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Comparative Genomic Analysis as a Tool for Biological Discovery

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Biology is a discipline rooted in comparisons. Comparative physiology has assembled a detailed catalogue of the biological similarities and differences between species, revealing insights into how life has adapted to fill a wide-range of environmental niches. For example, the oxygen and carbon dioxide carrying capacity of vertebrate has evolved to provide strong advantages for species respiring at sea level, at high elevation or within water. Comparative- anatomy, -biochemistry, -pharmacology, -immunology and –cell biology have provided the fundamental paradigms from which each discipline has grown.

Genomics is the most recent branch of biology to employ comparison-based strategies. At the foundation of the evolutionary relationship of all life on earth is conserved genetic information in the form of DNA sequence, which is assumed to underlie homologous functional and anatomical similarities between species. Technological progress in DNA cloning and sequencing has resulted in the generation of a large dataset of genomic sequence information. In the past two years, draft genome sequence has become available for six vertebrates: human, mouse, rat, zebrafish and two pufferfish (*Fugu rubripes* and *Tetraodon nigroviridis*) (Lander *et al.*, 2001; Venter *et al.*, 2001; Aparicio *et al.*, 2002; Waterston *et al.*, 2002). The sudden wealth of sequence data has allowed whole genome alignments to compare and contrast the evolution and content of vertebrate genomes. Such comparative strategies have identified pockets of DNA sequences conserved over evolutionary time, and such evolutionary conservation has been a powerful guide in sorting functional from non-functional DNA (Duret & Bucher, 1997; Hardison *et al.*, 1997; Hardison, 2000; Loots *et al.*, 2000; Pennacchio & Rubin, 2001; Gottgens *et al.*, 2002). Accordingly, this review focuses on the biological insights derived from comparative sequence-based studies and their increasing utility as the amount of genome

sequence data increases. Details on various computational tools can be found in several recent reviews (Pennacchio & Rubin, 2001; Frazer *et al.*, 2003; Pennacchio & Rubin, 2003b; Ureta-Vidal *et al.*, 2003).

The Power of Varying Evolutionary Distance in Comparative Genomics

The utility of comparative sequence analysis is based on the hypothesis that important biological sequences are conserved between species due to functional constraints. To derive insights into biology through comparative sequence analysis the first challenge is the choice of species to compare. The ideal pair-wise comparison is between two organisms that share a common physiology or biology. For example, human/mouse sequence comparisons have been useful in mapping the regulation of genes involved in lipid metabolism that are shared between the two organisms, but conversely such comparisons are not useful for understanding the regulation of a lipid gene found only in the primate lineage. These larger concerns must also be balanced with the amount of actual sequence conservation between two organisms, too much conservation and the functional regions are obscured, too little conservation and they are hidden. Thus a balance of biological relevance and sequence analysis provides the best opportunity for the identification of conserved sequences that appear to be evolving under evolutionary constraints in a background of sequence that has randomly diverged due to genetic drift.

In recent years, the availability of sequence from numerous species has allowed multiple species comparisons to aid in calibrating the ideal evolutionary distance required for the optimal identification of functionally conserved sequences (Koop & Hood, 1994; Hood *et al.*, 1995; Dubchak *et al.*, 2000; Pennacchio & Rubin, 2001; Gottgens *et al.*, 2002). In

this review we adopt a human-centric focus of comparative genomics, describing strategies where sequence-based analyses alone have been used to better understand functional sequences in the human genome. Examples are provided of the most commonly used vertebrate genomes in cross-species sequence comparisons (Figure 1), highlighting the uniqueness, usefulness and limitations of each.

Human-Mouse Sequence Comparisons

The evolutionary distance between humans and mice place these species at a strategic position for the identification of shared functionally conserved sequences. It has been estimated that the rate of divergence in independently evolving vertebrate genomes is on average 0.1-0.5% per million years, supporting that the ~80 million years separating humans and mice from their last common ancestor is sufficient for functionally important sequences to be identified (Tautz, 2000). A number of recent studies have reported the identification of functional sequences solely through the use of human-mouse genomic comparisons, thereby further validating this assumption (Loots *et al.*, 2000; Pennacchio *et al.*, 2001; Gottgens *et al.*, 2002; Kappen & Yaworsky, 2003). The most standard applications of human/mouse comparative sequence analyses involves 1) the annotation of previously undefined genes, 2) the identification of large (80-1000 bps) functional gene-regulatory elements, and 3) the detailed characterization of transcription factor binding sites ("phylogenetic footprints") present in larger conserved non-coding regions.

1- Identification of new genes

The first application of human-mouse comparative genomics relates to the discovery of new genes within the human and mouse genomes, which have previously been invisible to extensive computational and experimental investigation. Since coding sequences of active genes are commonly under strong negative selection, human-mouse sequence comparisons are expected to unveil sequences corresponding to previously unidentified genes, thus expanding the complete gene catalogue of each organism. The discovery of the apolipoprotein A5 gene (*APOA5*) exemplifies this principle. Solely through the use of human-mouse genomic sequence comparisons, this evolutionary paralog of the neighboring *APOA4* gene was identified based on its high degree of sequence conservation within a previously well-studied cluster of apolipoproteins (Pennacchio *et al.*, 2001). Transcripts from this corresponding interval were identified in human and mouse liver tissue, serving as evidence that these conserved sequences correspond to a previously missed gene. Further studies in transgenic and knockout mice revealed that the newly described *APOA5* gene is a pivotal determinant of plasma triglyceride levels (Pennacchio *et al.*, 2001). In addition, these findings were extended to human physiology when strong genetic associations between common *APOA5* polymorphisms and plasma triglyceride levels were uncovered in a wide range of studies (reviewed in (Pennacchio & Rubin, 2003a)). Similar strategies will be useful in identifying un-annotated genes that are still predicted to exist in the human and mouse genomes.

