Programmed DNA elimination of germline development genes in songbirds

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Summary

Genomes can vary within individual organisms. Programmed DNA elimination leads to dramatic changes in genome organisation during the germline–soma differentiation of ciliates[1], lampreys[2], nematodes[3,4], and various other eukaryotes[5]. A particularly remarkable example of tissue-specific genome differentiation is the germline-restricted chromosome (GRC) in the zebra finch which is consistently absent from somatic cells[6]. Although the zebra finch is an important animal model system[7], molecular evidence from its large GRC (>150 megabases) is limited to a short intergenic region[8] and a single mRNA[9]. Here, we combined cytogenetic, genomic, transcriptomic, and proteomic evidence to resolve the evolutionary origin and functional significance of the GRC. First, by generating tissue-specific *de-novo* linked-read genome assemblies and re-sequencing two additional germline and soma samples, we found that the GRC contains at least 115 genes which are paralogous to single-copy genes on 18 autosomes and the Z chromosome. We detected an amplification of ≥38 GRC-linked genes into high copy numbers (up to 185 copies) but, surprisingly, no enrichment of transposable elements on the GRC. Second, transcriptome and proteome data provided evidence for functional expression of GRC genes at the RNA and protein levels in testes and ovaries. Interestingly, the GRC is enriched for genes with highly expressed orthologs in chicken gonads and gene ontologies involved in female gonad development. Third, we detected evolutionary strata of GRC-linked genes. Genes such as *bicc1* and *trim71* have resided on the GRC for tens of millions of years, whereas dozens have become GRC-linked very recently. The GRC is thus likely widespread in songbirds (half of all bird species) and its rapid evolution may have contributed to their diversification. Together, our results demonstrate a highly dynamic evolutionary history of the songbird GRC leading to dramatic germline–soma genome differences as a novel mechanism to minimize genetic conflict between germline and soma.

Text

Not all cells of an organism must contain the same genome. Some eukaryotes exhibit dramatic differences between their germline and somatic genomes, resulting from programmed DNA elimination of chromosomes or fragments thereof during germline–soma differentiation[5]. Here we present the first comprehensive analyses of a germline-restricted chromosome (GRC). The zebra finch (*Taeniopygia guttata*) GRC is the largest chromosome of this songbird[6] and likely comprises >10% of the genome (>150 megabases)[7,10]. Cytogenetic evidence suggests the GRC is inherited through the female germline, expelled late during spermatogenesis, and eliminated from the soma during early embryo development[6,11]. Previous analyses of a 19-kb intergenic region suggested that the GRC contains sequences with high similarity to regular chromosomes ('A chromosomes')[8].

In order to reliably identify sequences as GRC-linked, we used a single-molecule sequencing technology not applied in birds before that permits reconstruction of long haplotypes through linked reads[12]. We generated separate haplotype-resolved *de-novo* genome assemblies for the germline and soma of a male zebra finch (testis and liver; 'Seewiesen'; Supplementary Table 1). We further used the linked-read data to compare read coverage and haplotype barcode data in relation to the zebra finch somatic reference genome ('taeGut2')[7], allowing us to identify sequences that are shared, amplified, or unique to the germline genome in a fashion similar to recent studies on cancer aneuploidies[13]. We also re-sequenced the germline and soma from two male zebra finches from another population ('Spain'; testis and muscle) using short reads.

We first established the presence of the GRC in the three germline samples. Cytogenetic analysis using fluorescence *in-situ* hybridization (FISH) with a new GRC probe showed that the GRC is present exclusively in the germline and eliminated during spermatogenesis as hypothesised (Fig. 1a-b, Extended Data Fig. 1)[6,11]. We compared germline/soma sequencing coverage by mapping reads from all three sampled zebra finches onto the reference genome assembly (regular 'A chromosomes'), revealing consistently germline-increased coverage for single-copy regions, similar to programmed DNA elimination of short genome fragments in lampreys[2] (Fig. 1c-d). A total of 92 regions (41 with >10 kb length) on 13 chromosomes exhibit >4-fold increased germline coverage in Seewiesen relative to the soma (Fig. 1e, Supplementary Table 2). Such a conservative coverage cut-off provides high confidence in true GRC-amplified regions. We obtained nearly identical confirmatory results with another sequencing technology using the Spanish birds (Fig. 1f). Notably, the largest block of testis-increased coverage spans nearly 1 Mb on chromosome 1 and overlaps with the previously[8] FISH-verified intergenic region 27L4 (Fig. 1e-f).

Our linked-read and re-sequencing approach allowed us to determine the sequence content of the GRC. The GRC is effectively a non-recombining chromosome as it recombines with itself after duplication, probably to ensure stable inheritance during female meiosis[8]. We predicted that the GRC would be highly enriched in repetitive elements, similar to the female-specific avian W chromosome (repeat density >50%)[14]. Surprisingly, neither assembly-based nor read-based repeat quantifications detected a significant enrichment in transposable elements or satellite repeats in the germline samples relative to the soma samples (Supplementary Text, Supplementary Table 3). Instead, most germline coverage peaks lie in single-copy regions of the reference genome including 38 genes (Fig. 1e-f, Table 1), suggesting that these peaks stem from highly similar GRC-amplified paralogs with high copy numbers (up to 185 copies per gene; Supplementary Table 4). GRC linkage of these regions is further supported by sharing of linked-read barcodes between different amplified chromosomal regions in germline but not soma (Fig. 1g-h), suggesting that these regions reside on the same haplotype (Extended Data Fig. 2). We additionally identified 245 GRC-linked genes through germline-specific single-nucleotide polymorphisms (SNPs) present in read mapping of all three germline samples onto zebra finch reference genes (up to 402 SNPs per gene; Supplementary Table 4). As a control, we used the same methodology to screen for soma-specific SNPs and found no such genes. We conservatively consider the 38 GRC-amplified genes and those with at least 5 germline-specific SNPs as our highest-confidence set (Table 1). We also identified GRC-linked genes using germline–soma assembly subtraction and coverage differences (Fig. 1i); however, all were already found via coverage or SNP evidence (Table 1). Together with the *napa* gene recently identified in transcriptomes (Fig. 1j)[9], our complementary approaches yielded 115 high-confidence GRC-linked genes with paralogs located on 18 autosomes and the Z chromosome (Table 1; all 267 GRC genes in Supplementary Table 4).

We next tested whether the GRC is functional and thus probably physiologically important using transcriptomics and proteomics. We sequenced RNA from the same tissues of the two Spanish birds used for genome re-sequencing and combined these with published testis and ovary RNA-seq data from North American domesticated zebra finches[9,15]. Among the 115 high-confidence genes, 6 and 32 were transcribed in testes and ovaries, respectively (Table 1). Note, these are only genes where we could reliably separate reads from GRC-linked and A-chromosomal paralogs by GRC-specific SNPs in the transcripts (Fig. 2a-b, Extended Data Fig. 3, Supplementary Table 5). We next verified translation of GRC-linked genes through protein mass spectrometry data for 7 testes and 2 ovaries from another population ('Sheffield'). From 83 genes with GRC-specific amino acid changes, we identified peptides from 5 GRC-linked genes in testes and ovaries (Fig. 2c-d, Extended Data Fig. 4, Table 1, Supplementary Table 6). We hence established that many GRC-linked genes are transcribed and translated in adult male and female gonads, extending previous RNA evidence for a single gene[9] and refuting the hypothesis from cytogenetic studies that the GRC is silenced in the male germline[16,17]. Instead, we hypothesise that the GRC has important functions during germline development, which is supported by a significant enrichment in gene ontology terms related to reproductive developmental processes among GRC-linked genes (Fig. 2e, Supplementary Table 7). We further found that the GRC is significantly enriched in genes that are also germline-expressed in GRC-lacking species with available RNA expression data from many tissues[18] (Fig. 2f, Supplementary Table 8). Specifically, we found that 22 and 6 out of 65 chicken orthologs of high-confidence GRC-linked genes are most strongly expressed in chicken testis and ovary, respectively.

