An Interspecific Functional Complementation Test in *Drosophila* for Introductory Genetics Laboratory Courses

LIDON MONFERRER AND RUBEN ARTERO

From the Department of Genetics, Valencia University, Doctor Moliner 50, Burjasot 46100, Valencia, Spain.

Address correspondence to Ruben Artero at the address above, or e-mail: ruben.artero@uv.es.

Introductory genetics courses often include evolutionary genetics concepts such as sequence homology and functional conservation. It is usually assumed that two sequences showing homology (i.e., sharing a common ancestral sequence) perform the same molecular function. The correlation, however, does not always hold true, and evidence for functional conservation must come from functional studies. In this study we describe a genetics laboratory class that demonstrates functional conservation between the *Drosophila* protein Muscleblind (Mbl) and its human ortholog MBNL1. We use the Gal4/UAS system to express MBNL1 in a *Drosophila* mutant background and measure the in vivo activity of the human protein by rescue of *mbl* mutant phenotype in embryos. As a control, ubiquitous expression of *Drosophila* MblC, one of the four protein isoforms encoded by the gene, increased by 71% the viability of *mbl* mutant embryos and greatly reduced the hypercontracted abdomen of mutant larvae. In a parallel experiment, human MBNL1 provided a robust rescue of the embryonic lethality (78%) and improved abdomen hypercontraction as well. Under both conditions, rescued larvae die as first instars, probably due to overexpression effects, lack of alternative protein isoforms, or incomplete expression in critical tissues such as the nervous system. The use of two constructs in the rescue experiment (UAS-mblC and UAS-MBNL1) and the incomplete rescue prompt several questions for students. The fact that a human protein works in a *Drosophila* cellular context illustrates the use of an in vivo test to prove functional conservation.

A recurring limitation of sequence comparison of genes between species is that some of the genes may not be functionally equivalent despite the fact that they may show high similarity at the sequence level. In fact, sequence conservation does not necessarily imply the existence of functional conservation, as demonstrated, for example, in the sex determination pathways in *Drosophila* and mammals (for a comparison see Marin and Baker 1998). The HuD protein shows a remarkable homology (up to 50% identity over half of the protein sequences) to the *Drosophila* proteins Elav and Sex- lethal (Szabo et al. 1991), two pre-messenger RNA (mRNA) splicing factors involved in the neuronal and sex determination pathways, respectively. HuD proteins, however, have not been so far implicated in the human sex determination pathway. Similarly, the vertebrate family of myogenic regulatory factors represented by *MyoD*, *Myf-5, myogenin*, and *MRF4* are expressed exclusively in skeletal muscle and can induce a skeletal muscle differentiation program when expressed in a wide range of cultured cells (reviewed in Weintraub 1993). A single myogenic regulatory factor family member has been identified in invertebrate organisms. In *Drosophila*, for example, this family is represented by the Nautilus protein. Analysis of mutations that eliminate nautilus function, coupled with germ line clones in which both maternal and zygotic Nautilus have been removed, indicates that Nautilus is required for the differentiation of a limited subset of muscles (Balagopalan et al. 2001). Such limited phenotype rules out a general myogenic function for nautilus equivalent to the general roles myogenic regulatory factors play in vertebrates. Functional conservation, therefore, must be established through functional studies of individual genes. In *Drosophila*, one such approach is to use a human wild-type complementary DNA (cDNA) to substitute for (or rescue) a loss-of-function mutation in the *Drosophila* gene counterpart; muscleblind (*mbl*) provides a good case study for this type of functional test.