2 - Identification of gene regulatory sequences

While it is intuitive that comparative sequence analysis is suitable to identify exons based on conservation, its ability to uncover conserved gene regulatory sequences is less

obvious due to the small size of transcription factor binding sites (~6-12 bps in size). Nevertheless, the architecture of the majority of characterized enhancers in metazoan genomes is thought to be determined by a combination of multiple transcription-factor binding sites, arranged in a modular fashion within large clusters. Thus, the size of these enhancer elements is expected to be similar to many exons. An early study of human-mouse comparative sequence analysis as a starting point to identify gene regulatory elements was performed on a human interleukin gene cluster, which has long been known to harbor genes involved in several human inflammatory conditions (Noguchi *et al.*, 1997; Rioux *et al.*, 2001). In this work, human-mouse comparisons revealed a highly conserved 401-bp noncoding sequence within a genomic interval containing the interleukin -4, -5 and -13 genes (Loots *et al.*, 2000). Subsequent deletion of this conserved noncoding sequence from mice revealed inappropriate expression of all three interleukins upon T_H2 cytokine stimulation (Mohrs *et al.*, 2001), thus demonstrating that the 401-bp conserved element corresponds to a regulatory element able to coordinately modulate the expression of three interleukin genes spread over 120 kb of sequence. This coordinated expression of interleukins had been previously proposed, but several studies using traditional approaches failed to uncover the sequence so clearly revealed by a comparative approach (Noguchi *et al.*, 1997; Lacy *et al.*, 2000).

Human-mouse sequence comparisons are also expected to represent a powerful piece of the puzzle in the decoding of gene-regulatory sequence. A paucity of published studies reporting the identification of functional gene regulatory sequences through traditional approaches highlight the difficulty in defining functional noncoding sequences. With the recent availability of large amounts of human-mouse genomic sequence, cross-species

comparisons are poised to dramatically increase our ability to decipher noncoding DNA. Nevertheless, a handful of characterized enhancers - originally identified through laborious experimental strategies - have been retrospectively shown to be highly conserved between humans and mice. Several studies using standard enhancer-trapping strategies identified and characterized three regulatory sequences within a segment 1.5 to 3.0 kb upstream of the human pancreatic duodenal homeobox 1 (*PDX-1*) gene promoter (Sharma *et al.*, 1996; Ben-Shushan *et al.*, 2001). As shown in figure 2, both exons of the *PDX-1* gene as well as several noncoding sequences are well conserved between humans and mice in this interval. Inspection of the sequence upstream of *PDX-1* shows three distinct segments of sequence conservation located approximately 1.6 to 2.8 kb upstream of the promoter, corresponding to the three enhancers previously shown to regulate *PDX-1* expression. These sequences, easily highlighted by direct genomic comparisons, would likely have been prioritized for characterization of biological function based solely on a comparative strategy. Similar strategies will likely identify many human gene regulatory elements in the genome.

3- Identification of Transcription Factor Binding Sites through "Phylogenetic Footprinting"

Human-mouse sequence conservation has also proved a useful guide in the detailed characterization of regulatory elements identified through cross-species sequence comparisons. For instance, an enhancer responsible for the expression of nestin in the ventral midbrain neuro-epithelium of mice was recently identified through the use of human/mouse/rat genomic comparisons (Kappen & Yaworsky, 2003). To deduce the

critical transcription-factor binding sites responsible for the activity of this enhancer, further analysis by "phylogenetic footprinting" of conserved sequence coupled with reporter gene assays were employed. "Phylogenetic footprinting" utilizes multi-species sequence alignments to identify highly conserved motifs at a fine-scale (6-12bps) comparable to the size of transcription factor binding sites (Gumucio *et al.*, 1996). Following the identification of such "footprints" in the nestin enhancer, nucleotide substitutions were introduced into two sites of a transcription factor-binding site (RXR- β) that was a candidate for mediating the enhancer activity. Transgenic mice harboring these mutations lost the tissue-specific gene expression compared to the normal version of these binding sites indicating that "phylogenetic footprinting" of the enhancer had identified transcription factor binding sites of biological importance (Kappen & Yaworsky, 2003). Thus in addition to the identification of enhancer elements, comparative genomics is useful for the detailed characterization of their composition.

Though a wealth of important examples may reinforce the notion that humans and mice occupy a privileged position for cross-species sequence comparisons, they alone cannot capture all biologically active sequences. First, it has been well established that the degree of sequence conservation is heterogeneous among different genomic segments in human and mouse. For instance, the T-cell receptor locus has been shown to be extremely conserved in human-mouse (Koop & Hood, 1994), while the alpha globin locus has been found to be highly divergent (Hardison *et al.*, 1991). Such intra-species variation is due to wide-ranging differences in the human-mouse nucleotide substitution rates across the genome. The result is a set of genomic regions with vast amounts of conservation (though likely not functional), and a set lacking significant conservation

(though still containing functional elements). Such an observation carries significant implications for cross-species sequence comparisons since this strategy assumes that natural selection has constrained functional sequences to evolve at slower rates than non-functional sequences. In practice, human-mouse comparisons are not always feasible for deriving biological insights for a given genomic region.

To study regions of the human genome where human-mouse sequence comparisons are not ideal, examination of species occupying different evolutionary distances may be useful. In regions that are too well conserved between human-mouse, the comparison of human to more distantly related species is warranted (i.e. birds, reptiles, amphibians), while in regions that are poorly conserved between human-mouse, the comparison of human to closer species can be beneficial (i.e. primates, dogs, rabbits). In the remainder of this review, we will describe the utility of human genomic comparisons to species other than mouse.