The observation that all identified GRC-linked genes have A-chromosomal paralogs allowed us to decipher the evolutionary origins of the GRC. We utilised phylogenies of GRC-linked genes and their A-chromosomal paralogs to infer when these genes copied to the GRC, similarly to the inference of evolutionary strata of sex chromosome differentiation[19]. First, the phylogeny of the intergenic 27L4 locus of our germline samples and a previous GRC sequence[8] demonstrated stable inheritance among the sampled zebra finch populations (Fig. 3a). Second, 37 gene trees of GRC-linked genes with germline-specific SNPs and available somatic genome data from other birds identify at least five evolutionary strata (Fig. 3b-f, Extended Data Fig. 5, Table 1), with all but stratum 3 containing expressed genes (*cf*. Fig. 2a-d). Stratum 1 emerged during early songbird diversification, stratum 2 before the diversification of estrildid finches, and stratum 3 within estrildid finches (Fig. 3g). The presence of at least 7 genes in these three strata implies that the GRC is tens of millions of years old and likely present across songbirds (Extended Data Fig. 5), in line with a recent cytogenetics preprint[20]. Notably, stratum 4 is specific to the zebra finch species and stratum 5 to the Australian zebra finch subspecies (Fig. 3g), suggesting piecemeal addition of genes from 18 autosomes and the Z chromosome over millions of years of GRC evolution (Fig. 3h). The long-term residence of expressed genes on the GRC implies that they have been under selection, such as *bicc1* and *trim71* on GRC stratum 1 whose human orthologs are important for embryonic cell differentiation[21]. Additionally, we detected evidence for purifying selection on GRC-linked genes from older and younger strata using ratios of non-synonymous to synonymous substitutions (dN/dS; Supplementary Table 9), again implying that the GRC is an important chromosome with a long evolutionary history.

Here we provided the first evidence for the origin and functional significance of a GRC. Notably, our analyses suggest that the GRC emerged during early songbird evolution and we predict it to be present in half of all bird species. The species-specific addition of dozens of genes on stratum 5 implies that the rapidly evolving GRC likely contributed to reproductive isolation during the massive diversification of songbirds[22]. It was previously hypothesised that GRCs are formerly parasitic B chromosomes that became stably inherited after acquiring essential functions for the host[23,24]. Our evidence for an enrichment of germline-expressed genes on the zebra finch GRC is reminiscent of nematodes and lampreys where short genome fragments containing similar genes are eliminated during germline–soma differentiation[2-4]. All these cases constitute extreme mechanisms of gene regulation through germline–soma gene removal rather than transcriptional repression[3,5,10]. Consequently, we hypothesise that the GRC became indispensable for its host by the acquisition of germline development genes. The aggregation of developmental genes on a single eliminated chromosome constitutes a novel mechanism to ensure germline-specific gene expression in multicellular organisms. This may allow adaptation to germline-specific functions free of deleterious effects on the soma which would otherwise arise from antagonistic pleiotropy.

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Tables and Figures

Table 1 | The 115 high-confidence genes on GRC with information on their A-chromosomal origin in the reference genome taeGut2, number of testis-specific SNPs, methods supporting their GRC linkage, testis/ovary RNA expression of the GRC paralog, testis/ovary protein expression of the GRC paralog, and evolutionary stratum on the GRC.

Gene symbol	Chr.	Start	End	SNPs	Method	RNA evidence	Protein evidence	GRC stratum
AAGAB	10	19608548	19634367	10	SNPs			S5
ADGRL2	8	14047115	14171612	10	SNPs			
ADGRL3	4	14919933	15404594	8	SNPs	ovary		
AKIRIN2	3	78683482	78688947	6	SNPs	ovary		S5
ALDH18A1	6	36280145	36301392	17	SNPs			S4
ALG13	4A	18474239	18501426	19	SNPs	ovary		
ARMC6	28	4942046	4946063	5	SNPs			
ATP2A2	15	2841010	2879975	8	SNPs			
BICC1	6	6355408	6434911	402	SNPs	ovary		S1
BMP15	4A	15596686	15598225	29	SNPs, coverage	ovary		S5
BMPR1B	4	18997710	19024248	47	SNPs, coverage			S5
CCND3	26_random			14	SNPs			
CD164	3	69169111	69174605	38	SNPs, coverage	ovary		
COPS2	10	10200701	10222248	1	SNPs, coverage	ovary		
CPEB1	10	3114181	3137661	114	SNPs	ovary		
CSNK1A1L	Un	135422201	135425792	NA	coverage			
CXCL14	13	9423543	9433139	12	SNPs			S5
DDX49	28	4913058	4918451	5	SNPs	ovary		
DIS3L	10	19097281	19112154	13	SNPs	ovary		S5
DNAAF5	14	13758049	13780402	NA	coverage			
DNAH5	2	81235805	81361091	7	SNPs			
DPH6	5	31543945	31606965	13	SNPs, coverage			
EFNB1	4A	5764021	5807953	86	SNPs	ovary		S5
ELAVL4	8	21034240	21098310	364	SNPs	ovary		

EPPK1	Un			52	SNPs			
FBXO16	3	112541865	112568948	6	SNPs			
FEM1B	10	19886491	19891616	9	SNPs	ovary		S5
FIG4	3	69023384	69073678	17	SNPs			S5
FRS3	26_random			42	SNPs, coverage			S5
GBE1	1	105820640	105934310	4	SNPs, coverage			
INTS9	3	112259951	112313512	NA	coverage			
LIAS	4	48132714	48139736	42	SNPs			S2
LIN54	4	13615974	13637371	17	SNPs			
LINC02027	1	106086596	106087033	NA	coverage			
LMBRD2	Z	41646446	41665840	NA	coverage			
LOC100223190	Z	69149414	69156994	41	SNPs			
LOC100224235	Un			5	SNPs			S5
LOC100225322	1A	47543094	47544622	6	SNPs	ovary		
LOC100227189	Un	150797142	150801997	NA	coverage			
LOC100228170	Un	55540047	55541360	NA	coverage			
LOC101233087	Z	47991391	47994344	7	SNPs			
LOC101233688	5	937818	939059	5	SNPs			S5
LOC101233767	18	8034939	8038005	11	SNPs			
LOC101233800	Un			16	SNPs			
LOC101234253	10	19184028	19186114	7	SNPs	ovary		S5
LOC105758464	23	46808	60360	14	SNPs			S5
LOC105758894	26_random			5	SNPs			