Analysis of the *Drosophila mbl* transcription unit reveals a complex splicing pattern giving rise to four mRNAs, which share 5’ sequences but differ in their 3’-ends due to the use of alternative exons. The mRNAs *mblA, mblB, mblC*, and *mblD* give rise to four open reading frames of 203, 316, 243, and 84 amino acids, respectively. Protein isoforms MblA, MblB, and MblC share the first 179 amino terminal residues, whereas MblD shares the first 63 amino acids.
Mbl isoforms A, B, and C contain two copies of a Cys-His zinc finger structure with a typical spacing of Cx5CxxCxxH (Begemann et al. 1997). Homozygous mutant embryos and mosaic analysis reveal an involvement of mbl in the terminal differentiation of muscles and eye photoreceptor cells, respectively. In muscles, mbl homozgygous mutant embryos show defects in the sarcomeric Z-bands, signs of muscle hypercontraction at the ultrastructural level, and a severe reduction in the tendon-like material normally present in indirect muscle attachments to the epidermis. Altogether, these cellular phenotypes lead to a characteristically paralyzed and contracted larva (Artero et al. 1998). The mbl pattern of expression in the imaginal discs and in the embryonic somatic, visceral, and pharyngeal musculature is consistent with the mutant phenotypes. However, mbl is not restricted to muscle tissues. It is also detected in the central nervous system (CNS) and larval photoreceptors. Homology searches with the Drosophila mbl sequence reveal three human muzsclelnklk-1ke orthologs: MBNL1, 2, and 3, which themselves undergo alternative splicing giving rise to several protein isoforms (Fardaei et al. 2002; Miller et al. 2000). Protein alignments strongly suggest that human MBNL proteins arose by tandem gene duplication because they contain four, instead of two, zinc fingers (Figure 1A). The involvement of MBNL1 as an alternative splicing factor during cardiac Troponin T primary transcript splicing, antagonizing the activity of the CUG-BP and ETR-3 like factors (CELF) family member CUG-BP in exon choice, has been recently reported (Ho et al. 2004).

Involvement of the human MBNL1–3 proteins in the toxic effect brought about by long microsatellite expansions in noncoding sequences is now a well-established fact (reviewed in Ranum and Day 2004). Such repeat expansions are found in Myotonic Dystrophy (DM) type 1 (DM1) patients, who carry long CTG expansions in the 3'UTR of the DM protein kinase (DMPK), and type 2 (DM2) patients, who carry up to thousands of CCTG repeats in the first intron of the ZNF9 gene. According to the current model of pathogenesis, the presence of the CUG repeats in the 3'-untranslated region of the DMPK transcripts aberrantly recruits MBNL proteins (Fardaei et al. 2002; Miller et al. 2000) and may interfere with DMPK expression itself. Sequestration of MBNL seems to alter alternative splicing of downstream target genes (Mankodi et al. 2002).

To address the extent to which the human MBNL and Mbl proteins are functionally exchangeable, we used the Gal4 system to express the human MBNL1 protein in mbl mutant embryos. In these experiments, we detected a robust rescue. We have incorporated such rescue experiments into a genetics laboratory setting to illustrate functional conservation and describe here the experimental details involved and the complete results.

Materials and Methods

Drosophila Stocks

Stocks were maintained at 25°C on a standard yeast/glucose medium supplemented with β-hydroxybenzoic acid methyl ester (as a mold inhibitor) and live yeast. Eggs were collected on standard apple juice agar plates (Stern and Sucena 2000). To generate Gal4-responsive target genes, the coding sequence of cDNA KIAA0428 (MBNL1) and mblC were cloned into the polylinker of vector pUAST downstream from five GAL4-binding sites (Upstream Activating Sequences; UAS) and from lbp70 TATA and upstream of the SV40 terminator to generate constructs UAS-MBNL1.KIAA0428 and UAS-mblC, respectively (Garcia-Casado et al. 2002). cDNA KIAA0428 encodes for protein isoform NM_021038 of MBNL1 according to the description by Fardaei et al. (2002). To generate the driver stock daughtercall-Gal4 (da-Gal4), a promoter-Gal4 fusion construct was generated by cloning a 3.2-kb KpnI-BglII fragment (encompassing the da promoter) into the polylinker of Gal4-containing vector pGaTB. The resulting construct was then excised and ligated into a P-element transformation vector (Roessel and Brand 2000; Wodarz et al. 1995). da-Gal4 gives a more or less uniform expression of the yeast Gal4 transcription factor and was obtained from the Bloomington stock center. mbl/loss-of-function mutations mblE16 and mblE27 derive from imprecise P element excisions and are described in the studies of Artero et al. (1998) and Begemann et al. (1997), respectively. The first two exons, which include the start codon, are deleted in mblE27 (Begemann et al. 1997). Relevant phenotypic markers are y+, for yellow body color, and w1118 for white eye color. For a complete description of mutant gene phenotypes used as markers see FlyBase (The FlyBase Consortium 2003). To perform the mutant rescue experiment we built fly stocks homozygous in the third chromosome for the da-Gal4 driver or the UAS-MBNL1.KIAA0428 construct (UAS-mblC in the positive control stock). These stocks were also heterozygous for the mblE16 or mblE27 mutation on the second chromosome, which were kept over a CyO,y+ balancer. All stocks were built in a y1w1118 mutant background. As a derivative during stock building, we also synthesized y1118; mblE16/CyO, y+ and y1118; mblE27/CyO,y+ stocks that were used to characterize the ground mutant state. General recommendations to culturing Drosophila flies, sources of supplies, small equipment, and media recipes can be found in the Bloomington Drosophila stock center Web site at Indiana University (http://flystocks.bio.indiana.edu/working-with-docs.html). Fly stocks are available from the authors.