Human-Chicken Sequence Comparisons

The shared ancestor that gave rise to birds and mammals existed approximately 300 million years ago during the vertebrate radiation (Kumar & Hedges, 1998), placing the distance between humans and chickens at approximately 3-4 times that of humans and mice (Figure 1). A deeper phylogenetic relationship suggests that the majority of neutrally evolving sequences in humans and birds will have diverged significantly more than those between humans and mice. In general, conserved DNA between humans and birds is more likely to be functional than that found between humans and mice. While no

entire avian genome sequence is currently available, small genomic fragments of chicken DNA have been sequenced for comparative studies.

As an example, the identification and characterization of a human cardiac-specific enhancer regulating the homeobox gene *Nkx2-5* was aided by the addition of orthologous chicken sequence. Initial examination of the region 10 kb upstream of *Nkx2-5* between humans and mice revealed five conserved noncoding sequences, but the addition of the orthologous chicken sequence revealed that only one of these five was also conserved in chicken. Functional studies in transgenic mice confirmed that this segment corresponds to a cardiac-specific enhancer regulating *NKx2-5* expression (Lien *et al.*, 2002). Further dissection of this enhancer through "phylogenetic footprinting" revealed the precise transcription factor-binding sites responsible for the enhancer activity, aided by having the chicken genomic sequence. While human-mouse enhancer sequence comparisons revealed between 90 -100% identity throughout the segment, making the identification of conserved "footprints" difficult, human-mouse-chicken enhancer sequence comparison decreased the overall conservation in the region to 70% and revealed four *Smad* binding sites were conserved in all three species. A combination of mouse transgenics and mutagenesis later confirmed that one of the conserved *Smad* sites mediates the enhancer activation of *Nkx2-5* in the developing heart (Lien *et al.*, 2002).

An interesting observation from these data is that genome sequences obtained from organisms with little or no use as model organisms for experimental biology represent extremely important resources for annotating the human genome. This underscores the importance of prioritizing the choices for sequencing further vertebrate genomes based

not simply on a hierarchical list of experimentally suitable models, but also on a composite of factors that take into account the potential uses of the data generated for applications such as comparative genomics. Indeed, while the chicken, honeybee and chimpanzee are not standard experimental models, their genomes have been prioritized for the next round of DNA sequencing (Boguski, 2002).

Human-Fish Sequence Comparisons

Human-fish comparisons also provide a useful evolutionary position for comparative sequence-based discovery. Several species of fish have been fully sequenced, which include working drafts for zebrafish, and the two pufferfish; *Fugu rubripes* and *Tetraodon nigroviridis* (Aparicio *et al.*, 2002). The phylogenetic relationship between fish and humans dates back 400-450 million years, making fish the most distant vertebrates with available genomic sequence for comparison with humans (Figure 1). Although this large evolutionary distance implies that only a fraction of the functional sequences in the human genome are still shared, comparison has revealed that a significant fraction of known human genes are also conserved in fish. Importantly, the annotation of conserved sequences between the human and *Fugu rubripes* genomes led to the rapid identification of over 1,000 previously unidentified human genes (Abrahams *et al.*, 2002; Aparicio *et al.*, 2002). While the majority of conserved orthologous sequences between human and fugu represent coding sequences, thousands of conserved sequences that do not appear to correspond to genes are also present. This suggests that human-fugu genomic comparisons may result in the discovery of functionally important noncoding sequences in the human genome.

One of the most attractive features of fugu for its use in cross-species sequence comparisons is the compact size of its genome, totaling a mere 365 million bp (one-eighth the size of the human genome) (Brenner *et al.*, 1993). This compactness predicts that regulatory sequences shared between humans and fugu will be found much closer to a given fugu gene than its human ortholog, thus human-fugu comparisons may identify distant regulatory elements in the human genome (Gilligan *et al.*, 2002). A recent comparison of a 3.7-million bp sequence from human with fugu identified 195 kb of sequence with orthology revealing several genes in the region shared between the two species (Bagheri-Fam *et al.*, 2001). Moreover, eight conserved sequences which were not predicted to be exons, were identified within 750 kb of the human *SOX9* transcription factor gene. In the fugu genome these conserved sequences are located within less than 80 kb of *SOX9*, suggesting that these may represent distant sequences that regulate *SOX9* expression (Bagheri-Fam *et al.*, 2001). Thus, the use of *Fugu rubripes* sequences in genomic comparisons may be a powerful tool for the identification of both local and distant regulatory elements.

Inter-Primate Sequence Comparisons

Finally, comparison of the human sequence to that of other primate species is a likely strategy to identify functional regions of the human genome. The overall strategy previously described for cross-species sequence comparisons is based on using species of relatively distant phylogenetic positions to maximize the identification of functionally conserved sequences in the human genome. However, this strategy is limited in that it

does not allow studies aimed at identifying primate-specific genes or regulatory sequences. For instance, the comparison of the human and mouse genomes identified ~1% of mouse genes without a human ortholog (Waterston *et al.*, 2002). In addition, this estimate does not take into account the numerous examples where tandem duplications lead to the formation and expansion of gene families in one species but not the other. To this end, only 80% of human/mouse genes have a 1:1 orthologous relationship (Waterston *et al.*, 2002). Therefore, there is a need to develop strategies to characterize the catalog of the 20% of genes and regulatory elements that do not have a true ortholog in both humans and mice. For these studies, comparing human sequences to that of closer evolutionary species, such as primates, may prove essential. However, the use of primate sequences for cross-species sequence comparisons poses a paradox: while primates are likely to share most genes present in the human genome, their close phylogenetic relationship results in high levels of sequence identity between orthologous sequences. For example, humans, chimpanzees and gorillas shared a common ancestor approximately 6.0 – 8.0 million years ago and their average rate of sequence conservation is 98 – 99% even in non-coding intervals (Hacia, 2001).