LOC105758976	2	34301994	34306899	16	SNPs			
LOC105759101	3	76396180	76401262	21	SNPs			
LOC105759167	4A	15573874	15574621	5	SNPs			
LOC105759195	4	14453003	14473747	18	SNPs			
LOC105759199	4	20714525	20720872	11	SNPs			
LOC105759260	5	1874731	1886007	32	SNPs			S5
LOC105759646	Un			7	SNPs			
LOC105759655	Un			8	SNPs			
LOC105759660	Un			18	SNPs			
LOC105759665	Un			5	SNPs			
LOC105759692	Un			12	SNPs			
LOC105759919	Un			8	SNPs			
LOC105760011	Un			7	SNPs			
LOC105760123	Un			18	SNPs			
LOC105760228	Un			14	SNPs			
LOC105760286	Un			18	SNPs			
LOC105760461	Un			10	SNPs			
LOC105760874	Z	60949696	60953194	19	SNPs	testis		
LOC105760936	16_random			12	SNPs			
LUC7L3	Un	35019850	35021569	NA	coverage			
MED20	26_random	110500	113183	28	SNPs, coverage			S5

MSH4	8	27964612	27983306	30	SNPs			S4
NAPA	NA			NA	Biederman et al. 2018		both	
NEUROG1	13	9450787	9451086	6	SNPs			
NFYA	26	4725655	4735626	7	SNPs			S5
NRBP2	2	156379345	156398225	48	SNPs			
PCSK4	28	4059367	4063775	21	SNPs			
PGC	26_random			24	SNPs			
PHKA1	4A	15562688	15593666	16	SNPs			
PIM1	26	603349	607242	50	SNPs	testis		
PIM3	1A	18426716	18430551	81	SNPs	ovary		
PMM1	1A	49038672	49047011	NA	coverage			
PRDM1	3	70624594	70644625	12	SNPs			
PRKAR1A	18	2200317	2211579	NA	coverage			
PRKAR1B	14	13784578	13872733	NA	coverage			
PRPSAP1	18	8008870	8033058	7	SNPs, coverage	ovary		S5
PSIP1	Z	59887174	59919902	57	SNPs, coverage	ovary		S3
PUF60	2	156354670	156376091	63	SNPs	ovary		
RFC1	4	48169638	48202709	77	SNPs	ovary		S2
RNF157	18	8048721	8062403	NA	coverage			
RNF17	1	45827734	45870640	69	SNPs	ovary	testis	S4
RNF20	Z_random			9	SNPs, subtraction	both		
ROBO1	1	107094521	107228509	19	SNPs, coverage	ovary		S5
ROBO2	1	107529365	107979302	25	SNPs			
RXRA	17	8320685	8355067	14	SNPs			S5
SCRIB	2	156239884	156325797	83	SNPs	ovary		S5
SECISBP2L	10	10159176	10193647	60	SNPs, coverage	ovary	both	S5

SHC4	10	10124441	10151124	11	SNPs, coverage			S4
SPHK1	18	7991834	7994408	2	SNPs, coverage	testis		
SRRT	Un			16	SNPs	both		
SUGP2	28	4930094	4937971	33	SNPs	ovary	both	S5
SURF4	17	7682661	7693000	50	SNPs	ovary		S3
TFEB	26_random	20475	21840	11	SNPs			S5
TIAM2	3	54800961	54890499	NA	coverage			
TRIM71	2	60893878	60907039	159	SNPs, subtraction			S1
UBE2O	18	7960889	7981633	NA	coverage			
UGDH	4	48113314	48126079	136	SNPs, coverage, subtraction	ovary	ovary	S2
UNC5C	4	19035187	19126466	13	SNPs, coverage			
Unnamed	Un	124574513	124575553	NA	coverage			
Unnamed	Un	127129819	127130503	NA	coverage			
Unnamed	16_random	26580	73126	NA	coverage			
Unnamed	Un	130103514	130104264	NA	coverage			
Unnamed	Un	50859565	50860210	NA	coverage			
Unnamed	Un	115355883	115358154	NA	coverage			
Unnamed	Un	124578595	124579326	NA	coverage			
VEGFA	3	31631385	31652650	34	SNPs, coverage	both		
WDR19	4	48204115	48240398	34	SNPs	ovary		S5
ZWILCH	10	19199771	19206407	8	SNPs	ovary		S5

Note: We were able to place only some genes on evolutionary strata due to our strict criteria for evaluating the maximum likelihood gene trees. The remaining genes lacked sequence information from several of the other sampled somatic genomes or had poorly resolved tree topologies.