Rescue Experiment

To perform rescue experiments, we crossed driver and UAS stocks carrying different mbl alleles in order to complement any spurious second site lethal mutation in the mbl chromosome (Figure 1B). Flies were allowed to mate en masse for 2 days and were then transferred to laying pots at 25°C. The first-day egg collections were discarded, and, starting on the second day, a 12-h egg collection regimen was established. Eggs were transferred to a fresh agar plate in which they were pooled in groups of five for an approximate total number of a hundred. Agar plates were incubated at 25°C until the next day, when newly hatched larvae were scored for wild type or yellow mouth hooks and discarded or transferred to new agar
plates, respectively. Plates were scored a few times during the day for at least 2 days.

Preparation of Cuticles from First-Instar Larvae

To assess abdomen hypercontraction, larval cuticles were prepared according to Stern and Sucena (2000). Briefly, collect embryos in tap water through an egg sieve, dechorionate in 50% bleach for 2–3 min (fully dechorionated eggs appear shiny instead of dull white), and wash thoroughly with running tap water. Using a paint brush, transfer embryos from the basket to an eppendorf tube containing 1 ml methanol, wash twice, and devitellinize in 0.5 ml methanol plus 0.5 ml heptane by vigorously shaking the tube for 30 s. Devittelized embryos sink to the bottom of the tube. Using a Pasteur pipette, remove the interface and all floating debris. Wash embryos twice (or more) in 1 ml methanol and allow them to settle, subsequently discarding all floating debris. Substitute methanol with 0.1% Triton X-100 and wash once. Transfer embryos with a glass pipette (contained in a small amount of liquid) to a clean slide. Using a folded piece of filter paper, wick up most of the liquid, carefully avoiding the embryos. Place a drop of Hoyer’s medium onto the embryos when most of the liquid has been removed, and put a coverslip onto the preparation. Capillary action will draw the mountant to the edge of the coverslip. Use the minimum amount of Hoyer’s medium so that none is squeezed out at the edges. Place the slide at 60°C for at least 1 h, although it may be preferable to let the slides cook for one to several days. Hoyer’s mountant clears and preserves tissue, and be- may be preferable to let the slides cook for one to several days.

Data Analysis

Phenotypes observed in the progeny fall into three classes: viable larvae with wild-type mouth hooks (and cuticle pigmentation), rescued mbl mutant larvae with yellow mouth hooks, and unhatched embryos. The last class includes unfertilized embryos and embryos homozygous for the balancer or for the mbl mutation, in which expression of UAS-mblC or UAS-MBNL1E16;UAS-mblC was unable to rescue lethality. Because it is not feasible to properly sort these classes out, we made use of viable larvae to quantify the degree of rescue. We reasoned that heterozygous larvae (dark mouth hooks) would account for two-thirds of viable larvae if all mbl mutant larvae were rescued. Hence, we calculate the expected one-third number of mbl mutant larvae, if rescue was complete, from the number of heterozygous larvae. Rescue was measured as the ratio (in percentage) of actual larvae with yellow mouth hooks to expected mbl mutant larvae. In general, the number of viable larvae (heterozygous for mbl and rescued) in different experiments was approximately 5% lower than the number of empty chorions on the agar plate. Missing larvae were assumed to fall into the heterozygous class because these larvae were far more active than the rescued ones. This discrepancy was probably due to the heterozygous larvae that managed to escape from the plate or got inside the media and were not visible. Exact details of all methods are available upon request from the authors.