Recently, a strategy named “phylogenetic shadowing” was introduced to overcome the excessive sequence identity shared by primates, making their use in cross-species sequence comparisons possible (Boffelli *et al.*, 2003). The foundation of this approach is to analyze orthologous sequence from numerous primate species to increase the evolutionary distance of the sequence comparisons. Rather than performing only pairwise comparisons between human-mouse, human-chicken, or human-fugu, “phylogenetic shadowing” compares a dozen or more different primate species. The summation of

these primate comparisons robustly identifies regions of increased variation and "shadows" representing conserved segments (Figure 3A).

As a proof of principle, "phylogenetic shadowing" proved successful for the identification of both exons as well as putative gene regulatory elements (Boffelli *et al.*, 2003). In this study, 13-17 primate sequences of several orthologous genomic segments were generated and compared. For a single exon from four independent genes, highly conserved "shadows" coincided strongly with these functionally important protein-encoding regions (Figure 3B for one example). In addition, analysis of the human apolipoprotein (a) gene (*apo(a)*) revealed highly conserved intervals embedded within the upstream promoter region, and functional studies of these "phylogenetic shadows" compared to more variable flanking DNA supported their role in regulating *apo(a)* expression (Boffelli *et al.*, 2003). The success of this approach suggests a genome-wide comparison of a handful of primate species will aid in the identification of both human exons and gene regulatory elements.

Conclusions and Future Perspective

Comparative genomics is a relatively new field that complements a long history of comparison-based disciplines in biology. The recent development of a large dataset of vertebrate genomic sequences has aided in global gene predictions as well as in the identification of sequences important in gene regulation. In addition, vertebrate comparative sequence analysis is poised to contribute to the exploration of the genetic bases for differences and similarities among species. In combination with areas of study

such as comparative-physiology or comparative-biochemistry, we are likely to finally understand the genetic explanation for how species have adapted to perform their shared or unique biological functions.

The number of cross-species sequence comparisons will undoubtedly increase in use as additional genomes are sequenced. While we currently have access to a handful of vertebrate genome sequences and our tools for dealing with these data sets are rapidly improving, the computational challenges ahead are formidable. Current efforts have focused primarily on pair-wise comparisons to annotate and explore a single species of interest (such as humans), but future methods will require the simultaneous analysis of sequence data from numerous species in the form of multiple alignments in order to catalog the evolutionary extent of sequence conservation and divergence. In addition, the area of high-throughput experimental biology is a quickly evolving field with vast opportunities to exploit comparative sequence data.

For the biologist, the application of cross-species sequence analyses requires flexibility. It should be emphasized that no single pair-wise comparison is sufficient to capture all biologically functional sequences based on conservation. Thus, the primary decision in the process of designing a comparative genomic-based study is the biological question under investigation and which two (or more) species are most appropriate for comparison. While there is no clear way to predict which repertoire of species is ideally suited for each cross-species comparison, the analysis of aligned human-mouse orthologous sequences provides an initial starting point for most biological studies. However, for example, if the study aims to identify regulatory elements of a primate-

specific gene, it will not be useful to compare human-mouse or other lower vertebrates. In contrast, the study of basic vertebrate biological processes may be aided by distant species sequence comparisons (human-bird, human-amphibian, human-fish, etc). Therefore, the biologist must make logical predictions about which species to compare and should readily adopt additional species as warranted based on their initial comparative analysis. The five currently available vertebrate genome sequences are immediate resources for the community and additional vertebrate genomes are in the pipeline.

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Figure Legends:

Figure 1. Phylogenetic tree of a subset of vertebrate species. The approximate divergence time of each of the eight vertebrate species whose genome sequences are currently available are represented (not drawn to scale). Diploid genome sizes are indicated in million base-pairs.

Figure 2. Human versus mouse *PDX-1* genomic sequence comparison. VISTA Genome Browser output in which human is the reference sequence with percent similarity to mouse plotted on the vertical axis (<http://pipeline.lbl.gov>). Vertical arrows correspond to highly conserved noncoding sequences that coincide with previously defined gene regulatory elements. Gene orientation (arrows) and exon location (rectangles) are provided above each panel.

Figure 3. Phylogenetic shadowing of primate species. **A)** The alignment and comparison of sequences from multiple species sequences reveal which sequences have been conserved in most species, making them likely candidates for being functionally relevant. **B)** A sequence variation plot of numerous aligned primate sequences flanking an exon of the LXR- α gene. On the x-axis 1200 bps of sequence is depicted, while on the y-axis the percent variation is plotted. Note the lack of sequence diversity ("phylogenetic shadow") corresponds closely with the functional exon interval.

References:

Abrahams, B. S., Mak, G. M., Berry, M. L., Palmquist, D. L., Saionz, J. R., Tay, A., Tan, Y. H., Brenner, S., Simpson, E. M. & Venkatesh, B. (2002). Novel vertebrate genes and putative regulatory elements identified at kidney disease and NR2E1/fierce loci.

Genomics **80**, 45-53.

Aparicio, S., Chapman, J., Stupka, E., Putnam, N., Chia, J. M., Dehal, P., Christoffels, A., Rash, S., Hoon, S., Smit, A., Gelpke, M. D., Roach, J., Oh, T., Ho, I. Y., Wong, M., Detter, C., Verhoef, F., Predki, P., Tay, A., Lucas, S., Richardson, P., Smith, S. F., Clark, M. S., Edwards, Y. J., Doggett, N., Zharkikh, A., Tavtigian, S. V., Pruss, D., Barnstead, M., Evans, C., Baden, H., Powell, J., Glusman, G., Rowen, L., Hood, L., Tan, Y. H., Elgar, G., Hawkins, T., Venkatesh, B., Rokhsar, D. & Brenner, S. (2002). Whole-genome shotgun assembly and analysis of the genome of *Fugu rubripes*. *Science* **297**, 1301-1310.