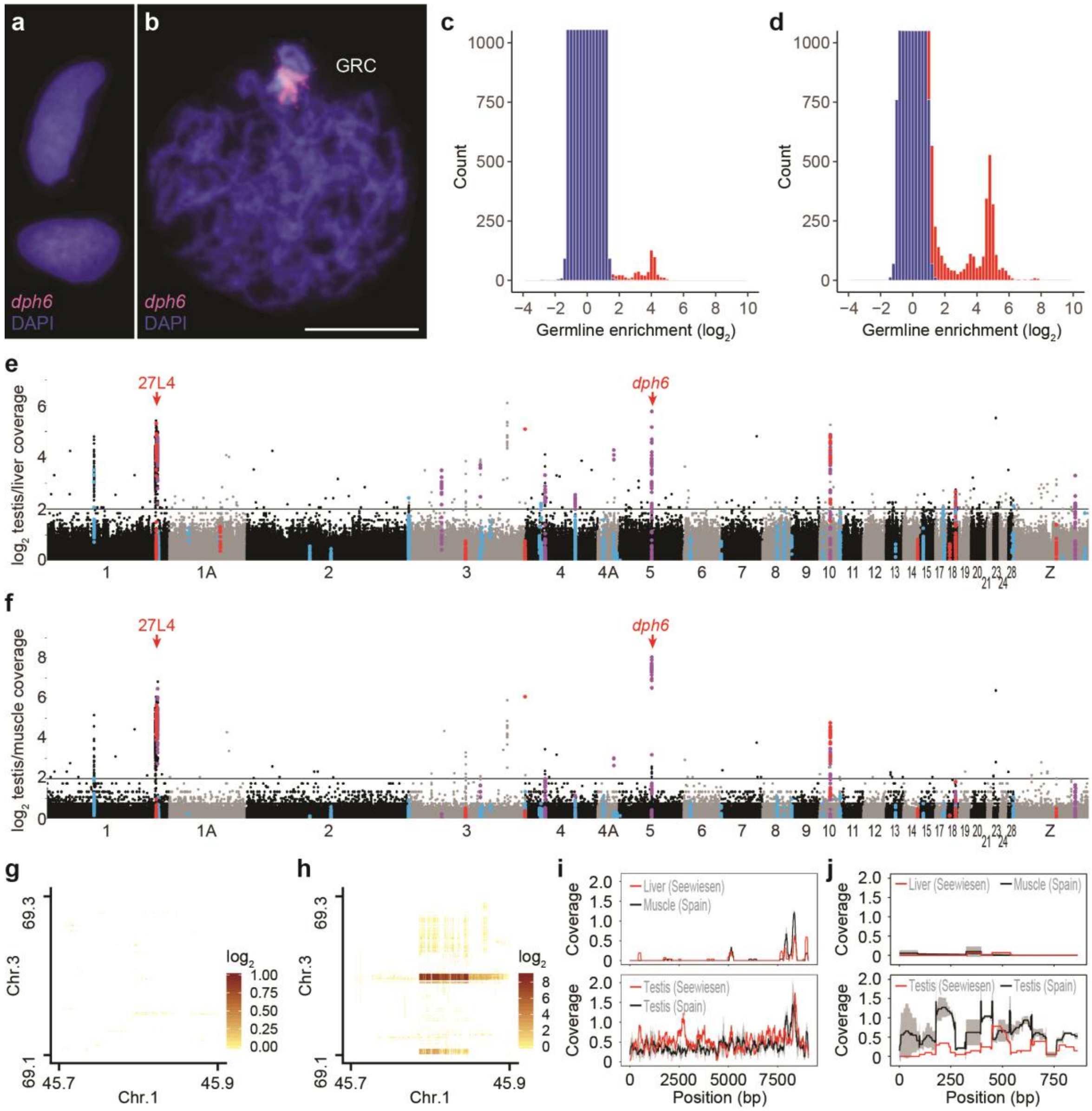


Figure 1 | The zebra finch germline-restricted chromosome contains genes copied from many A chromosomes. **a**-**b**, Cytogenetic evidence for GRC absence in muscle (**a**) and GRC presence in the testis (**b**) of the same bird (Spain_1) using fluorescence *in-situ* hybridization (FISH) of our new GRC-ampliconic probe *dph6* (selected due its high germline/soma coverage ratio; *cf*. panels e-f). The scale bar indicates 10 µm. **c**-**d**, Comparison of germline/soma coverage ratios (red) for 1 kb windows with an expected symmetrical distribution (blue) indicates enrichment of single-copy regions in the germline, similar to lamprey[2] both in Spain (average of Spain_1 and Spain_2 coverage; PCR-free short reads) and Seewiesen (linked reads) samples. Y-axis is truncated for visualisation. **e**-**f**, Manhattan plot of germline/soma coverage ratios in 1 kb windows across chromosomes of the somatic reference genome taeGut2. Colours indicate high-confidence GRC-linked genes and their identification (red: coverage, blue: SNPs, purple: both; Table 1). Note that the similarities between Seewiesen (**e**) and Spain_1/Spain_2 averages (**f**) constitute independent biological replicates for GRC-ampliconic regions, as the data are based on different domesticated populations and different library preparation methods. Red arrows denote two FISH-verified GRC-amplified regions (*cf*. panel b)[8]. Only chromosomes >5 Mb are shown for clarity. **g**-**h**, Linked-read barcode interaction heatmaps of an inter-chromosomal rearrangement on the GRC absent in Seewiesen liver (**g**) but present in Seewiesen testis (**h**). **i**-**j**, Coverage plots of two examples of GRC-linked genes that are divergent from their A-chromosomal paralog, *trim71* (**i**) and *napa* (**j**)[9], and thus have very low coverage (normalized by total reads and genome size) in soma.

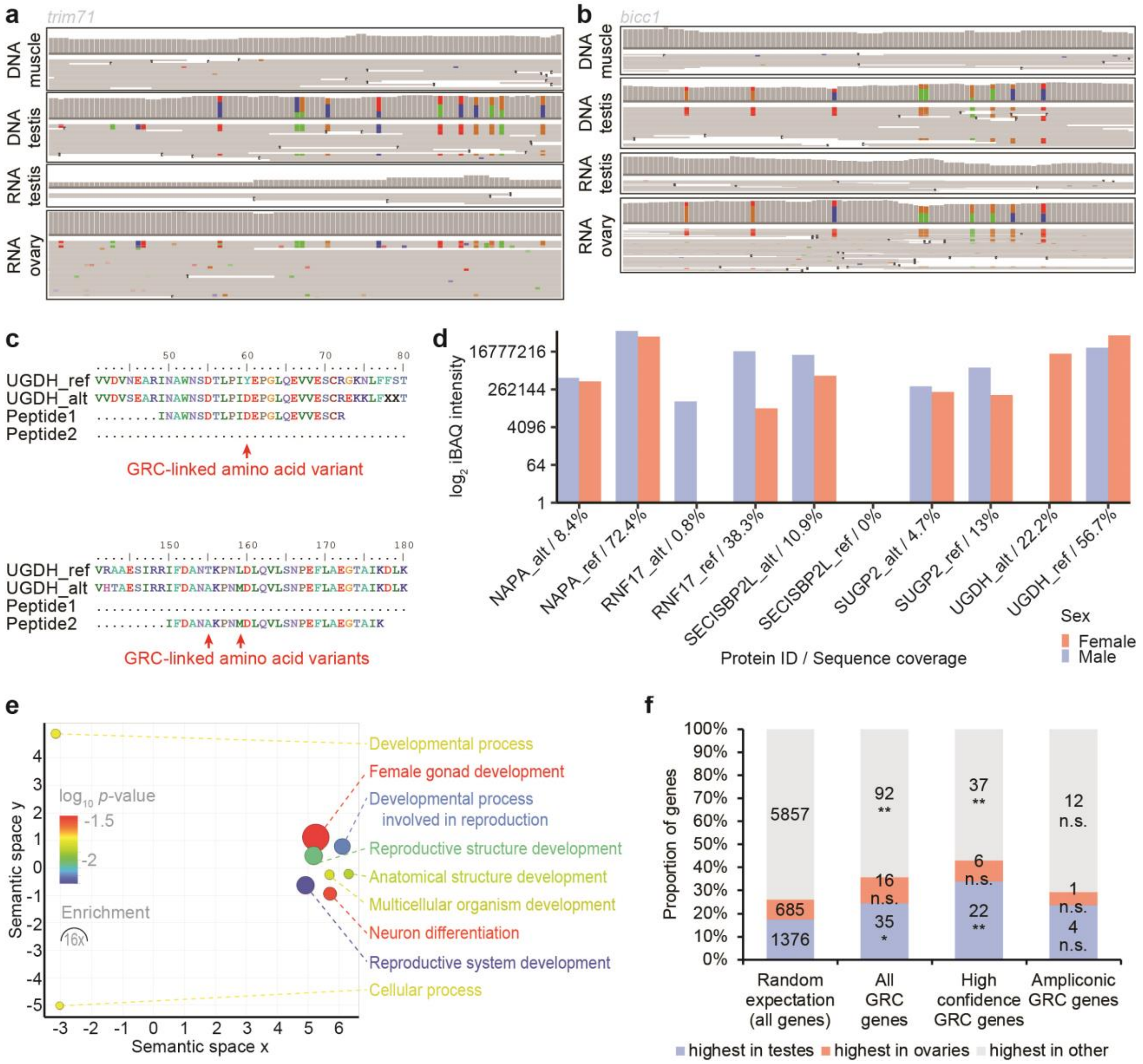


Figure 2 | The zebra finch germline-restricted chromosome is expressed in male and female gonads. a-b, Comparison of coverage and read pileups for DNAseq from Spain_1 and Spain_2 testis/muscle, RNAseq data from Spain_1 and Spain_2 testis, and available ovary RNAseq data[9]. Shown are 100-bp regions within *trim71* (**a**) and *bicc1* (**b**). Colours indicate SNPs deviating from the reference genome taeGut2. **c**, Example alignment of proteomics data showing peptide expression of the GRC-linked paralog of *ugdh* (alternative or 'alt'; *cf.* reference or 'ref'). **d**, Proteomic evidence for GRC protein expression ('alt') in comparison to their A-chromosomal paralog ('ref') in 7 sampled testes and 2 sampled ovaries. Only GRC paralogs of RNF17 and UGDH appear to be expressed in a sex-specific manner. **e**, Gene ontology term enrichment analysis of the 115 high-confidence GRC-linked genes (77 mapped gene symbols). Colours indicate the $\log_{10}$ of the false discovery rate *p*-value, circle sizes denote fold enrichment above expected values. **f**, Expression evidence for orthologs of three different sets of GRC genes in testes, ovaries, or other tissues in chicken[18]. Randomization tests show a significant enrichment for germline-expressed genes among the 115 high-confidence GRC genes and all 267 GRC genes, but not the 38 ampliconic GRC genes.

