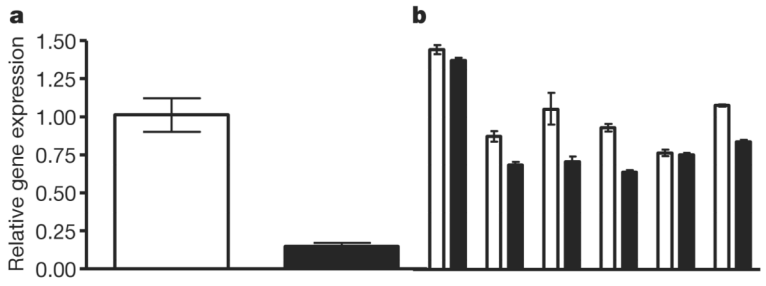

Figure 3. miR-124 in the auditory forebrain is suppressed by exposure to new song

TaqMan assays comparing samples from the auditory lobule of adult male zebra finches in silence (open bars) or 30 min after onset of new song playback (filled bars). **a**, Comparison of two sample pools, each containing auditory forebrains of 20 birds. **b**, Comparisons of paired individual subjects, $n = 6$ pairs ($P = 0.03$, Wilcoxon paired test). Error bars denote s.e.m. of triplicate TaqMan assays. Parallel TaqMan analyses of the small RNA *RNU6B* were performed with all samples and showed no significant effect of treatment for this control RNA.

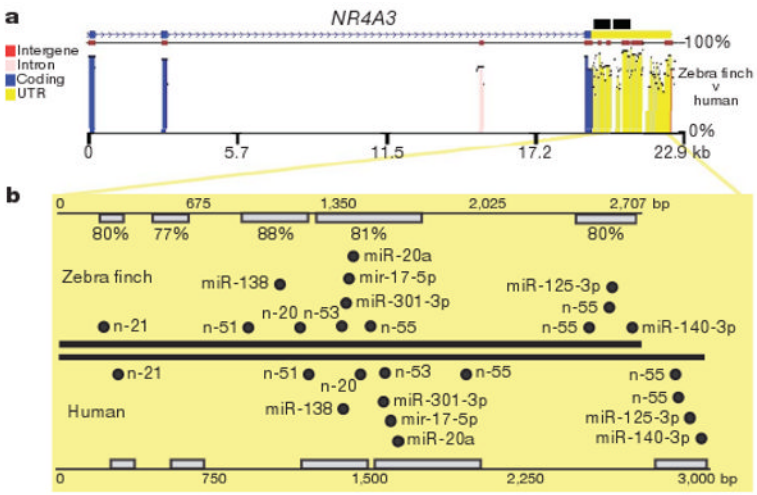

Figure 4. Conserved *NR4A3 3′* UTR is a potential region for microRNA integration

a, zPicture alignment of 3′ portion of zebra finch to human gene[35] showing UTR region of high similarity beyond the coding exons. Dark red bars, regions with the highest sequence conservation; black rectangles, position of song-regulated ESTs[27] within the conserved UTR but outside the Ensembl gene model (ENSTGUG00000008853). **b**, Alignment of zebra finch and human 3′ UTR sequences showing the per cent sequence identity for each evolutionarily conserved region. Dots indicate positions of conserved new (‘n-’) or established (‘miR-’) microRNA-binding sites in both species within these regions.