Bagheri-Fam, S., Ferraz, C., Demaille, J., Scherer, G. & Pfeifer, D. (2001). Comparative genomics of the SOX9 region in human and *Fugu rubripes*: conservation of short regulatory sequence elements within large intergenic regions. *Genomics* **78**, 73-82.

- Ben-Shushan, E., Marshak, S., Shoshkes, M., Cerasi, E. & Melloul, D. (2001). A pancreatic beta -cell-specific enhancer in the human PDX-1 gene is regulated by hepatocyte nuclear factor 3beta (HNF-3beta), HNF-1alpha, and SPs transcription factors. *J Biol Chem* **276**, 17533-17540.
- Boffelli, D., McAuliffe, J., Ovcharenko, D., Lewis, K. D., Ovcharenko, I., Pachter, L. & Rubin, E. M. (2003). Phylogenetic shadowing of primate sequences to find functional regions of the human genome. *Science* **299**, 1391-1394.
- Boguski, M. S. (2002). Comparative genomics: the mouse that roared. *Nature* **420**, 515-516.
- Brenner, S., Elgar, G., Sandford, R., Macrae, A., Venkatesh, B. & Aparicio, S. (1993). Characterization of the pufferfish (Fugu) genome as a compact model vertebrate genome. *Nature* **366**, 265-268.
- Dubchak, I., Brudno, M., Loots, G. G., Pachter, L., Mayor, C., Rubin, E. M. & Frazer, K. A. (2000). Active conservation of noncoding sequences revealed by three-way species comparisons. *Genome Res* **10**, 1304-1306.
- Duret, L. & Bucher, P. (1997). Searching for regulatory elements in human noncoding sequences. *Curr Opin Struct Biol* **7**, 399-406.

- Frazer, K. A., Elnitski, L., Church, D. M., Dubchak, I. & Hardison, R. C. (2003). Cross-species sequence comparisons: a review of methods and available resources. *Genome Res* **13**, 1-12.
- Gilligan, P., Brenner, S. & Venkatesh, B. (2002). Fugu and human sequence comparison identifies novel human genes and conserved non-coding sequences. *Gene* **294**, 35.
- Gottgens, B., Barton, L. M., Chapman, M. A., Sinclair, A. M., Knudsen, B., Grafham, D., Gilbert, J. G., Rogers, J., Bentley, D. R. & Green, A. R. (2002). Transcriptional regulation of the stem cell leukemia gene (SCL)--comparative analysis of five vertebrate SCL loci. *Genome Res* **12**, 749-759.
- Gumucio, D. L., Shelton, D. A., Zhu, W., Millinoff, D., Gray, T., Bock, J. H., Slightom, J. L. & Goodman, M. (1996). Evolutionary strategies for the elucidation of cis and trans factors that regulate the developmental switching programs of the beta-like globin genes. *Mol Phylogenet Evol* **5**, 18-32.
- Hacia, J. G. (2001). Genome of the apes. *Trends Genet* **17**, 637-645.
- Hardison, R., Krane, D., Vandenberg, D., Cheng, J. F., Mansberger, J., Taddie, J., Schwartz, S., Huang, X. Q. & Miller, W. (1991). Sequence and comparative analysis of the rabbit alpha-like globin gene cluster reveals a rapid mode of evolution in a G + C-rich region of mammalian genomes. *J Mol Biol* **222**, 233-249.

Hardison, R. C. (2000). Conserved noncoding sequences are reliable guides to regulatory elements [In Process Citation]. *Trends Genet* **16**, 369-372.

Hardison, R. C., Oeltjen, J. & Miller, W. (1997). Long human-mouse sequence alignments reveal novel regulatory elements: a reason to sequence the mouse genome. *Genome Res* **7**, 959-966.

Hood, L., Rowen, L. & Koop, B. F. (1995). Human and mouse T-cell receptor loci: genomics, evolution, diversity, and serendipity. *Ann N Y Acad Sci* **758**, 390-412.

Kappen, C. & Yaworsky, P. J. (2003). Mutation of a putative nuclear receptor binding site abolishes activity of the nestin midbrain enhancer. *Biochim Biophys Acta* **1625**, 109-115.

Koop, B. F. & Hood, L. (1994). Striking sequence similarity over almost 100 kilobases of human and mouse T-cell receptor DNA. *Nat Genet* **7**, 48-53.

Kumar, S. & Hedges, S. B. (1998). A molecular timescale for vertebrate evolution. *Nature* **392**, 917-920.

Lacy, D. A., Wang, Z. E., Symula, D. J., McArthur, C. J., Rubin, E. M., Frazer, K. A. & Locksley, R. M. (2000). Faithful expression of the human 5q31 cytokine cluster in transgenic mice. *J Immunol* **164**, 4569-4574.

