PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B

Phil. Trans. R. Soc. B (2008) **363**, 1453–1461
doi:10.1098/rstb.2007.2235
Published online 11 January 2008

The animal in the genome: comparative genomics and evolution

Richard R. Copley*

Wellcome Trust Centre for Human Genetics, Roosevelt Drive, Oxford OX3 7BN, UK

Comparisons between completely sequenced metazoan genomes have generally emphasized how similar their encoded protein content is, even when the comparison is between phyla. Given the manifest differences between phyla and, in particular, intuitive notions that some animals are more complex than others, this creates something of a paradox. Simplistic explanations have included arguments such as increased numbers of genes; greater numbers of protein products produced through alternative splicing; increased numbers of regulatory non-coding RNAs and increased complexity of the *cis*-regulatory code. An obvious value of complete genome sequences lies in their ability to provide us with inventories of such components. I examine progress being made in linking genome content to the pattern of animal evolution, and argue that the gap between genomic and phenotypic complexity can only be understood through the totality of interacting components.

Keywords: comparative genomics; evolution; Metazoa; transcription factors; ultraconserved regions

> Deus ex machina: A power, event, person, or thing that comes in the nick of time to solve a difficulty; providential interposition…
>
> Oxford English Dictionary

1. INTRODUCTION

Complete genome sequences provide limits to our imaginations. Even just a few years before the human genome was available in rough draft form, it was widely believed to encode at least 50 000 genes (Fields *et al.* 1994; Nature Genetics Editorial 2000). In contrast, the initial publications estimated 25–40 000 protein-coding genes (Lander *et al.* 2001; Venter *et al.* 2001), and since then estimates have generally carried a downward momentum, most recently approaching 20 000 (Goodstadt & Ponting 2006; Pennisi 2007). Although this number is higher than 16 000 or so found in invertebrate chordates (Dehal *et al.* 2002), it is roughly the same total as the nematode worm *Caenorhabditis elegans* (Hillier *et al.* 2005). Whether or not these low numbers of protein-coding genes for vertebrates stand the test of time, the sense of unease surrounding the lack of correlation between organismal complexity (often measured in numbers of distinct cell types) and protein-coding gene count is evident from the framing of the 'G-value paradox' by Hahn & Wray (2002), and the various explanations that have been put forward to ease it, including, for example, miRNAs (Sempere *et al.* 2006), non-protein-coding DNA (Taft *et al.* 2007) and alternative splicing (Kim *et al.* 2007).

Similar gene counts are, of course, a crude measure of biological complexity. There is no reason why two genomes should not encode very different sets of protein-coding genes, but still have similar overall totals. Within the field of animal evolution and the evolution of development (evo–devo), however, the G-value paradox has a particular resonance. Studies in different animal phyla have repeatedly shown the reuse of a core set of developmental genes, the so-called 'toolkit' (Carroll *et al.* 2005), with the HOX genes in particular taking on an iconic significance. Broadly, toolkit genes come from a handful of transcription factor families, defined by the presence of particular structural domains such as the helix-turn-helix (HTH), including the homeobox genes; zinc fingers (ZnFs); leucine zippers and the helix-loop-helix (HLH). As well as transcription factors, there are seven well-conserved pathways responsible for intercellular signalling (Pires-daSilva & Sommer 2003), many of which appear to be present in sponges, the earliest branching clade of animals (Nichols *et al.* 2006). An extreme interpretation of these data is provided by Davidson (2006): 'if we focus explicitly on the genes encoding transcription factors, and […] signalling systems required for developmental spatial regulation, there is almost no qualitative variation among the genomes of bilaterians'.

Given all this, where in the genome do the phenotypic differences between animal taxa arise? The undoubted conservation of the protein-coding developmental genes has, particularly in the evo–devo field with its morphological concerns, focused attention on *cis*-enhancer elements affecting transcription (Carroll *et al.* 2005; Davidson 2006; Simpson 2007; Wray 2007), although there are alternative views emphasizing the importance of different kinds of regulatory elements (Alonso & Wilkins 2005) and different protein classes, such as structural genes (Hoekstra & Coyne 2007). As well as the presence of particular genes, the role of gene loss, especially with regard to secondarily simplified organisms such as tunicates and nematodes, is also likely to be of major significance. Below I outline some major themes being

*copley@well.ox.ac.uk

One contribution of 17 to a Discussion Meeting Issue 'Evolution of the animals: a Linnean tercentenary celebration'.

developed by large-scale genome comparisons, principally of nematodes, insects and vertebrates. My aim is not to present an exhaustive account, but to highlight areas where functionally relevant species-specific differences may arise, within apparently conserved systems. Although I concentrate on the evolution of the systems regulating animal development, this is not to lose sight of the things being regulated: the proteins involved in making nematode cuticles, or asynchronous flight muscles in insects, or the human brain and adaptive immune system, to name but a few, are what make it necessary to evolve those systems.

2. GENE DUPLICATION

Usefully summarizing the differences and similarities between more than 10 000 protein-coding genes from several species at once is not necessarily straightforward. Although pairwise similarities between sequences are easy to compute, they suffer from the imposition of arbitrary cut-offs and are less easy to interpret than measures that explicitly reflect phylogeny. Genes in different species are most obviously compared by grouping into sets of orthologues (i.e. genes related by speciation events) and paralogues (genes related by intra-genome duplication events). Closely related species share large numbers of orthologues: 93% of dog (*Canis familiaris*) and 82% of the marsupial *Monodelphis domesticus* gene predictions have orthologues in human (Goodstadt *et al.* 2007). The Linnean hierarchy, however, is not necessarily a good guide of genomic relatedness by this definition of similarity. Within the nematodes, 65% of *C. elegans* genes share an orthologue with *Caenorhabditis briggsae*, despite their being from the same genus (Stein *et al.* 2003). For more distantly related genomes, orthologue counts can drop rapidly. This may be as much a sign of difficulties in reliably assigning gene orthology on a large scale, as a real indication of the extents of the conserved cores.

Paralogues often arise via tandem duplication of genes, giving rise to localized clusters of functionally related genes. As these are the regions where gene content is evolving most rapidly between closely related species, the functions of these genes are of special interest for understanding animal-specific differences. For the most part, for any two closely related vertebrate genomes, the functional classes of genes duplicated in this way are similar—olfaction and chemosensation, reproduction and effectors of the immune response—although the duplications have occurred independently in each lineage (Emes *et al.* 2003). These large groups of paralogues often show evidence of adaptive evolution in their amino acid sequences, suggesting that new functions have been selected for (Emes *et al.* 2004*a*,*b*).