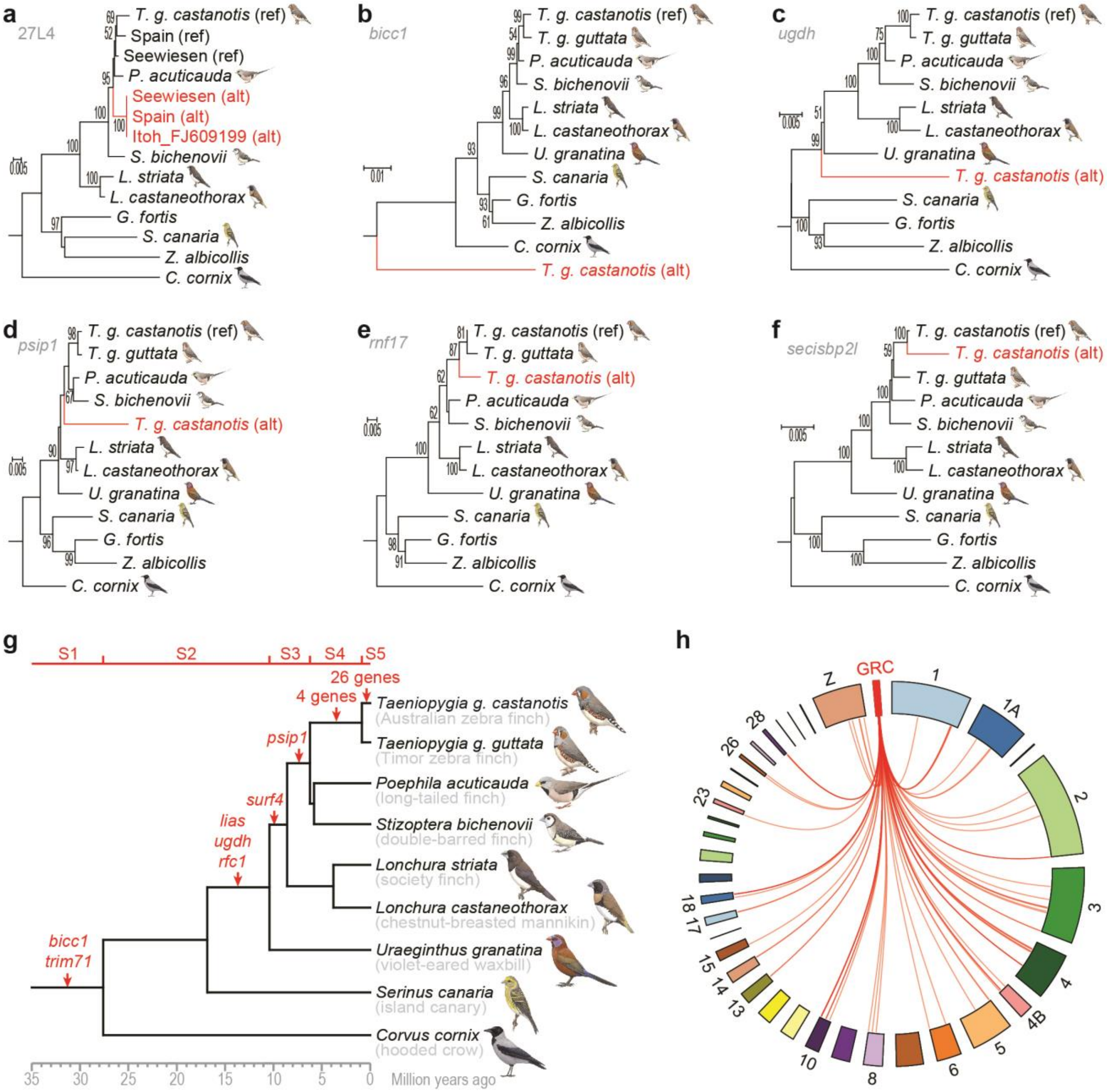
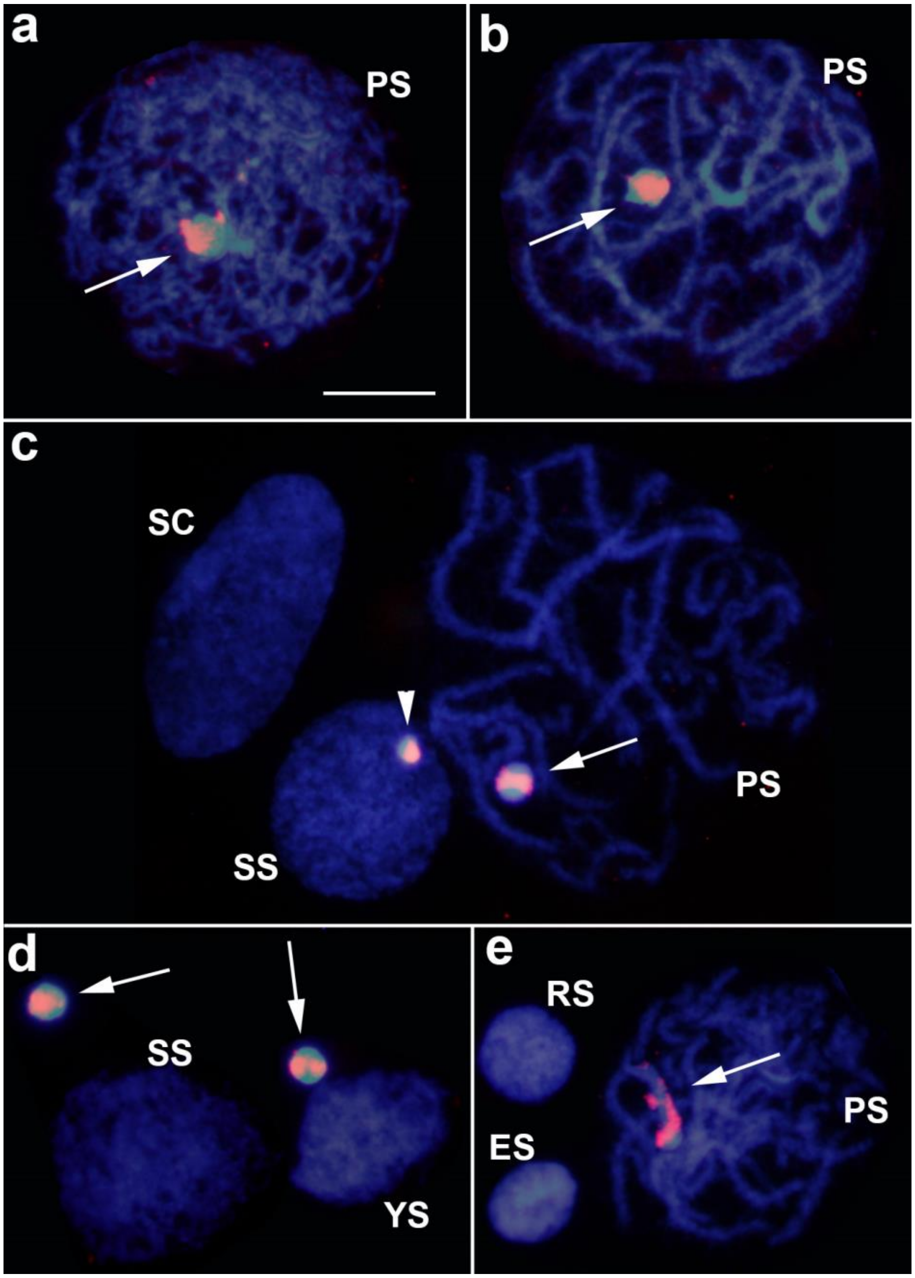
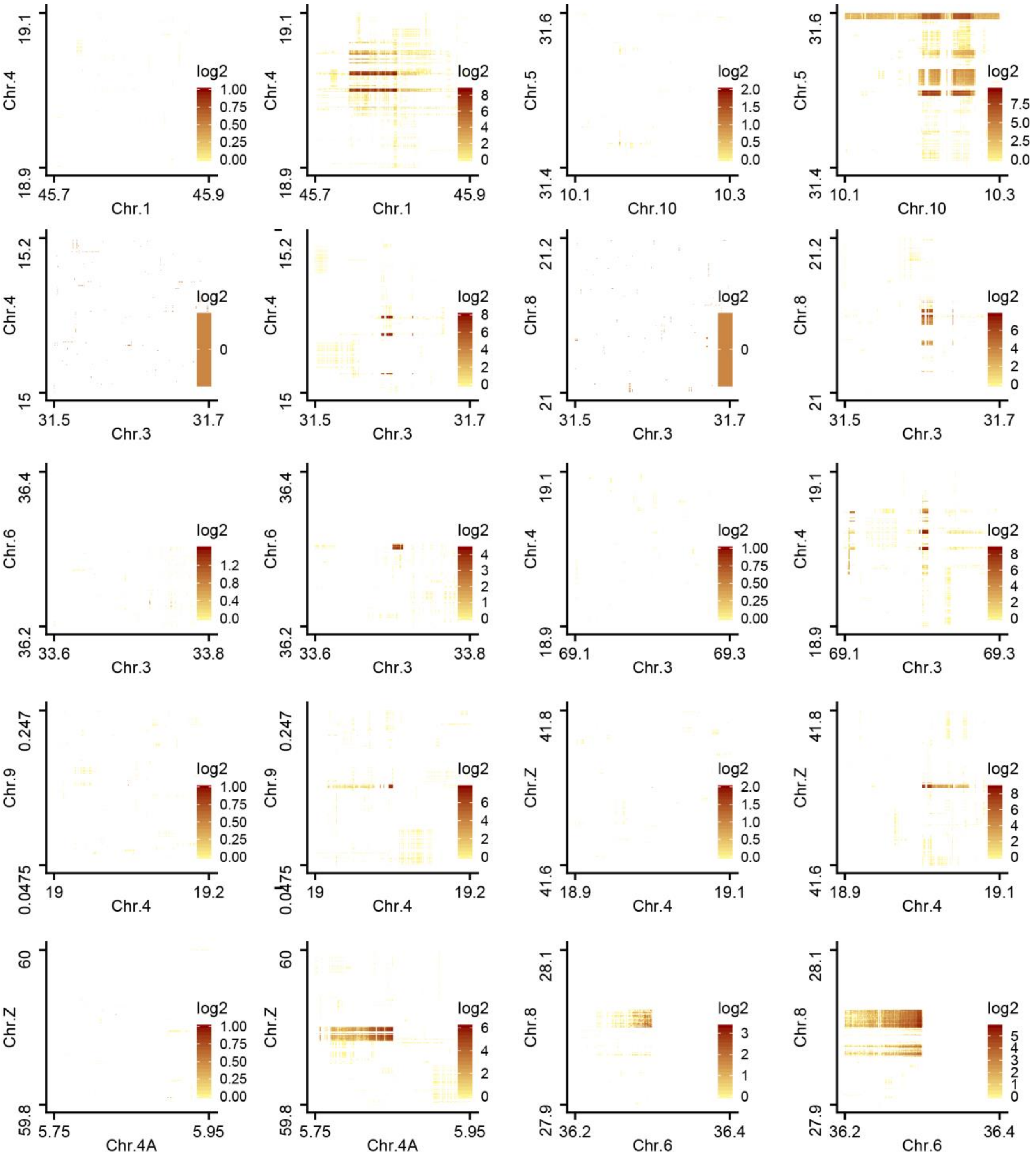