Lander, E. S., Linton, L. M., Birren, B., Nusbaum, C., Zody, M. C., Baldwin, J., Devon, K., Dewar, K., Doyle, M., FitzHugh, W., Funke, R., Gage, D., Harris, K., Heaford, A., Howland, J., Kann, L., Lehoczky, J., LeVine, R., McEwan, P., McKernan, K., Meldrim, J., Mesirov, J. P., Miranda, C., Morris, W., Naylor, J., Raymond, C., Rosetti, M., Santos, R., Sheridan, A., Sougnez, C., Stange-Thomann, N., Stojanovic, N., Subramanian, A., Wyman, D., Rogers, J., Sulston, J., Ainscough, R., Beck, S., Bentley, D., Burton, J., Clee, C., Carter, N., Coulson, A., Deadman, R., Deloukas, P., Dunham, A., Dunham, I., Durbin, R., French, L., Grafham, D., Gregory, S., Hubbard, T., Humphray, S., Hunt, A., Jones, M., Lloyd, C., McMurray, A., Matthews, L., Mercer, S., Milne, S., Mullikin, J. C., Mungall, A., Plumb, R., Ross, M., Shownkeen, R., Sims, S., Waterston, R. H., Wilson, R. K., Hillier, L. W., McPherson, J. D., Marra, M. A., Mardis, E. R., Fulton, L. A., Chinwalla, A. T., Pepin, K. H., Gish, W. R., Chissoe, S. L., Wendl, M. C., Delehaunty, K. D., Miner, T. L., Delehaunty, A., Kramer, J. B., Cook, L. L., Fulton, R. S., Johnson, D. L., Minx, P. J., Clifton, S. W., Hawkins, T., Branscomb, E., Predki, P., Richardson, P., Wenning, S., Slezak, T., Doggett, N., Cheng, J. F., Olsen, A., Lucas, S., Elkin, C., Uberbacher, E., Frazier, M., Gibbs, R. A., Muzny, D. M., Scherer, S. E., Bouck, J. B., Sodergren, E. J., Worley, K. C., Rives, C. M., Gorrell, J. H., Metzker, M. L., Naylor, S. L., Kucherlapati, R. S., Nelson, D. L., Weinstock, G. M., Sakaki, Y., Fujiyama, A., Hattori, M., Yada, T., Toyoda, A., Itoh, T., Kawagoe, C., Watanabe, H., Totoki, Y., Taylor, T., Weissenbach, J., Heilig, R., Saurin, W., Artiguenave, F., Brottier, P., Bruls, T., Pelletier, E., Robert, C., Wincker, P., Smith, D. R., Doucette-Stamm, L.,

Rubinfeld, M., Weinstock, K., Lee, H. M., Dubois, J., Rosenthal, A., Platzer, M., Nyakatura, G., Taudien, S., Rump, A., Yang, H., Yu, J., Wang, J., Huang, G., Gu, J., Hood, L., Rowen, L., Madan, A., Qin, S., Davis, R. W., Federspiel, N. A., Abola, A. P., Proctor, M. J., Myers, R. M., Schmutz, J., Dickson, M., Grimwood, J., Cox, D. R., Olson, M. V., Kaul, R., Shimizu, N., Kawasaki, K., Minoshima, S., Evans, G. A., Athanasiou, M., Schultz, R., Roe, B. A., Chen, F., Pan, H., Ramser, J., Lehrach, H., Reinhardt, R., McCombie, W. R., de la Bastide, M., Dedhia, N., Blocker, H., Hornischer, K., Nordsiek, G., Agarwala, R., Aravind, L., Bailey, J. A., Bateman, A., Batzoglou, S., Birney, E., Bork, P., Brown, D. G., Burge, C. B., Cerutti, L., Chen, H. C., Church, D., Clamp, M., Copley, R. R., Doerks, T., Eddy, S. R., Eichler, E. E., Furey, T. S., Galagan, J., Gilbert, J. G., Harmon, C., Hayashizaki, Y., Haussler, D., Hermjakob, H., Hokamp, K., Jang, W., Johnson, L. S., Jones, T. A., Kasif, S., Kasprzyk, A., Kennedy, S., Kent, W. J., Kitts, P., Koonin, E. V., Korf, I., Kulp, D., Lancet, D., Lowe, T. M., McLysaght, A., Mikkelsen, T., Moran, J. V., Mulder, N., Pollara, V. J., Ponting, C. P., Schuler, G., Schultz, J., Slater, G., Smit, A. F., Stupka, E., Szustakowski, J., Thierry-Mieg, D., Thierry-Mieg, J., Wagner, L., Wallis, J., Wheeler, R., Williams, A., Wolf, Y. I., Wolfe, K. H., Yang, S. P., Yeh, R. F., Collins, F., Guyer, M. S., Peterson, J., Felsenfeld, A., Wetterstrand, K. A., Patrinos, A., Morgan, M. J., Szustakowki, J., de Jong, P., Catanese, J. J., Osoegawa, K., Shizuya, H., Choi, S. & Chen, Y. J. (2001). Initial sequencing and analysis of the human genome. *Nature* **409**, 860-921.

- Lien, C. L., McAnally, J., Richardson, J. A. & Olson, E. N. (2002). Cardiac-specific activity of an Nkx2-5 enhancer requires an evolutionarily conserved Smad binding site. *Dev Biol* **244**, 257-266.
- Loots, G. G., Locksley, R. M., Blankespoor, C. M., Wang, Z. E., Miller, W., Rubin, E. M. & Frazer, K. A. (2000). Identification of a coordinate regulator of interleukins 4, 13, and 5 by cross-species sequence comparisons. *Science* **288**, 136-140.
- Mohrs, M., Blankespoor, C. M., Wang, Z. E., Loots, G. G., Afzal, V., Hadeiba, H., Shinkai, K., Rubin, E. M. & Locksley, R. M. (2001). Deletion of a coordinate regulator of type 2 cytokine expression in mice. *Nat Immunol* **2**, 842-847.
- Noguchi, E., Shibasaki, M., Arinami, T., Takeda, K., Maki, T., Miyamoto, T., Kawashima, T., Kobayashi, K. & Hamaguchi, H. (1997). Evidence for linkage between asthma/atopy in childhood and chromosome 5q31-q33 in a Japanese population. *Am J Respir Crit Care Med* **156**, 1390-1393.
- Pennacchio, L. A., Olivier, M., Hubacek, J. A., Cohen, J. C., Cox, D. R., Fruchart, J. C., Krauss, R. M. & Rubin, E. M. (2001). An apolipoprotein influencing triglycerides in humans and mice revealed by comparative sequencing. *Science* **294**, 169-173.
- Pennacchio, L. A. & Rubin, E. M. (2001). Genomic strategies to identify mammalian regulatory sequences. *Nat Rev Genet* **2**, 100-109.