The recurrent nature of duplications within particular functional classes, coupled with the observed diversifying selection suggests that they are a standard adaptive genomic response to environmental challenges. Does similar rapid duplication occur in the kinds of genes, such as transcription factors, that might be implicated in development? A growing number of examples are known. Perhaps most dramatically, in mice a set of 32 tandemly duplicated homeoboxes have arisen from apparently one or two genes in the common ancestor of humans and rodents; they are believed to play a role in germ cell development and embryonic stem cell differentiation (Maclean *et al.* 2005; Jackson *et al.* 2006).

ZnF containing transcription factors have undergone independent rounds of gene duplication in insects and tetrapods. In insects a set of ZnFs is found to co-occur with a ZnF-associated domain (ZAD; Chung *et al.* 2007); this ZAD class is found in approximately 100 and 150 copies in *Drosophila melanogaster* and the mosquito *Anopheles gambiae*, respectively; there is only a single copy in vertebrates (Chung *et al.* 2007). In *D. melanogaster*, many are expressed in the female germ line, suggesting a role in oocyte development or embryogenesis (Chung *et al.* 2007). An analogous story is found with Krüppel-associated box (KRAB) containing Zn fingers in tetrapods. Successive independent tandem duplication events have occurred in different mammalian lineages, leading to over 400 copies in the human genome (Huntley *et al.* 2006). The KRAB domain itself appears to have been co-opted from a progenitor sequence conserved throughout eukaryotes (Birtle & Ponting 2006), however, it has evolved so much as to make this similarity difficult to detect; clearly identifiable KRAB domains are specific to tetrapods. Their functions are largely unknown, and have not been tied to any general aspects of tetrapod-specific biology. As such, why the family as a whole has expanded is a puzzle.

Nematodes too exhibit lineage-specific expansions of particular transcription factor families, most notably, the nuclear hormone receptors (NHRs). The *C. elegans* genome encodes 284, far more than the 48 in human and 21 in *D. melanogaster*. The bulk of these (greater than 200) have arisen from an apparently nematode-specific expansion of a unique gene (Lander *et al.* 2001; Robinson-Rechavi *et al.* 2005). Once more, the reasons for such a dramatic lineage-specific expansion of a particular transcription factor family, and any links to taxon-specific biology, are obscure, although it has been speculated that *C. elegans* relies less on combinatorial reuse of different transcription factors (Antebi 2006). A less dramatic lineage-specific expansion occurs in the case of the T-box-containing transcription factors: there are 21 in *C. elegans*, with 17 arising from a lineage-specific expansion when compared with *D. melanogaster* and humans. Ascertaining when and in which taxa these duplications found in *C. elegans* took place is currently frustrated by a lack of nematode genome sequences—currently, only those of *C. elegans* and *C. briggsae* have been published. These T-box genes as a set map to several genomic locations, suggesting that they have arisen over a more protracted time scale than the examples discussed above; some, at least, have known roles in the development of *C. elegans* (Poole & Hobert 2006).

3. THE INVENTION OF NEW GENES

A number of gene families appear to be metazoan novelties, with no clear sequence similarity to other genes outside the Metazoa, but present in the more basal animal phyla, such as cnidarians and sponges.

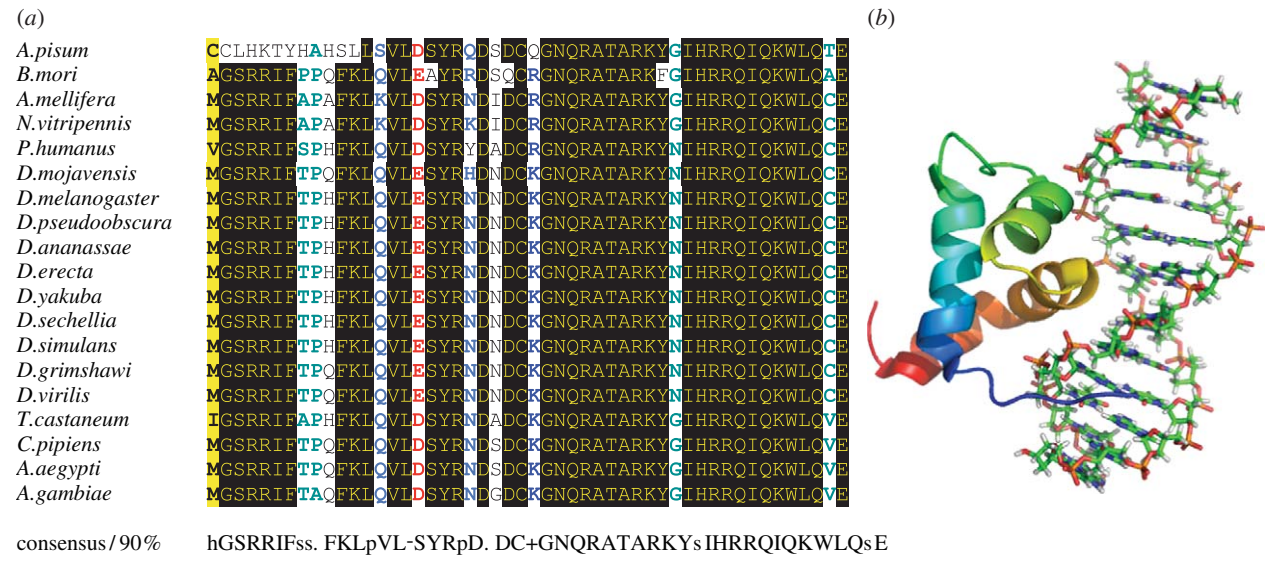


Figure 1. The DNA-binding domain of brinker is conserved within insects, but has no significantly similar sequences in other taxa. (*a*) The alignment shows the conserved core from selection of insect species. *Drosophila* species sequences were taken from the UCSC web browser (http://genome.ucsc.edu), *Anopheles* and *Aedes* from ENSEMBL (http://www.ensembl.org), other predictions were made from sequences at the NCBI. GI Accessions: N.vit 146253130, T.cas 73486274, C.pip 145464888, P.hum 145365328, A.mel 63051942, B.mor 91842977 and A.pis 47522326. (*b*) The three-dimensional structure of the aligned region when binding DNA. The structure was taken from the PDB file 2glo.