Results

Characterization of muscleblind Mutations

To properly measure rescue, we require a defined and easy to detect mutant phenotype. A late embryonic lethal phenotype fulfills such requirements because we can detect even a slight improvement by an increase in the mutant viability. Among the available mbl loss-of-function mutations, we chose mblE16 and mblE27 because they behave as very late embryonic lethal mutations and were classified as putative nulls in previous reports (Artero et al. 1998; Begemann et al. 1997). However, no detailed account of the viability of mbl mutant embryos was available, and specifically, we did not know the percentage of embryos that actually failed to hatch in the transheterozygous mutant combination.

Following standard fly genetics procedures (see Materials and Methods), we built stocks that allowed direct genotyping of larvae by mouth hook pigmentation. Using this approach, we detected less than 1% of mblE16 homozygous mutant larvae in our experiments (data not shown) and virtually 0% larvae of the mblE16/mblE27 genotype. The fact that the transheterozygous condition gave a stronger phenotype than the mblE27 homozygous condition indicates that the mblE16 allele is stronger than mblE27 and possibly a null. During the course of this characterization, we also defined the extent of abdomen hypercontraction of mbl mutant embryos as an addition to viability in the measure of mutant rescue (compare Figure 2B,C).

Ubiquitous mblC Expression Provides Strong Rescue

The Gal4 system has been extensively used in mutant rescue experiments in which a given cloned gene is expressed in a mutant background and the resulting phenotype is compared with the original mutant (see for example Mason et al. 2003). Briefly, the Gal4 system is a method for ectopic gene expression in Drosophila that allows selective activation of any cloned gene in a variety of tissue and cell-specific patterns (Brand and Perrimon 1993; Roessel and Brand 2000). The method uses two constructs introduced into the fly by germ line transformation. The first construct is the yeast Gal4 transcription factor, which inserts near a defined genomic enhancer that drives its expression or fused to a defined enhancer. The second is the cDNA of interest cloned downstream of the Gal4 target sequences. By simply crossing both types of flies, a cDNA is expressed in the tissues dictated by the enhancer upstream of the Gal4 construct.
Figure 1. Sequence comparison between Mbl and MBNL1 and general rescue strategy. (A) Sequence comparison between MblC and the NM_021038 protein (KIAA0428 cDNA) isoforms encoded by mbl and MBNL1, respectively. Numbers refer to the position of the first residue of the lane in the corresponding protein sequence. The Drosophila protein contains pairs of zinc finger motifs (boxed) that are duplicated in the human protein. These regions are conserved the most, with up to 85% of amino acids identical in the first zinc finger. Also conserved in the zinc fingers are casein kinase II phosphorylation sites (residues 31–34, 80–83, and 212–215 in the human protein; shown in lower case) and protein kinase C phosphorylation sites (residues 59–61; 182–184, and 366–368 in MBNL1KIAA0428; shown in bold upper case). Regions outside the zinc fingers are less conserved but N-myristoylation sites (residues 106–111; 115–120; 128–133 in MblC and residues 273–278 and 329–334 in MBNL1KIAA0428; in bold lower case) and sumoylation sites (residues 283–286 in the human protein and 201–204 in MblC; shown doubly underlined) can be found in both proteins. Specific to the human protein is a tyrosine kinase phosphorylation (residues 63–71; 231–239; shown underlined). In this alignment the degree of conservation is denoted by an asterisk (*) for identical, a colon (:) for highly conservative, and a period (.) for weakly conservative residue changes. Dashes (-) indicate regions with no significant conservation of residues. (B) Targeted gene expression using the Gal4/UAS system. The Gal4 coding sequence, encoding a transcriptional activator from yeast, is fused to daughterless enhancers that activate gene expression throughout the embryo. The construct is introduced into the Drosophila genome with an artificial P-element genetically marked with a Drosophila white minigene. Lines
(Figure 1C). (See Supplementary Data for a color version of Figure 1).

To test transgene rescue under our experimental conditions, we first used a Drosophila mbl construct in a control rescue. We chose the UAS-mblC construct for these experiments because mbl primary transcripts undergo developmentally regulated alternative splicing and mblC was isolated from an embryonic cDNA library. It was therefore predicted that the expression of the wild-type MblC protein isoform would rescue the embryonic lethality associated with mbl mutations. Because no Gal4 driver reproducing the complete endogenous mbl pattern of expression has been described, we attempted the rescue with the da-Gal4 line. da-Gal4 is expressed in the two most prominent tissues where mbl is expressed, the CNS and the somatic and visceral musculature, among other embryonic tissues. The driver line would therefore express the UAS-mblC construct in all (or most) cells where the protein is required, as well as in ectopic places.