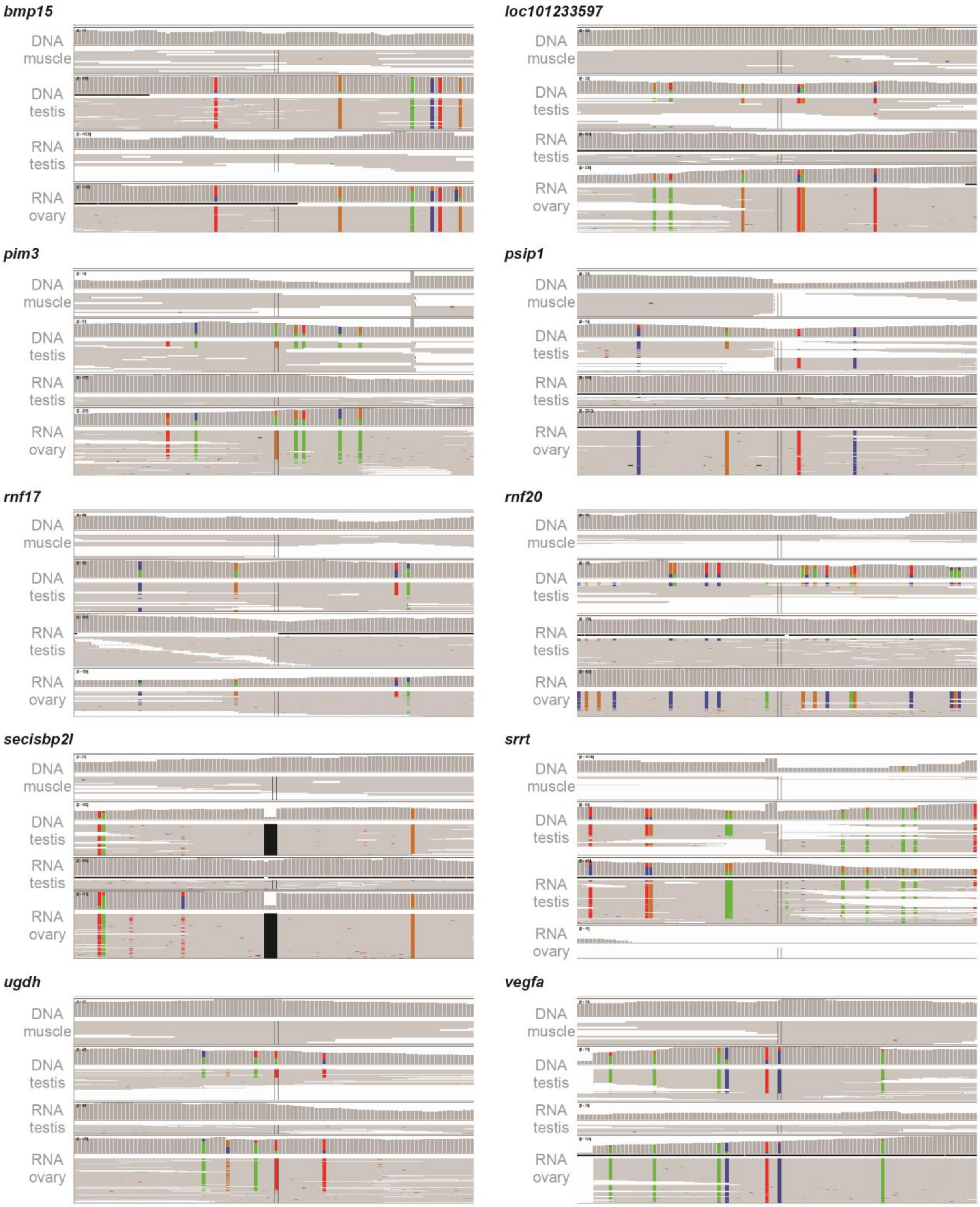
Figure 3 | The zebra finch germline-restricted chromosome is ancient and highly dynamic. a, Phylogeny of the intergenic 27L4 locus previously sequenced by Itoh et al.[8] suggests stable inheritance of the GRC paralog (alternative or 'alt' in red; *cf.* reference or 'ref') among the sampled zebra finches. **b-f**, Phylogenies of GRC-linked genes ('alt', in red; most selected from expressed genes) diverging from their A-chromosomal paralogs ('ref') before/during early songbird evolution (**b**; *bicc1*, stratum 1; *cf.* Extended Data Fig. 5), during songbird evolution (**c**; *ugdh*, stratum 2), during estrildid finch evolution (**d**; *psip1*, stratum 3), in the ancestor of the zebra finch species (**e**; *rnf17*, stratum 4), and in the Australian zebra finch subspecies (**f**; *secisbp2l*; stratum 5). The maximum likelihood phylogenies in panels a-f (only bootstrap values ≥50% shown) include available somatic genome data from estrildid finches and other songbirds. **g**, Species tree of selected songbirds showing the emergence of evolutionary strata (S1–S5) on the GRC (red gene names). Molecular dates are based on previous phylogenies[22,25]. Bird illustrations were used with permission from Lynx Edicions. **h**, Circos plot indicating A-chromosomal origin of high-confidence GRC-linked genes from 18 autosomes and the Z chromosome. Note that A-chromosomal paralogs of 37 genes remain unplaced on chromosomes in the current zebra finch reference genome taeGut2.