These include key families involved in animal development, such as T-box and SMAD transcription factors, and signalling molecules such as WNTs and fibroblast growth factors (FGFs; Putnam *et al.* 2007). Was the invention of such families a prerequisite for the evolution of the Metazoa, and were analogous protein inventions required for the evolution of particular taxa, such as insects and vertebrates? Analysis of three-dimensional structures (i.e. the protein fold itself) suggests a more subtle transition than large-scale evolution of new protein folds. In many cases, examination of protein three-dimensional structural similarities shows that these genes have distant homologues in non-metazoan genomes. The MH1 (DNA binding) domain of SMADs, for instance, is probably homologous to a family of homing endonucleases found in all kingdoms of life (Grishin 2001); the T-box shares structural similarities indicative of homology with a variety of other transcription factors, such as STAT DNA-binding domains, which are found in other eukaryotes (Murzin *et al.* 1995; Soler-Lopez *et al.* 2004); and the signalling domain of metazoan hedgehog proteins shares detailed similarities with members of a family of bacterial peptidases, suggesting that they too are likely to be homologous (Murzin *et al.* 1995). In these cases, the novel families are likely to be cases of rapid sequence evolution, accompanying functional shifts, within stem lineages leading to the Metazoa. Sparse sequence sampling of non-fungal and metazoan eukaryotic genomes may contribute to the apparent co-origin of these protein domains with the animals.

As this type of domain evolution is occurring from pre-existing domain types, the process fits within a standard framework of accelerated point mutation and selection for new functions. The invention of the domain type is not a key innovation in itself; rather, it can be seen as the extension of functional diversification of subfamilies of the kind that is apparent when comparing more closely related species. The fact that so many new domain types are found to be coincident within the origin of metazoans suggests that the selective pressures giving rise to this kind of accelerated sequence evolution were greater in the metazoan stem lineage.

An example of a more recent domain innovation is found in the *Drosophila* gene *brinker*, which plays a key role in the establishment of dorsoventral patterning. Although the protein-coding sequence of its DNA-binding domain is well conserved in insects, using current sequence databases it shows no significant sequence similarity to proteins from any other taxa (figure 1), although there is weak (non-significant) similarity to pogo-like transposases, and the structure, which is only folded when complexed with DNA, suggests similarity to various transcription factors (Cordier *et al.* 2006).

4. EVOLUTION OF TRANSCRIPTION FACTORS: THE ANIMAL IN THE ORTHOLOGUE

Lineage-specific duplication followed by sequence divergence provides one route to species-specific biology, but what scope is there for lineage-specific functional shifts within orthologous genes? In the absence of gene duplication, it is hard to imagine how the DNA specificity of a particular factor might be significantly changed in such a way that it targets new genes, without deleterious consequences. The modular structure of proteins, however, suggests that other routes of functional evolution are available. A protein may have pleiotropic effects, but that is not the same as saying that every amino acid in the protein will be directly involved in all those effects. A recent illustrative example from the hox gene *Ultrabithorax*, is of an insect-specific 'QA' protein motif, found outside the homeodomain. The region is involved in limb repression; the effects of deleting the motif are strong in some

tissues but close to undetectable in others (Hittinger *et al.* 2005). Clearly, changes in the protein-coding sequences of transcription factors, apart from their more obvious DNA-binding residues, must be integrated into our understanding of the evolution of developmental regulation.

The majority of residues in metazoan transcription factors do not fall within regions of well-defined globular structure, with many belonging to so-called 'intrinsically disordered' regions—regions that may form a structure when complexed with other macromolecules (Liu *et al.* 2006; Minezaki *et al.* 2006). The specific sequences of these regions are typically not obviously conserved between paralogues; because they are unique to particular families they are not covered in domain databases such as SMART and Pfam (Finn *et al.* 2006; Letunic *et al.* 2006). The lack of extreme conservation between distant species has sometimes masked the fact that within closely related species, these regions are conserved. Comparisons of orthologous sequences from closely related genomes (e.g. vertebrates or drosophilids) often show that substantial proportions of these non-domain sequences are undergoing strong purifying selection—they accumulate many more synonymous nucleotide changes than non-synonymous changes—and are thus functional. For the large part, precisely what these biological functions are is unknown; two possibilities, however, suggest themselves. Firstly, they may have relatively uninteresting non-specific effects, such as facilitating folding of the major domain (e.g. by reducing aggregation) or acting as spacers between globular domains. Secondly, and more interestingly from the point of view of animal evolution, they may include short linear peptide motifs that mediate protein–protein interactions (Dyson & Wright 2005; Neduva & Russell 2005; Neduva *et al.* 2005).

There are numerous examples of regulatory motifs found outside of transcription factor domains. Many hox proteins include a YPWM-like hexapeptide motif that interacts with other homeodomain-containing proteins (In der Rieden *et al.* 2004); *Drosophila* ftz orthologues have lost this motif but acquired an LXXLL motif coupled to a new role in segmentation (Lohr & Pick 2005); and an N-terminal SSYF-like motif believed to be involved in transcriptional activation is conserved across Hox orthologues and paralogues from different phyla (Tour *et al.* 2005). Interaction motifs can be coupled with signalling pathways to create cell-type specificity. They can, for instance, be regulated by phosphorylation, such that the phosphorylation status governs what interactions can be made (e.g. Sapkota *et al.* 2007), or alternative splicing can result in protein–protein interaction motifs being included or excluded from particular cell types, providing additional layers of regulatory complexity that are likely to be species specific (Neduva & Russell 2005).

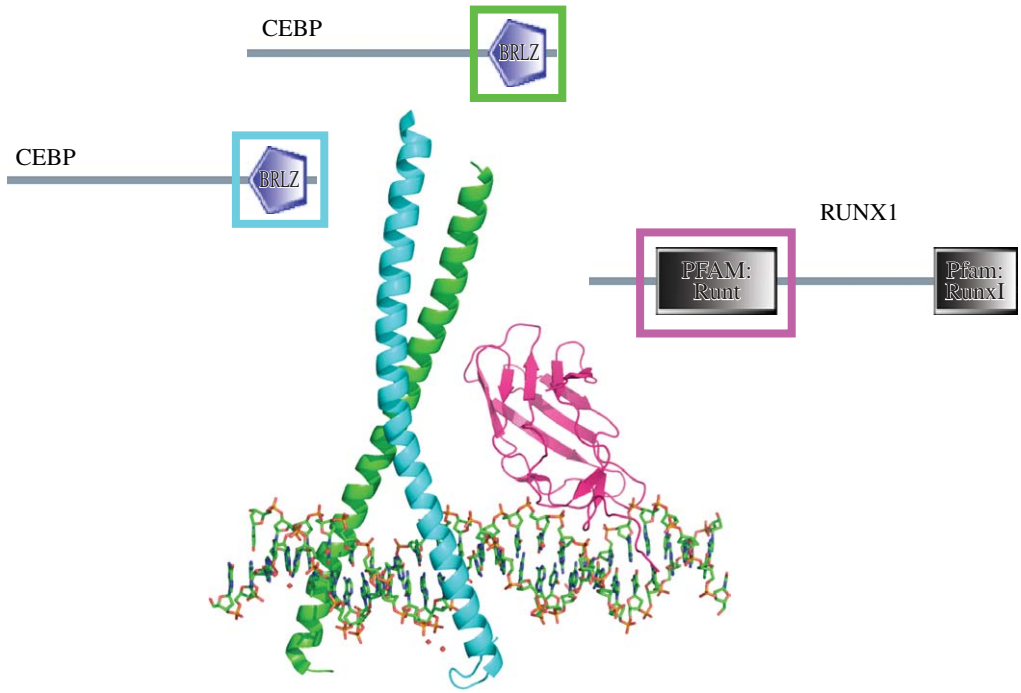
The challenge of identifying small regulatory motifs means that their species distributions, and how their presence might produce taxon-specific differences in protein functions, have not been well studied. Examples that tie cleanly to one taxonomic group are less common, but an interesting case has been proposed in bilaterian orthologues of the Brachyury gene. These possess an N-terminal motif that is not found in non-bilaterian Metazoa (Marcellini 2006), which instead have a well-defined EH1-like motif (Copley 2005). The bilaterian motif is believed to be responsible for an interaction with Smad1, and hence to link gastrulation to bilateral pattern formation (Marcellini 2006).