Rescue was determined by measuring the percentage of hatching and reduction in the hypercontraction phenotype observed in mutant larvae. Expression of MblC throughout the embryo increased viability of mblE27/mblE16 mutant embryos from 0% to approximately 71.43% (Figure 2A; SD 1.07%) and notably reduced the abdomen hypercontraction (compare Figure 2C,D). Such a robust rescue demonstrates that the Gal4/UAS system can properly deliver the MblC protein in the cells and that the embryonic lethality can be complemented; that is, there is no second site mutation besides mbl that would preclude rescue by the human MBNL1 protein. In addition, it points to MblC as performing most of the mbl function in the embryo because other protein isoforms are apparently dispensable for embryo viability.

MBNL1, a Human Protein, Rescues a Drosophila Mutation

In humans, up to 18 Muscleblind protein isoforms have been described by the combination of alternative splicing of primary transcripts at the level of individual genes and the existence of three different genes: MBNL1, 2, and 3 (Fardaei et al. 2002; reviewed in Pascual et al., in press). MBNL1 transcripts are expressed the highest in skeletal muscle, and it has been proposed that MBNL1 regulates terminal muscle differentiation (Fardaei et al. 2002; Squillace et al. 2002). Because mutations in Drosophila mbl chiefly affect differentiation of body wall musculature (Artero et al. 1998), we decided to test a MBNL1 protein isoform in our mutant rescue experiments. A multiple alignment between MblC and all five MBNL1 protein isoforms (Fardaei et al. 2002) revealed that variable sequences lie outside the first pair of zinc fingers. Because the sequence similarity between Mbl and MBNL1 proteins is almost completely restricted to the zinc finger region itself, we have not been able to detect significant differences in the degree of similarity between MblC and MBNL1 protein expressing the Gal4 protein are crossed to lines carrying a target gene of interest (MBNL1KIAA0428 in this rescue experiment), subcloned downstream from five Gal4-binding sites (the UAS; circles). These constructs are also introduced into the genome flanked by P-element ends and carrying a genetic marker. In the progeny of such a cross, Gal4 binds, and hence activates, the transcription of the target gene only in those cells and tissues where Gal4 is expressed. (C) Crosses involved in the rescue experiment showing the driver stock (containing the da-Gal4), the UAS stock (containing the UAS construct), and the expected genotypes of the offspring. Heterozygous and mbl mutant genotypes are readily genotyped by mouth hook pigmentation. As an example, the panels to the right show mouth parts (arrowheads) from y+ and y- larvae. Cyo, y+—bearing individuals show wild-type mouth hook pigmentation, whereas homozygous mutant larvae show a yellow pigment because of the y- mutant background.
isoforms. Protein isoform NM_021038, encoded by the full-length cDNA KIAA0428 from MBNL1, was therefore chosen because of its availability in the laboratory, and transgenic flies carrying a UAS-MBNL1_{KIAA0428} construct were generated.

In parallel with the UAS-mblC control experiment, we used the da-Gal4 driver and the UAS-MBNL1_{KIAA0428} construct to ubiquitously express MBNL1 in mbl mutant embryos. In these experiments, we found that the percentage of viable mbl mutant embryos was on average 78.14% (Figure 2A; SD 9.66%), which was greater than the increase in viability provided by MblC. The difference between both rescues, however, was not dependent on genotype because a Fisher’s exact test supports that they are independent ($P = .48$; for details about this statistic see Uitenbroek 1997). Moreover, the highly contracted abdomen of mbl mutant larva was greatly improved by the expression of the human MBNL1 protein (compare Figure 2C,F). Rescued larvae wandered freely in the plate and were responsive to stimulus but noticeably less active than heterozygous larvae. It is worth noting that despite the fact that a large proportion of mutant embryos were able to hatch when MblC or MBNL1 was expressed ubiquitously in the embryo, rescued larvae remained first instars for up to approximately 50 h and never molted to second instars.

In summary, we conclude that the human MBNL1 protein encoded by the splice isoform KIAA0428 can perform the same molecular functions as Drosophila Mbl in the embryo because it rescues the lethality associated with loss-of-function mutations in the mbl locus. This result demonstrates that both proteins are functional homologues.