Pennacchio, L. A. & Rubin, E. M. (2003a). Apolipoprotein A5: A newly identified gene impacting plasma triglyceride levels in humans and mice. *Arteriosclerosis, Thrombosis, and Vascular Biology* **23**, 529-534.

Pennacchio, L. A. & Rubin, E. M. (2003b). Comparative genomic tools and databases: providing insights into the human genome. *J Clin Invest* **111**, 1099-1106.

Rioux, J. D., Daly, M. J., Silverberg, M. S., Lindblad, K., Steinhart, H., Cohen, Z., Delmonte, T., Kocher, K., Miller, K., Guschwan, S., Kulbokas, E. J., O'Leary, S., Winchester, E., Dewar, K., Green, T., Stone, V., Chow, C., Cohen, A., Langelier, D., Lapointe, G., Gaudet, D., Faith, J., Branco, N., Bull, S. B., McLeod, R. S., Griffiths, A. M., Bitton, A., Greenberg, G. R., Lander, E. S., Siminovitch, K. A. & Hudson, T. J. (2001). Genetic variation in the 5q31 cytokine gene cluster confers susceptibility to Crohn disease. *Nat Genet* **29**, 223-228.

Sharma, S., Leonard, J., Lee, S., Chapman, H. D., Leiter, E. H. & Montminy, M. R. (1996). Pancreatic islet expression of the homeobox factor STF-1 relies on an E-box motif that binds USF. *J Biol Chem* **271**, 2294-2299.

Tautz, D. (2000). Evolution of transcriptional regulation. *Curr Opin Genet Dev* **10**, 575-579.

Ureta-Vidal, A., Ettwiller, L. & Birney, E. (2003). Comparative genomics: genome-wide analysis in metazoan eukaryotes. *Nat Rev Genet* **4**, 251-262.

Venter, J. C., Adams, M. D., Myers, E. W., Li, P. W., Mural, R. J., Sutton, G. G., Smith, H. O., Yandell, M., Evans, C. A., Holt, R. A., Gocayne, J. D., Amanatides, P., Ballew, R. M., Huson, D. H., Wortman, J. R., Zhang, Q., Kodira, C. D., Zheng, X. H., Chen, L., Skupski, M., Subramanian, G., Thomas, P. D., Zhang, J., Gabor Miklos, G. L., Nelson, C., Broder, S., Clark, A. G., Nadeau, J., McKusick, V. A., Zinder, N., Levine, A. J., Roberts, R. J., Simon, M., Slayman, C., Hunkapiller, M., Bolanos, R., Delcher, A., Dew, I., Fasulo, D., Flanigan, M., Florea, L., Halpern, A., Hannenhalli, S., Kravitz, S., Levy, S., Mobarry, C., Reinert, K., Remington, K., Abu-Threideh, J., Beasley, E., Biddick, K., Bonazzi, V., Brandon, R., Cargill, M., Chandramouliswaran, I., Charlab, R., Chaturvedi, K., Deng, Z., Di Francesco, V., Dunn, P., Eilbeck, K., Evangelista, C., Gabrielian, A. E., Gan, W., Ge, W., Gong, F., Gu, Z., Guan, P., Heiman, T. J., Higgins, M. E., Ji, R. R., Ke, Z., Ketchum, K. A., Lai, Z., Lei, Y., Li, Z., Li, J., Liang, Y., Lin, X., Lu, F., Merkulov, G. V., Milshina, N., Moore, H. M., Naik, A. K., Narayan, V. A., Neelam, B., Nuskern, D., Rusch, D. B., Salzberg, S., Shao, W., Shue, B., Sun, J., Wang, Z., Wang, A., Wang, X., Wang, J., Wei, M., Wides, R., Xiao, C., Yan, C., Yao, A., Ye, J., Zhan, M., Zhang, W., Zhang, H., Zhao, Q., Zheng, L., Zhong, F., Zhong, W., Zhu, S., Zhao, S., Gilbert, D., Baumhueter, S., Spier, G., Carter, C., Cravchik, A., Woodage, T., Ali, F., An, H., Awe, A., Baldwin, D., Baden, H., Barnstead, M., Barrow, I., Beeson, K., Busam, D., Carver, A., Center, A., Cheng, M. L., Curry, L., Danaher, S., Davenport, L., Desilets, R., Dietz, S., Dodson, K., Doup, L., Ferriera, S., Garg, N., Gluecksmann, A., Hart, B., Haynes, J., Haynes, C., Heiner, C., Hladun, S., Hostin, D., Houck, J., Howland, T., Ibegwam, C.,