5. ENHANCERS: TRANSCRIPTION FACTOR-BINDING SITES AND ULTRACONSERVED REGIONS

Theoretical considerations have led to an intense focus on transcription factor-binding sites (TFBSs) as a major molecular source of morphological novelty (Wray *et al.* 2003; Carroll *et al.* 2005; Davidson 2006; Wray 2007), although see (Hoekstra & Coyne 2007) for a critique. Individual TFBSs show rapid turnover in comparisons of closely related genomes, with many being lineage specific (Dermitzakis & Clark 2002; Moses *et al.* 2006). This dynamic nature may not be revealed in the phenotype—patterns of gene expression may be conserved even though regulatory sequences change at the molecular level (Ludwig *et al.* 2000; Romano & Wray 2003; Fisher *et al.* 2006). On the other hand, the gain and the loss of individual TFBSs have been implicated in several recent cases of morphological evolution, in both vertebrates and invertebrates (reviewed in Simpson (2007) and Wray (2007)). The relationship between individual TFBSs and enhancer function is clearly not straightforward, beyond the fact that clustering of individual binding sites can identify some enhancer regions (Markstein *et al.* 2002). Cases of functional linkages between particular transcription factors have been proposed, for example, between dorsal, twist, Su(H) and an unidentified motif in neurogenic ectoderm formation in Diptera (Markstein *et al.* 2004), and even a coupling originating prior to the origin of Bilateria, of hairy and E(spl) promoting neural cell fate (Rebeiz *et al.* 2005).

Comparisons of vertebrate genomes have revealed large regions (more than 100 nucleotides) of extreme conservation of non-coding sequences (conserved non-coding elements (CNEs); Bejerano *et al.* 2004). These regions are often found near transcription factors and other developmental genes (Sandelin *et al.* 2004). Outside of the vertebrates, there is evidence for similar regions occurring near developmental genes in flies (Glazov *et al.* 2005) and nematodes (Vavouri *et al.* 2007). Although in many cases the conserved regions are even found near orthologous genes, there is no evidence that they are homologous; they appear to have evolved independently in each of the phyla (Vavouri *et al.* 2007). Experimental evidence from vertebrates shows that many instances have roles as tissue-specific enhancer elements (Woolfe *et al.* 2005; Pennacchio *et al.* 2006).

The length and lack of inter-phylum conservation of CNEs is in contrast to individual TFBSs. The DNA specificity of orthologous transcription factors is usually well conserved over large phylogenetic distances, but typical TFBSs are short, of the order of 6–10 nucleotides. An obvious possibility is that longer CNEs are composed of overlapping or adjacent TFBSs. This would suggest a tight packing of transcription factor




```
Human       GCAACCACAGAGTTTGGAAATCTT
Chimp       ........................
Rhesus      ........................
Mouse       .G...T.....A.........A..
Rat         .G...T..............A..
Rabbit      ........................
Dog         ........................
Cow         ........................
Elephant    ..........T..........-..
Tenrec      ..........G.............
```

Figure 2. Adjacent TFBSs cause extended regions of DNA sequence conservation. Structure of CEBPβ homodimer and Runx-1 (Tahirov *et al.* 2001). Three transcription factors (2xCEBPB and RUNX1) bind in a region of 25 nucleotides conserved throughout placental mammals. The DNA-binding domains represented as three-dimensional structures are boxed and colour coded in the schematic of the proteins. In each case, the majority of the protein is not represented in the structure; these regions could interact with other transcription factors, activators and repressors. The human sequence coordinates are chromosome 5, bases 149 446 373–149 446 396 of the NCBI build 36. The alignment is taken from the UCSC web browser http://genome.ucsc.edu.

proteins on the genomic DNA of these CNEs. There is direct evidence for this: some fragments of highly conserved non-coding sequences are present in crystal structures of transcription factor complexes. An atomic model based on known crystal structures of the interferon-β enhancer, for example, shows 50 consecutive nucleotides in contact with eight different proteins; these nucleotides are well conserved in mammalian species (Panne *et al.* 2007; see figure 2 for another example). Given that such structures exist, it is not such a leap to imagine 16 proteins binding to 100 nucleotides, or even bigger complexes. This suggests a model where CNE enhancer regions controlling orthologous genes in different phyla are controlled by multiple TFBSs, although not necessarily the same transcription factors or in the same orientation. Moreover, the tight packing of transcription factors on the genomic DNA suggests that the proteins themselves may be co-adapted to interact with each other and aid the cooperative formation of enhancer complexes. Previously, Ruvinsky & Ruvkun (2003) have presented experimental evidence that enhancers and transcription factors co-evolve in this way, with neuronal and muscle-specific enhancer elements from *D. melanogaster* failing to drive expression in homologous tissue types in *C. elegans*, and Dover and co-workers have argued for coevolution of bicoid protein and hunchback regulatory regions (McGregor *et al.* 2001; Shaw *et al.* 2002).

If protein–protein interactions between transcription factors are often required for the formation of enhancer complexes, close analysis of transcription factor sequence and structure may reveal evidence for co-adaptation of proteins, such as the Hox hexapeptide motif, through which homeotic proteins form complexes with TALE class homeodomains (LaRonde-LeBlanc & Wolberger 2003). We might expect instances of co-adapted transcription factor combinations to be taxon specific, to match the taxon specificity of enhancer sequences.