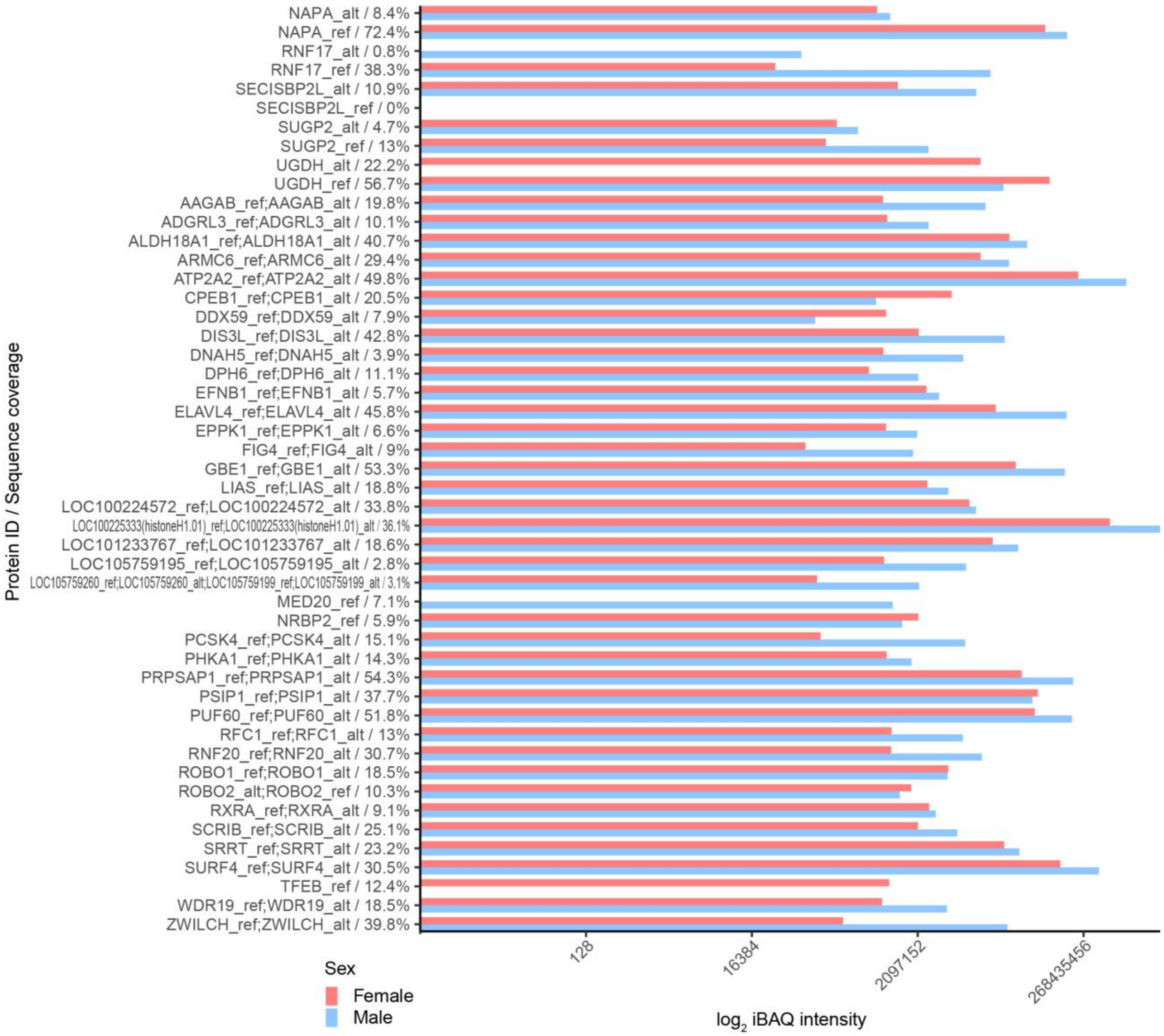
Extended Data Figure 1 | FISH analysis in testis cells of the Spain_1 zebra finch individual using the *dph6* probe (red) counterstained with DAPI (blue). Note the presence of primary (PS) and secondary (SS) spermatocytes, young spermatids (YS) and maturing spermatids at round (RS) and elongating (ES) stages. Also note that the *dph6* probe hybridizes with only part of the GRC chromosome (arrow), and this is apparent in PS at leptotene-zygotene (**a**), pachytene (**b**-**c**, **e**) and in GRCs which failed to integrate into the main nucleus of SS or YS cells (**d**), with no FISH signal in somatic cells (SC) indicating GRC absence in somatic structural testis cells (**c**). The half size of GRC in the SS cell in panel c, compared with that in the PS next to it and that those lying outside nuclei in panel d, indicates that GRC sometimes divides equationally in the first meiotic division (resulting in the half sized GRC body in panel c) but, in most cases, it divides reductionally yielding the large sized GRCs in panel d. Note that RS and ES nuclei in panel e lack FISH signal, indicating GRC absence. All photographs were made at the same magnification, and the scale bar in panel a indicates 10 µm.