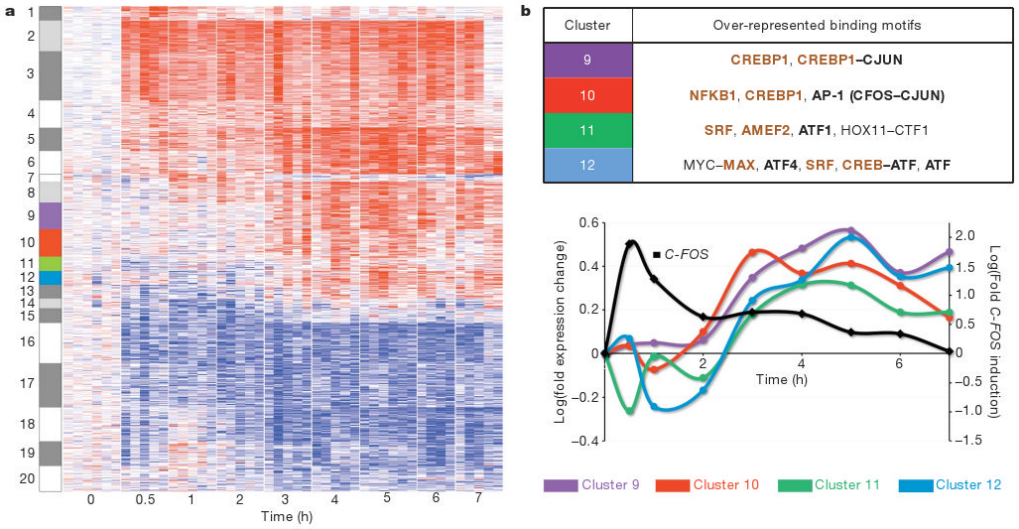

Figure 5. Transcriptional control network in area X engaged by singing

a, Clustered (1–20) temporal expression profiles of 807 genes (rows) that change with time and amount of singing; red, increases; blue, decreases; white, no change relative to average 0-h control. Grey/coloured bars on left, clusters with enrichment of specific promoter motifs ($P < 0.01$). **b**, Enriched transcription-factor-binding motifs (abbreviations) found in the promoters of late response genes, clusters 9–12 (coloured as in **a**); bold, binding sites for known activity-dependent transcription factors (for example, CREBP1) or transcription factor complexes (for example, CREBP1–CJUN); black, sites for post-translationally activated transcription factors; brown, sites for transcriptionally activated transcription factors including by singing (for example, in cluster 1). Graph shows time course of average expression of all genes in the late response clusters, normalized to average 0 h for that cluster. Also plotted is the average expression of the *C-FOS* transcription factor mRNA, which binds to the AP-1 site over-represented in the promoters of cluster 10 genes.

Table 1

Structural features of the song responsive genome

	All genes analysed	Novel up	Novel down	Habituate up	Habituate down
All ESTs	17,877	145	461	1,531	1,774
Mapped loci	15,009	125	435	1,217	1,112
Ensembl genes	8,438	136	301	1,138	1,136
Mobile element content*					
Number with mobile elements	688	2	40	32	38
Percentage mobile elements	4	1	9	2	2
P-value	2	0.18	1.4×10^{-5}	0.005	0.004
Coding and non-coding content†					
mRNA transcripts (% (*P*-value))	59	86 (0.05)	32 (1×10^{-10})	65 (0.05)	71 (0.001)
EST loci mapped to introns (% (*P*-value))	6	1 (0.05)	21 (1×10^{-10})	3 (0.001)	6
Intergenic loci (% (*P*-value))	33	12 (0.001)	45 (0.05)	31	21 (0.001)
Protein-coding gene territories‡					
Mean gene length (kb)	30.4	21.7	78.8	34.8	31.2
Intergenic length (kb)	57.4	42.3	108.0	64.9	55.3
Territory size (kb)	87.8	64.1	186.8	99.7	86.4
P-value	–	3.9×10^{-3}	1.7×10^{-28}	9.3×10^{-10}	1.4×10^{-4}

A microarray made from non-redundant brain-derived ESTs[34] was used to define four subgroups of RNAs that show different responses in auditory forebrain to song exposures (novel up and down, habituated up and down)[27]. These ESTs were mapped to genome positions as described (Supplementary Note 3).

*All ESTs were analysed for mobile element content using RepeatMasker (Supplementary Note 2). *P*-value is for the comparison to all genes (Fisher's exact test).

†All ESTs that could be mapped uniquely to the genome assembly were assessed for overlap with Ensembl annotations of mRNA transcripts (protein coding and UTRs), intronic regions, or intergenic regions. *P*-value is for comparison to all mapped loci (Fisher's exact test). Results are the percentage with *P* values in parentheses where shown.

‡The size of each unique protein-coding gene territory was determined by combining the length of the Ensembl gene model with its intergenic spacing. The *P*-value is for the comparison to all genes, using a two-tailed Wilcoxon rank sum test.

Table 2
Song-suppressed ion channel genes under positive selection

Gene	Description	Branch $\Delta\omega$	Sites PS/total
CACNA1B	Voltage-dependent N-type calcium channel subunit α-1B	0.016	9/2,484
CACNA1G	Voltage-dependent T-type calcium channel subunit α-1G	0.044*	2/2,468
GRIA2	Glutamate receptor 2 precursor (GluR-2, AMPA 2)	0.231*	17/948
GRIA3	Glutamate receptor 3 precursor (GluR-3, AMPA 3)	−0.010	4/894
KCNC2	Potassium voltage-gated channel subfamily C member 2 (Kv3.2)	0.315*	32/654
TRPV1	Transient receptor potential cation channel subfamily V member 1	−0.067	3/876

These six genes are suppressed by song exposure (FDR = 0.05)[27] and they show evidence of positive selection in the zebra finch relative to chicken ($P < 10^{-3}$, Supplementary Note 3). Branch $\Delta\omega$ denotes the difference in the non-synonymous to synonymous substitution ratio (*dN/dS*) between zebra finch and other birds (chicken and the ancestral branch leading to chicken and zebra finch). Positive values indicate that the gene is rapidly evolving, whereas negative values indicate genes evolving more slowly. Sites PS/total denotes the number of individual sites with empirical Bayes posterior probability greater than 0.95 of $\omega > 1$ (positive selection) in the finch versus the total number of residues in the protein, from branch-site model analysis implemented in PAML. Note that genes can show overall slower evolution in the branch model yet show evidence of significant positive selection at specific sites.

* Gene-wide differences that were significant ($P < 0.05$) by a likelihood ratio test.