6. ALTERNATIVE SPLICING

Not all CNEs are associated with enhancer regions. There is good evidence that many are involved in regulating alternative splicing events, including the

alternative splicing of mRNAs of proteins which themselves regulate alternative splicing (Lareau *et al.* 2007; Ni *et al.* 2007). The presence of highly conserved control elements to regulate alternative splicing indicates that the functional consequences are of importance. Although large very conserved elements may be the exception rather than the rule, detailed comparative analyses have identified smaller conserved motifs regulating alternative splicing, for instance in nematodes (Kabat *et al.* 2006) and vertebrates (Sorek & Ast 2003; Yeo *et al.* 2005).

Alternative splicing is often touted as a mechanism by which proteomic complexity is increased. Although early reports suggested that levels of alternative splicing were comparable in vertebrates and invertebrates (Brett *et al.* 2002), more recent studies suggest that there is indeed more alternative splicing of transcripts in vertebrates (Kim *et al.* 2007), suggesting a link with increased phenotypic complexity. How relevant is alternative splicing for species-specific biology and morphological differences? Quantitatively, the gene products that appear to be most affected by alternative splicing are typically involved in nervous and immune system function (Modrek *et al.* 2001). There are, however, ample examples of alternatively spliced transcription factors—as many as 63% of mouse transcription factors have variant exons (Taneri *et al.* 2004). Although the differences in molecular roles of the alternatively spliced products are often unknown, the genes themselves include developmental classics such as members of Hox, SMAD and T-box families (Fan *et al.* 2004; Dunn *et al.* 2005; Noro *et al.* 2006), although they do not necessarily present obvious morphological correlates (Yoder & Carroll 2006). Alternative splicing of modular proteins is an obvious route through which functions can be changed, by including or excluding particular combinations of domains. In this regard, it is interesting that alternative splicing often affects intrinsically disordered regions outside known protein domains (Romero *et al.* 2006)—this again points to a critical role for finely tuned protein–protein interactions among transcriptional regulators.

There are few known cases of distant conservation of alternative splice variants of transcription factors; typically, examples are conserved within phyla at best. Widening the search to other classes of gene again suggests that splice variants are not conserved over long periods, although it should be remembered that transcript coverage of most species from which evidence of alternative splicing is obtained is very restricted. Perhaps the best counter-example is currently that of fibroblast growth factor receptor 2 (FGFR2), where an exon configuration diagnostic of mutually exclusive alternative splicing is found in both vertebrates and the sea urchin *Strongylocentrotus purpuratus* (Mistry *et al.* 2003). Examples of orthologous ion channel encoding genes showing similar alternative splicing patterns in *D. melanogaster*, *C. elegans* and humans are likely to be cases of parallel evolution (Copley 2004). The shared ability of vertebrates and at least insects and *C. elegans* to produce alternative transcripts in a regulated manner, but the absence of large numbers of conserved alternative splicing between protostomes and deuterostomes suggests that gene products have become alternatively spliced in parallel between different lineages, while at the same time hinting that the functions performed by alternative splice variants may, over time, be replaced by different genomic solutions.

7. SUMMARY

Although most major classes of protein involved in animal development may be conserved throughout the Metazoa, detailed comparative analysis of these gene types reveals a more dynamic picture, with frequent gene duplication, gene loss, couplings with new motifs and other processes such as alternative splicing and regulation by micro-RNAs, all of which are likely to be important for a full understanding of function. *Cis*-regulatory variation may well be revealed to be quantitatively the most common form of variation between species, but it seems probable that the cumulative effects of multiple *cis*-regulatory changes will have required that protein networks evolve to accommodate and correctly regulate changed enhancer structures.

Our knowledge of animal evolution and the picture presented here is currently based on a very small sampling of almost exclusively nematode, insect and vertebrate genomes. Although this situation is beginning to change, the fact that many important functional regions, especially those that do not encode proteins, are only revealed by having sets of closely related genome sequences, and that there are 35 or so animal phyla gives some idea of the enormity of the challenges ahead. The rapidly falling costs of genome sequencing do, however, give grounds for optimism.

I thank the organizers and participants of the Novartis Foundation symposium on Animal Evolution, 20 June 2007, for helpful discussions, two anonymous referees for perceptive comments, and the Wellcome Trust for support.

REFERENCES

Alonso, C. R. & Wilkins, A. S. 2005 The molecular elements that underlie developmental evolution. *Nat. Rev. Genet.* **6**, 709–715. (doi:10.1038/nrg1676)

Antebi, A. 2006 Nuclear hormone receptors in *C. elegans*. In *WormBook* (ed. The *C. elegans* Research Community). See www.wormbook.org/chapters/www_nuclearhormonerecep/nuclearhormonerecep.htm.

Bejerano, G., Pheasant, M., Makunin, I., Stephen, S., Kent, W. J., Mattick, J. S. & Haussler, D. 2004 Ultraconserved elements in the human genome. *Science* **304**, 1321–1325. (doi:10.1126/science.1098119)

Birtle, Z. & Ponting, C. P. 2006 Meisetz and the birth of the KRAB motif. *Bioinformatics* **22**, 2841–2845. (doi:10.1093/bioinformatics/btl498)

Brett, D., Pospisil, H., Valcarcel, J., Reich, J. & Bork, P. 2002 Alternative splicing and genome complexity. *Nat. Genet.* **30**, 29–30. (doi:10.1038/ng803)

Carroll, S. B., Grenier, J. K. & Weatherbee, S. D. 2005 *From DNA to diversity: molecular genetics and the evolution of animal design*. Oxford, UK: Blackwell Publishing.

Chung, H. R., Lohr, U. & Jackle, H. 2007 Lineage-specific expansion of the zinc finger associated domain ZAD. *Mol. Biol. Evol.* **24**, 1934–1943. (doi:10.1093/molbev/msm121)

Copley, R. R. 2004 Evolutionary convergence of alternative splicing in ion channels. *Trends Genet.* **20**, 171–176. (doi:10.1016/j.tig.2004.02.001)

Copley, R. R. 2005 The EH1 motif in metazoan transcription factors. *BMC Genom.* **6**, 169. (doi:10.1186/1471-2164-6-169)

Cordier, F., Hartmann, B., Rogowski, M., Affolter, M. & Grzesiek, S. 2006 DNA recognition by the brinker repressor—an extreme case of coupling between binding and folding. *J. Mol. Biol.* **361**, 659–672. (doi:10.1016/j.jmb.2006.06.045)

Davidson, E. H. 2006 *The regulatory genome*. London, UK: Academic Press.