Johnson, J., Kalush, F., Kline, L., Koduru, S., Love, A., Mann, F., May, D.,
McCawley, S., McIntosh, T., McMullen, I., Moy, M., Moy, L., Murphy, B.,
Nelson, K., Pfannkoch, C., Pratts, E., Puri, V., Qureshi, H., Reardon, M.,
Rodriguez, R., Rogers, Y. H., Romblad, D., Ruhfel, B., Scott, R., Sitter, C.,
Smallwood, M., Stewart, E., Strong, R., Suh, E., Thomas, R., Tint, N. N., Tse, S.,
Vech, C., Wang, G., Wetter, J., Williams, S., Williams, M., Windsor, S., Winn-
Deen, E., Wolfe, K., Zaveri, J., Zaveri, K., Abril, J. F., Guigo, R., Campbell, M.
J., Sjolander, K. V., Karlak, B., Kejariwal, A., Mi, H., Lazareva, B., Hatton, T.,
Narechania, A., Diemer, K., Muruganujan, A., Guo, N., Sato, S., Bafna, V.,
Istrail, S., Lippert, R., Schwartz, R., Walenz, B., Yooseph, S., Allen, D., Basu, A.,
Baxendale, J., Blick, L., Caminha, M., Carnes-Stine, J., Caulk, P., Chiang, Y. H.,
Coyne, M., Dahlke, C., Mays, A., Dombroski, M., Donnelly, M., Ely, D.,
Esparham, S., Fosler, C., Gire, H., Glanowski, S., Glasser, K., Glodek, A.,
Gorokhov, M., Graham, K., Gropman, B., Harris, M., Heil, J., Henderson, S.,
Hoover, J., Jennings, D., Jordan, C., Jordan, J., Kasha, J., Kagan, L., Kraft, C.,
Levitsky, A., Lewis, M., Liu, X., Lopez, J., Ma, D., Majoros, W., McDaniel, J.,
Murphy, S., Newman, M., Nguyen, T., Nguyen, N., Nodell, M., Pan, S., Peck, J.,
Peterson, M., Rowe, W., Sanders, R., Scott, J., Simpson, M., Smith, T., Sprague,
A., Stockwell, T., Turner, R., Venter, E., Wang, M., Wen, M., Wu, D., Wu, M.,
Xia, A., Zandieh, A. & Zhu, X. (2001). The sequence of the human genome.
Science **291**, 1304-1351.

Waterston, R. H., Lindblad-Toh, K., Birney, E., Rogers, J., Abril, J. F., Agarwal, P.,
Agarwala, R., Ainscough, R., Alexandersson, M., An, P., Antonarakis, S. E.,

Attwood, J., Baertsch, R., Bailey, J., Barlow, K., Beck, S., Berry, E., Birren, B.,
Bloom, T., Bork, P., Botcherby, M., Bray, N., Brent, M. R., Brown, D. G.,
Brown, S. D., Bult, C., Burton, J., Butler, J., Campbell, R. D., Carninci, P.,
Cawley, S., Chiaromonte, F., Chinwalla, A. T., Church, D. M., Clamp, M., Clee,
C., Collins, F. S., Cook, L. L., Copley, R. R., Coulson, A., Couronne, O., Cuff, J.,
Curwen, V., Cutts, T., Daly, M., David, R., Davies, J., Delehaunty, K. D., Deri, J.,
Dermitzakis, E. T., Dewey, C., Dickens, N. J., Diekhans, M., Dodge, S., Dubchak,
I., Dunn, D. M., Eddy, S. R., Elnitski, L., Emes, R. D., Eswara, P., Eyras, E.,
Felsenfeld, A., Fewell, G. A., Flicek, P., Foley, K., Frankel, W. N., Fulton, L. A.,
Fulton, R. S., Furey, T. S., Gage, D., Gibbs, R. A., Glusman, G., Gnerre, S.,
Goldman, N., Goodstadt, L., Grafham, D., Graves, T. A., Green, E. D., Gregory,
S., Guigo, R., Guyer, M., Hardison, R. C., Haussler, D., Hayashizaki, Y., Hillier,
L. W., Hinrichs, A., Hlavina, W., Holzer, T., Hsu, F., Hua, A., Hubbard, T., Hunt,
A., Jackson, I., Jaffe, D. B., Johnson, L. S., Jones, M., Jones, T. A., Joy, A.,
Kamal, M., Karlsson, E. K., Karolchik, D., Kasprzyk, A., Kawai, J., Keibler, E.,
Kells, C., Kent, W. J., Kirby, A., Kolbe, D. L., Korf, I., Kucherlapati, R. S.,
Kulbokas, E. J., Kulp, D., Landers, T., Leger, J. P., Leonard, S., Letunic, I.,
Levine, R., Li, J., Li, M., Lloyd, C., Lucas, S., Ma, B., Maglott, D. R., Mardis, E.
R., Matthews, L., Mauceli, E., Mayer, J. H., McCarthy, M., McCombie, W. R.,
McLaren, S., McLay, K., McPherson, J. D., Meldrim, J., Meredith, B., Mesirov, J.
P., Miller, W., Miner, T. L., Mongin, E., Montgomery, K. T., Morgan, M., Mott,
R., Mullikin, J. C., Muzny, D. M., Nash, W. E., Nelson, J. O., Nhan, M. N., Nicol,
R., Ning, Z., Nusbaum, C., O'Connor, M. J., Okazaki, Y., Oliver, K., Overton-
Larty, E., Pachter, L., Parra, G., Pepin, K. H., Peterson, J., Pevzner, P., Plumb, R.,

Pohl, C. S., Poliakov, A., Ponce, T. C., Ponting, C. P., Potter, S., Quail, M.,
Reymond, A., Roe, B. A., Roskin, K. M., Rubin, E. M., Rust, A. G., Santos, R.,
Sapojnikov, V., Schultz, B., Schultz, J., Schwartz, M. S., Schwartz, S., Scott, C.,
Seaman, S., Searle, S., Sharpe, T., Sheridan, A., Shownkeen, R., Sims, S., Singer,
J. B., Slater, G., Smit, A., Smith, D. R., Spencer, B., Stabenau, A., Stange-
Thomann, N., Sugnet, C., Suyama, M., Tesler, G., Thompson, J., Torrents, D.,
Trevaskis, E., Tromp, J., Ucla, C., Ureta-Vidal, A., Vinson, J. P., Von
Niederhausern, A. C., Wade, C. M., Wall, M., Weber, R. J., Weiss, R. B., Wendl,
M. C., West, A. P., Wetterstrand, K., Wheeler, R., Whelan, S., Wierzbowski, J.,
Willey, D., Williams, S., Wilson, R. K., Winter, E., Worley, K. C., Wyman, D.,
Yang, S., Yang, S. P., Zdobnov, E. M., Zody, M. C. & Lander, E. S. (2002).
Initial sequencing and comparative analysis of the mouse genome. *Nature* **420**,
520-562.