**Discussion**

We have used the Gal4/UAS system to provide experimental proof of functional conservation between two sequence orthologs: Drosophila mbl and human MBNL1. These experiments are reliable and easy to adapt to a genetics laboratory setting in which students learn the relevance of in vivo experiments to support bioinformatics findings. Moreover, students learn the importance of developmental and tissue-type transcription regulation as well as pre-mRNA alternative splicing control because constitutive expression of a Drosophila Mbl protein isoform (da-Gal4 \( \rightarrow \) UAS-mblC) cannot completely rescue a mbl loss-of-function mutation. We are currently implementing these rescue experiments in genetics laboratory courses using a green fluorescent protein (GFP)-tagged MblC protein so that students can actually watch the protein being expressed. To facilitate the understanding of complex developmental processes such as muscle development, relevant to the laboratory course, we recommend instructors and students visit the FlyMove Web site (Weigmann et al. 2003; http://flymove.uni-muenster.de).

We found that MBNL1 expression gave a slightly better rescue of viability than did the fly protein counterpart, although the difference was not statistically significant. In a genetics laboratory class, the difference in rescue may prompt the question of its statistical significance. Students can apply a Fisher’s exact test either to their individual results or to the results of the class as a whole to answer that question. If the difference was significant, several explanations could be put forward which further illustrate gene expression concepts. For example, during Drosophila germ line transformation, individual transgenes insert in different genomic locations. Such particular genomic environments influence the degree of expression of the transgene due to the presence of nearby genomic enhancers, silencers, insulators, and heterochromatin, a phenomenon usually referred to as a position effect. Alternatively, the human protein may indeed provide more mbl function than the Drosophila protein isoform MblC.

We note that the human protein is a duplicated version of the fly protein (it contains a duplicated set of zinc fingers; Figure 1A). If MblC in flies was to work as a dimer, the human version may be a natural “tethered” dimer thus working more efficiently at the same or lower concentrations.

Although we detect a strong improvement in viability and abdomen contraction, neither MblC nor MBNL1_{KIAA0428} expression rescued larvae beyond first instar. We believe that such partial rescue stems from a number of experimental limitations that also exemplify several aspects of gene regulation.

First, as we noted in the Introduction, mbl shows a complex expression pattern in the embryo, which includes the musculature (but not the heart), the Bolwig’s organ (a larval photoreceptor structure), and the CNS (Artero et al. 1998). We used a driver with general expression pattern, but we cannot make certain that the construct is expressed at high enough levels in all cells required in our rescue experiment. Conversely, ubiquitous expression leads to ectopic expression, which may also contribute to the larval lethality. Such developmental and tissue-type changes in gene expression, either individually or in combination, may explain the partial rescue.

Second, alternative splicing from the mbl locus gives rise to four protein isoforms at times and places not currently known and the function of which may not be redundant. Indeed, whereas MBNL1 and MBNL2 are involved in promoting muscle differentiation, MBNL3 appears to function as an inhibitor of terminal muscle differentiation (Squillace et al. 2002). In Drosophila, the single mbl gene carries out such control over the terminal muscle differentiation (Artero et al. 1998). It is likely, therefore, that different Mbl protein isoforms perform different functions at the molecular level and that a tight control over their developmental and tissue-type expression exists. MblC may be the predominant embryonic isoform and hence rescues the lethality of most of the mutant embryos, but during larval stages, other protein isoforms may be required. Similarly, the MBNL1_{KIAA0428} construct used expresses only one of the five alternatively spliced mRNAs derived from the gene (Fardaei et al. 2002). Other human MBNL1 protein isoforms could provide more mbl function during larval stages and perhaps extend the life of rescued larvae. Thus, developmental and tissue-type control over mbl alternative splicing may also contribute to the partial rescue observed.

Finally, it is also possible that not all molecular functions are conserved between Drosophila and human Mbl proteins so
that, even driven by the endogenous promoter, we could never get a complete rescue.

In summary, we describe here an example of functional conservation between a human and a fly protein, which also serves to illustrate gene expression concepts such as spatial and temporal control over gene transcription and pre-mRNA splicing. Experiments such as these are easy to adapt to a genetics laboratory course and, most importantly, expose students to the complexities of gene expression and cross-genomics comparisons.

Supplementary Data
A color version of Figure 1 is available at Journal of Heredity online (www.jhered.oxfordjournals.org).

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