Dehal, P. *et al*. 2002 The draft genome of *Ciona intestinalis*: insights into chordate and vertebrate origins. *Science* **298**, 2157–2167. (doi:10.1126/science.1080049)

Dermitzakis, E. T. & Clark, A. G. 2002 Evolution of transcription factor binding sites in mammalian gene regulatory regions: conservation and turnover. *Mol. Biol. Evol.* **19**, 1114–1121.

Dunn, N. R., Koonce, C. H., Anderson, D. C., Islam, A., Bikoff, E. K. & Robertson, E. J. 2005 Mice exclusively expressing the short isoform of Smad2 develop normally and are viable and fertile. *Genes Dev.* **19**, 152–163. (doi:10.1101/gad.1243205)

Dyson, H. J. & Wright, P. E. 2005 Intrinsically unstructured proteins and their functions. *Nat. Rev. Mol. Cell Biol.* **6**, 197–208. (doi:10.1038/nrm1589)

Emes, R. D., Goodstadt, L., Winter, E. E. & Ponting, C. P. 2003 Comparison of the genomes of human and mouse lays the foundation of genome zoology. *Hum. Mol. Genet.* **12**, 701–709. (doi:10.1093/hmg/ddg078)

Emes, R. D., Beatson, S. A., Ponting, C. P. & Goodstadt, L. 2004*a* Evolution and comparative genomics of odorant- and pheromone-associated genes in rodents. *Genome Res.* **14**, 591–602. (doi:10.1101/gr.1940604)

Emes, R. D., Riley, M. C., Laukaitis, C. M., Goodstadt, L., Karn, R. C. & Ponting, C. P. 2004*b* Comparative evolutionary genomics of androgen-binding protein genes. *Genome Res.* **14**, 1516–1529. (doi:10.1101/gr.2540304)

Fan, W., Huang, X., Chen, C., Gray, J. & Huang, T. 2004 TBX3 and its isoform TBX3+2a are functionally distinctive in inhibition of senescence and are over-expressed in a subset of breast cancer cell lines. *Cancer Res.* **64**, 5132–5139. (doi:10.1158/0008-5472.CAN-04-0615)

Fields, C., Adams, M. D., White, O. & Venter, J. C. 1994 How many genes in the human genome? *Nat. Genet.* **7**, 345–346. (doi:10.1038/ng0794-345)

Finn, R. D. *et al*. 2006 Pfam: clans, web tools and services. *Nucleic Acids Res.* **34**, D247–D251. (doi:10.1093/nar/gkj149)

Fisher, S., Grice, E. A., Vinton, R. M., Bessling, S. L. & McCallion, A. S. 2006 Conservation of RET regulatory function from human to zebrafish without sequence similarity. *Science* **312**, 276–279. (doi:10.1126/science.1124070)

Glazov, E. A., Pheasant, M., McGraw, E. A., Bejerano, G. & Mattick, J. S. 2005 Ultraconserved elements in insect genomes: a highly conserved intronic sequence implicated in the control of homothorax mRNA splicing. *Genome Res.* **15**, 800–808. (doi:10.1101/gr.3545105)

Goodstadt, L. & Ponting, C. P. 2006 Phylogenetic reconstruction of orthology, paralogy, and conserved synteny for dog and human. *PLoS Comput. Biol.* **2**, e133. (doi:10.1371/journal.pcbi.0020133)

Goodstadt, L., Heger, A., Webber, C. & Ponting, C. P. 2007 An analysis of the gene complement of a marsupial, *Monodelphis domestica*: evolution of lineage-specific genes and giant chromosomes. *Genome Res.* **17**, 969–981. (doi:10.1101/gr.6093907)

Grishin, N. V. 2001 Mh1 domain of SMAD is a degraded homing endonuclease. *J. Mol. Biol.* **307**, 31–37. (doi:10.1006/jmbi.2000.4486)

Hahn, M. W. & Wray, G. A. 2002 The G-value paradox. *Evol. Dev.* **4**, 73–75. (doi:10.1046/j.1525-142X.2002.01069.x)

Hillier, L. W., Coulson, A., Murray, J. I., Bao, Z., Sulston, J. E. & Waterston, R. H. 2005 Genomics in *C. elegans*: so many genes, such a little worm. *Genome Res.* **15**, 1651–1660. (doi:10.1101/gr.3729105)

Hittinger, C. T., Stern, D. L. & Carroll, S. B. 2005 Pleiotropic functions of a conserved insect-specific Hox peptide motif. *Development* **132**, 5261–5270. (doi:10.1242/dev.02146)

Hoekstra, H. E. & Coyne, J. A. 2007 The locus of evolution: evo devo and the genetics of adaptation. *Evolution* **61**, 995–1016. (doi:10.1111/j.1558-5646.2007.00105.x)

Huntley, S., Baggott, D. M., Hamilton, A. T., Tran-Gyamfi, M., Yang, S., Kim, J., Gordon, L., Branscomb, E. & Stubbs, L. 2006 A comprehensive catalog of human KRAB-associated zinc finger genes: insights into the evolutionary history of a large family of transcriptional repressors. *Genome Res.* **16**, 669–677. (doi:10.1101/gr.4842106)

In der Rieden, P. M. J., Mainguy, G., Woltering, J. M. & Durston, A. J. 2004 Homeodomain to hexapeptide or PBC-interaction-domain distance: size apparently matters. *Trends Genet.* **20**, 76–79. (doi:10.1016/j.tig.2003.12.001)

Jackson, M. *et al*. 2006 A murine specific expansion of the Rhox cluster involved in embryonic stem cell biology is under natural selection. *BMC Genom.* **7**, 212. (doi:10.1186/1471-2164-7-212)

Kabat, J. L., Barberan-Soler, S., McKenna, P., Clawson, H., Farrer, T. & Zahler, A. M. 2006 Intronic alternative splicing regulators identified by comparative genomics in nematodes. *PLoS Comput. Biol.* **2**, e86. (doi:10.1371/journal.pcbi.0020086)

Kim, E., Magen, A. & Ast, G. 2007 Different levels of alternative splicing among eukaryotes. *Nucleic Acids Res.* **35**, 125–131. (doi:10.1093/nar/gkl924)

Lander, E. S. *et al*. 2001 Initial sequencing and analysis of the human genome. *Nature* **409**, 860–921. (doi:10.1038/35057062)

Lareau, L. F., Inada, M., Green, R. E., Wengrod, J. C. & Brenner, S. E. 2007 Unproductive splicing of SR genes associated with highly conserved and ultraconserved DNA elements. *Nature* **446**, 926–929. (doi:10.1038/nature05676)