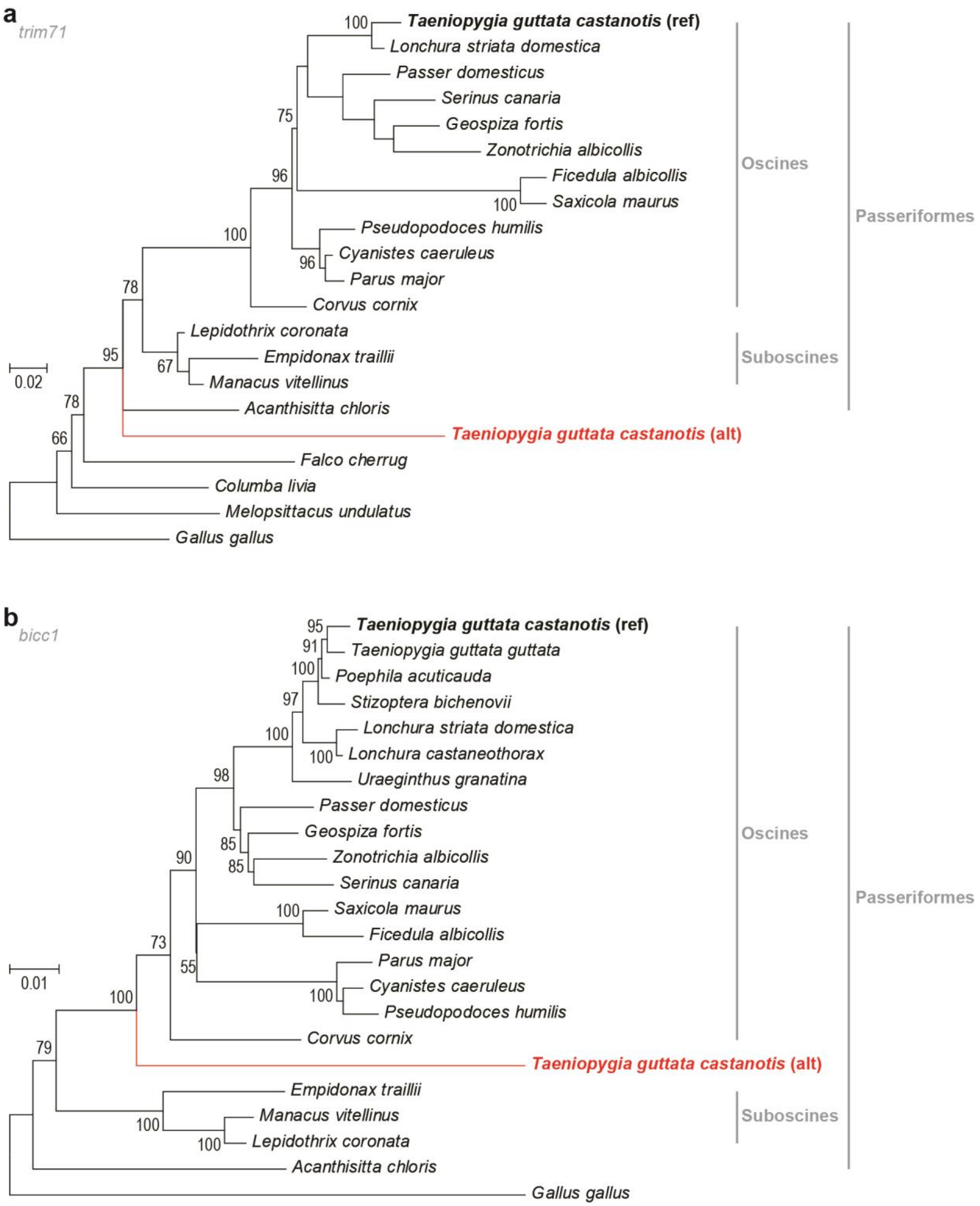
Extended Data Figure 2 | Testis-specific linked-read barcode sharing between A chromosomes indicates GRC haplotypes. Plots show side-by-side comparison of the inter-chromosomal barcode overlap for 200-kb regions for the liver and testis, respectively (chromosome position scale in Mb). With the exception of the interaction between chromosome 6 and chromosome 8 (bottom right) showing some background in the liver sample (potentially due to a shared A-chromosomal rearrangement), all inter-chromosomal structural variants were testis-specific and thus indicative of being on the same haplotype on the GRC. We exported barcode overlap matrices from the Loupe browser for testis-specific structural variants called by LongRanger and plotted them in R (v. 3.5.1). We reassigned 0 values to "NA" (shown in white on the plot) and $\log_2$-transformed all values.



Extended Data Figure 3 | Further examples for RNA expression of GRC-linked genes. Comparison of coverage and read pileups for DNAseq from Spain_1 and Spain_2 testis/muscle, RNAseq data from Spain_1 and Spain_2 testis, and available ovary RNAseq data[9]. Shown are 100-bp regions within 10 selected genes. Colours indicate SNPs deviating from the zebra finch reference genome taeGut2.



Extended Data Figure 4 | Proteomic evidence for functional GRC protein presence in zebra finch testes and ovaries. The five proteins listed at the top are also shown in Fig. 2d. GRC paralogs are denoted by the 'alt' suffix, where A-chromosomal paralogs are denoted by the 'ref' suffix. Sequence coverage corresponds to the peptide coverage percentage of the reference protein sequence. Entries of only one protein identification have sufficient evidence at the peptide level to differentiate between the GRC and A-chromosomal paralogs due to coverage of non-identical regions between the both reference sequences; entries of more than one protein identification contain evidence of presence based solely on identical regions, thus cannot be differentiated at the proteomic level. Entries of only one protein identification without the corresponding 'alt' or 'ref' variant contain evidence that span the non-identical region only, thus the alternate variant need not be called.



Extended Data Figure 5 | Gene trees of GRC-linked genes from stratum 1 and their A-chromosomal paralogs from broad taxon sampling imply GRC emergence in the ancestor of Passeriformes. a, Maximum likelihood gene tree of *trim71* (partitioned for codon positions) suggesting GRC linkage in the ancestor of Passeriformes. **b**, Maximum likelihood gene tree of *bicc1* (only 3' UTR) suggesting GRC linkage in the ancestor of oscine songbirds.