LaRonde-LeBlanc, N. A. & Wolberger, C. 2003 Structure of HoxA9 and Pbx1 bound to DNA: Hox hexapeptide and DNA recognition anterior to posterior. *Genes Dev.* **17**, 2060–2072. (doi:10.1101/gad.1103303)

Letunic, I., Copley, R. R., Pils, B., Pinkert, S., Schultz, J. & Bork, P. 2006 SMART 5: domains in the context of genomes and networks. *Nucleic Acids Res.* **34**, D257–D260. (doi:10.1093/nar/gkj079)

Liu, J., Perumal, N. B., Oldfield, C. J., Su, E. W., Uversky, V. N. & Dunker, A. K. 2006 Intrinsic disorder in transcription factors. *Biochemistry* **45**, 6873–6888. (doi:10.1021/bi0602718)

Lohr, U. & Pick, L. 2005 Cofactor-interaction motifs and the cooption of a homeotic Hox protein into the segmentation pathway of *Drosophila melanogaster*. *Curr. Biol.* **15**, 643–649. (doi:10.1016/j.cub.2005.02.048)

Ludwig, M. Z., Bergman, C., Patel, N. H. & Kreitman, M. 2000 Evidence for stabilizing selection in a eukaryotic enhancer element. *Nature* **403**, 564–567. (doi:10.1038/35000615)

Maclean II, J. A., Chen, M. A., Wayne, C. M., Bruce, S. R., Rao, M., Meistrich, M. L., Macleod, C. & Wilkinson, M. F. 2005 Rhox: a new homeobox gene cluster. *Cell* **120**, 369–382. (doi:10.1016/j.cell.2004.12.022)

Marcellini, S. 2006 When Brachyury meets Smad1: the evolution of bilateral symmetry during gastrulation. *Bioessays* **28**, 413–420. (doi:10.1002/bies.20387)

Markstein, M., Markstein, P., Markstein, V. & Levine, M. S. 2002 Genome-wide analysis of clustered dorsal binding sites identifies putative target genes in the *Drosophila* embryo. *Proc. Natl Acad. Sci. USA* **99**, 763–768. (doi:10.1073/pnas.012591199)

Markstein, M., Zinzen, R., Markstein, P., Yee, K. P., Erives, A., Stathopoulos, A. & Levine, M. 2004 A regulatory code for neurogenic gene expression in the *Drosophila* embryo. *Development* **131**, 2387–2394. (doi:10.1242/dev.01124)

McGregor, A. P., Shaw, P. J., Hancock, J. M., Bopp, D., Hediger, M., Wratten, N. S. & Dover, G. A. 2001 Rapid restructuring of bicoid-dependent hunchback promoters within and between dipteran species: implications for molecular coevolution. *Evol. Dev.* **3**, 397–407. (doi:10.1046/j.1525-142X.2001.01043.x)

Minezaki, Y., Homma, K., Kinjo, A. R. & Nishikawa, K. 2006 Human transcription factors contain a high fraction of intrinsically disordered regions essential for transcriptional regulation. *J. Mol. Biol.* **359**, 1137–1149. (doi:10.1016/j.jmb.2006.04.016)

Mistry, N., Harrington, W., Lasda, E., Wagner, E. J. & Garcia-Blanco, M. A. 2003 Of urchins and men: evolution of an alternative splicing unit in fibroblast growth factor receptor genes. *RNA* **9**, 209–217. (doi:10.1261/rna.2470903)

Modrek, B., Resch, A., Grasso, C. & Lee, C. 2001 Genome-wide detection of alternative splicing in expressed sequences of human genes. *Nucleic Acids Res.* **29**, 2850–2859. (doi:10.1093/nar/29.13.2850)

Moses, A. M., Pollard, D. A., Nix, D. A., Iyer, V. N., Li, X. Y., Biggin, M. D. & Eisen, M. B. 2006 Large-scale turnover of functional transcription factor binding sites in *Drosophila*. *PLoS Comput. Biol* **2**, e130. (doi:10.1371/journal.pcbi.0020130)

Murzin, A. G., Brenner, S. E., Hubbard, T. & Chothia, C. 1995 SCOP: a structural classification of proteins database for the investigation of sequences and structures. *J. Mol. Biol.* **247**, 536–540. (doi:10.1006/jmbi.1995.0159)

Nature Genetics Editorial 2000 The nature of the number. *Nat. Genet.* **25**, 127–128. (doi:10.1038/75946)

Neduva, V. & Russell, R. B. 2005 Linear motifs: evolutionary interaction switches. *FEBS Lett.* **579**, 3342–3345. (doi:10.1016/j.febslet.2005.04.005)

Neduva, V., Linding, R., Su-Angrand, I., Stark, A., de Masi, F., Gibson, T. J., Lewis, J., Serrano, L. & Russell, R. B. 2005 Systematic discovery of new recognition peptides mediating protein interaction networks. *PLoS Biol.* **3**, e405. (doi:10.1371/journal.pbio.0030405)

Ni, J. Z. *et al*. 2007 Ultraconserved elements are associated with homeostatic control of splicing regulators by alternative splicing and nonsense-mediated decay. *Genes Dev.* **21**, 708–718. (doi:10.1101/gad.1525507)

Nichols, S. A., Dirks, W., Pearse, J. S. & King, N. 2006 Early evolution of animal cell signaling and adhesion genes. *Proc. Natl Acad. Sci. USA* **103**, 12 451–12 456. (doi:10.1073/pnas.0604065103)

Noro, B., Culi, J., McKay, D. J., Zhang, W. & Mann, R. S. 2006 Distinct functions of homeodomain-containing and homeodomain-less isoforms encoded by homothorax. *Genes Dev.* **20**, 1636–1650. (doi:10.1101/gad.1412606)

Panne, D., Maniatis, T. & Harrison, S. C. 2007 An atomic model of the interferon-beta enhanceosome. *Cell* **129**, 1111–1123. (doi:10.1016/j.cell.2007.05.019)

Pennacchio, L. A. *et al*. 2006 *In vivo* enhancer analysis of human conserved non-coding sequences. *Nature* **444**, 499–502. (doi:10.1038/nature05295)

Pennisi, E. 2007 Genetics. Working the (gene count) numbers: finally, a firm answer? *Science* **316**, 1113. (doi:10.1126/science.316.5828.1113a)

Pires-daSilva, A. & Sommer, R. J. 2003 The evolution of signalling pathways in animal development. *Nat. Rev. Genet.* **4**, 39–49. (doi:10.1038/nrg977)

Poole, R. J. & Hobert, O. 2006 Early embryonic programming of neuronal left/right asymmetry in *C. elegans*. *Curr. Biol.* **16**, 2279–2292. (doi:10.1016/j.cub.2006.09.041)

Putnam, N. H. *et al*. 2007 Sea anemone genome reveals ancestral eumetazoan gene repertoire and genomic organization. *Science* **317**, 86–94. (doi:10.1126/science.1139158)

Rebeiz, M., Stone, T. & Posakony, J. W. 2005 An ancient transcriptional regulatory linkage. *Dev. Biol.* **281**, 299–308. (doi:10.1016/j.ydbio.2005.03.004)

Robinson-Rechavi, M., Maina, C. V., Gissendanner, C. R., Laudet, V. & Sluder, A. 2005 Explosive lineage-specific expansion of the orphan nuclear receptor HNF4 in nematodes. *J. Mol. Evol.* **60**, 577–586. (doi:10.1007/s00239-004-0175-8)

Romano, L. A. & Wray, G. A. 2003 Conservation of Endo16 expression in sea urchins despite evolutionary divergence in both *cis* and *trans*-acting components of transcriptional regulation. *Development* **130**, 4187–4199. (doi:10.1242/dev.00611)

Romero, P. R. *et al*. 2006 Alternative splicing in concert with protein intrinsic disorder enables increased functional diversity in multicellular organisms. *Proc. Natl Acad. Sci. USA* **103**, 8390–8395. (doi:10.1073/pnas.0507916103)

Ruvinsky, I. & Ruvkun, G. 2003 Functional tests of enhancer conservation between distantly related species. *Development* **130**, 5133–5142. (doi:10.1242/dev.00711)

Sandelin, A., Bailey, P., Bruce, S., Engstrom, P. G., Klos, J. M., Wasserman, W. W., Ericson, J. & Lenhard, B. 2004 Arrays of ultraconserved non-coding regions span the loci of key developmental genes in vertebrate genomes. *BMC Genom.* **5**, 99. (doi:10.1186/1471-2164-5-99)

Sapkota, G., Alarcon, C., Spagnoli, F. M., Brivanlou, A. H. & Massague, J. 2007 Balancing BMP signaling through integrated inputs into the Smad1 linker. *Mol. Cell* **25**, 441–454. (doi:10.1016/j.molcel.2007.01.006)

Sempere, L. F., Cole, C. N., McPeek, M. A. & Peterson, K. J. 2006 The phylogenetic distribution of metazoan microRNAs: insights into evolutionary complexity and constraint. *J. Exp. Zool. B: Mol. Dev. Evol.* **306**, 575–588. (doi:10.1002/jez.b.21118)

Shaw, P. J., Wratten, N. S., McGregor, A. P. & Dover, G. A. 2002 Coevolution in bicoid-dependent promoters and the inception of regulatory incompatibilities among species of higher Diptera. *Evol. Dev.* **4**, 265–277. (doi:10.1046/j.1525-142X.2002.02016.x)

Simpson, P. 2007 The stars and stripes of animal bodies: evolution of regulatory elements mediating pigment and bristle patterns in *Drosophila*. *Trends Genet.* **23**, 350–358. (doi:10.1016/j.tig.2007.04.006)

Soler-Lopez, M., Petosa, C., Fukuzawa, M., Ravelli, R., Williams, J. G. & Muller, C. W. 2004 Structure of an activated Dictyostelium STAT in its DNA-unbound form.

Mol. Cell **13**, 791–804. (doi:10.1016/S1097-2765(04)00130-3)

Sorek, R. & Ast, G. 2003 Intronic sequences flanking alternatively spliced exons are conserved between human and mouse. *Genome Res.* **13**, 1631–1637. (doi:10.1101/gr.1208803)

Stein, L. D. *et al.* 2003 The genome sequence of *Caenorhabditis briggsae*: a platform for comparative genomics. *PLoS Biol.* **1**, e45. (doi:10.1371/journal.pbio.0000045)

Taft, R. J., Pheasant, M. & Mattick, J. S. 2007 The relationship between non-protein-coding DNA and eukaryotic complexity. *Bioessays* **29**, 288–299. (doi:10.1002/bies.20544)

Tahirov, T. H. *et al.* 2001 Structural analyses of DNA recognition by the AML1/Runx-1 Runt domain and its allosteric control by CBFbeta. *Cell* **104**, 755–767. (doi:10.1016/S0092-8674(01)00271-9)

Taneri, B., Snyder, B., Novoradovsky, A. & Gaasterland, T. 2004 Alternative splicing of mouse transcription factors affects their DNA-binding domain architecture and is tissue specific. *Genome Biol.* **5**, R75. (doi:10.1186/gb-2004-5-10-r75)

Tour, E., Hittinger, C. T. & McGinnis, W. 2005 Evolutionarily conserved domains required for activation and repression functions of the *Drosophila* Hox protein Ultrabithorax. *Development* **132**, 5271–5281. (doi:10.1242/dev.02138)

Vavouri, T., Walter, K., Gilks, W. R., Lehner, B. & Elgar, G. 2007 Parallel evolution of conserved non-coding elements that target a common set of developmental regulatory genes from worms to humans. *Genome Biol.* **8**, R15. (doi:10.1186/gb-2007-8-2-r15)

Venter, J. C. *et al.* 2001 The sequence of the human genome. *Science* **291**, 1304–1351. (doi:10.1126/science.1058040)

Woolfe, A. *et al.* 2005 Highly conserved non-coding sequences are associated with vertebrate development. *PLoS Biol.* **3**, e7. (doi:10.1371/journal.pbio.0030007)

Wray, G. A. 2007 The evolutionary significance of *cis*-regulatory mutations. *Nat. Rev. Genet.* **8**, 206–216. (doi:10.1038/nrg2063)

Wray, G. A., Hahn, M. W., Abouheif, E., Balhoff, J. P., Pizer, M., Rockman, M. V. & Romano, L. A. 2003 The evolution of transcriptional regulation in eukaryotes. *Mol. Biol. Evol.* **20**, 1377–1419. (doi:10.1093/molbev/msg140)

Yeo, G. W., Van Nostrand, E., Holste, D., Poggio, T. & Burge, C. B. 2005 Identification and analysis of alternative splicing events conserved in human and mouse. *Proc. Natl Acad. Sci. USA* **102**, 2850–2855. (doi:10.1073/pnas.0409742102)

Yoder, J. H. & Carroll, S. B. 2006 The evolution of abdominal reduction and the recent origin of distinct abdominal-B transcript classes in Diptera. *Evol. Dev.* **8**, 241–251. (doi:10.1111/j.1525-142X.2006.00095.x)