AMERICAN SOCIETY FOR MICROBIOLOGY | mBio®

Stage-Specific Transcriptome and Proteome Analyses of the Filarial Parasite *Onchocerca volvulus* and Its *Wolbachia* Endosymbiont

Sasisekhar Bennuru,[a] James A. Cotton,[b] Jose M. C. Ribeiro,[c] Alexandra Grote,[d] Bhavana Harsha,[b] Nancy Holroyd,[b] Amruta Mhashilkar,[e] Douglas M. Molina,[f] Arlo Z. Randall,[f] Adam D. Shandling,[f] Thomas R. Unnasch,[e] Elodie Ghedin,[d,g] Matthew Berriman,[b] Sara Lustigman,[h] Thomas B. Nutman[a]

Laboratory of Parasitic Diseases, NIAID, NIH, Bethesda, Maryland, USA[a]; Wellcome Trust Sanger Institute, Wellcome Genome Campus, Hinxton, Cambridgeshire, United Kingdom[b]; Laboratory of Malaria and Vector Research, NIAID, NIH, Rockville, Maryland, USA[c]; Department of Biology, Center for Genomics and Systems Biology, New York University, New York, New York, USA[d]; University of South Florida, Tampa, Florida, USA[e]; Antigen Discovery Inc., Irvine, California, USA[f]; College of Global Public Health, New York University, New York, New York, USA[g]; New York Blood Center, New York, New York, USA[h]

ABSTRACT Onchocerciasis (river blindness) is a neglected tropical disease that has been successfully targeted by mass drug treatment programs in the Americas and small parts of Africa. Achieving the long-term goal of elimination of onchocerciasis, however, requires additional tools, including drugs, vaccines, and biomarkers of infection. Here, we describe the transcriptome and proteome profiles of the major vector and the human host stages (L1, L2, L3, molting L3, L4, adult male, and adult female) of *Onchocerca volvulus* along with the proteome of each parasitic stage and of its *Wolbachia* endosymbiont (*w*Ov). In so doing, we have identified stage-specific pathways important to the parasite's adaptation to its human host during its early development. Further, we generated a protein array that, when screened with well-characterized human samples, identified novel diagnostic biomarkers of *O. volvulus* infection and new potential vaccine candidates. This immunomic approach not only demonstrates the power of this postgenomic discovery platform but also provides additional tools for onchocerciasis control programs.

IMPORTANCE The global onchocerciasis (river blindness) elimination program will have to rely on the development of new tools (drugs, vaccines, biomarkers) to achieve its goals by 2025. As an adjunct to the completed genomic sequencing of *O. volvulus*, we used a comprehensive proteomic and transcriptomic profiling strategy to gain a comprehensive understanding of both the vector-derived and human host-derived parasite stages. In so doing, we have identified proteins and pathways that enable novel drug targeting studies and the discovery of novel vaccine candidates, as well as useful biomarkers of active infection.

Address correspondence to Sara Lustigman, slustigman@nybloodcenter.org, or Thomas B. Nutman, tnutman@niaid.nih.gov.

Onchocerciasis or river blindness, caused by infection with *Onchocerca volvulus*, is a neglected tropical disease (NTD) that is associated with significant morbidity and disability in the 17 million people currently estimated to be infected (1). Onchocerciasis was the first NTD targeted for control in 1974 by the World Health Organization and is now one of the six NTDs targeted for elimination (2). Currently, the strategy for elimination of *O. volvulus* focuses on controlling transmission through ivermectin-based mass drug administration (MDA) programs that have largely eliminated onchocerciasis in the Americas (3) and that have made significant progress toward that goal in some regions of Africa (4). However, according to recent reports, onchocerciasis cannot be eliminated through MDA with ivermectin solely (5) and may require an estimated 1.30 billion ivermectin treatments until 2045 (6). In addition, ivermectin is contraindicated in areas of coendemicity with *Loa loa*, where the risk of severe (occasionally fatal) adverse events is associated with high levels of circulating *L. loa* microfilariae (mf) (7). Furthermore, the

potential for ivermectin resistance (8), the lack of macrofilaricidal activity by ivermectin, and the long time line (>20 years) needed for transmission interruption (5, 6) have prompted research into the development of new tools (macrofilaricidal drugs, diagnostics, and vaccines), the basis of which relies on a fundamental understanding of the parasite's biology.

Humans are the only definitive hosts of *O. volvulus*. Because there are no existing small-animal models for propagating the life cycle (see Fig. S1 in the supplemental material) of *O. volvulus*, approaches that require parasite material from most stages have been difficult. For example, adult parasites must be obtained surgically from subcutaneous nodules, the mf from human skin, and the infective larvae must be obtained from infected blackflies—a process that, to date, requires feeding of newly hatched naive blackflies on infected humans with microfiladermia. Nevertheless, using relatively limited parasite material from most of the life cycle stages, we have comprehensively profiled the stage-specific transcriptomes of *O. volvulus*, as well as the proteomes of both *O. volvulus* and its *Wolbachia* endosym-

Bennuru et al.
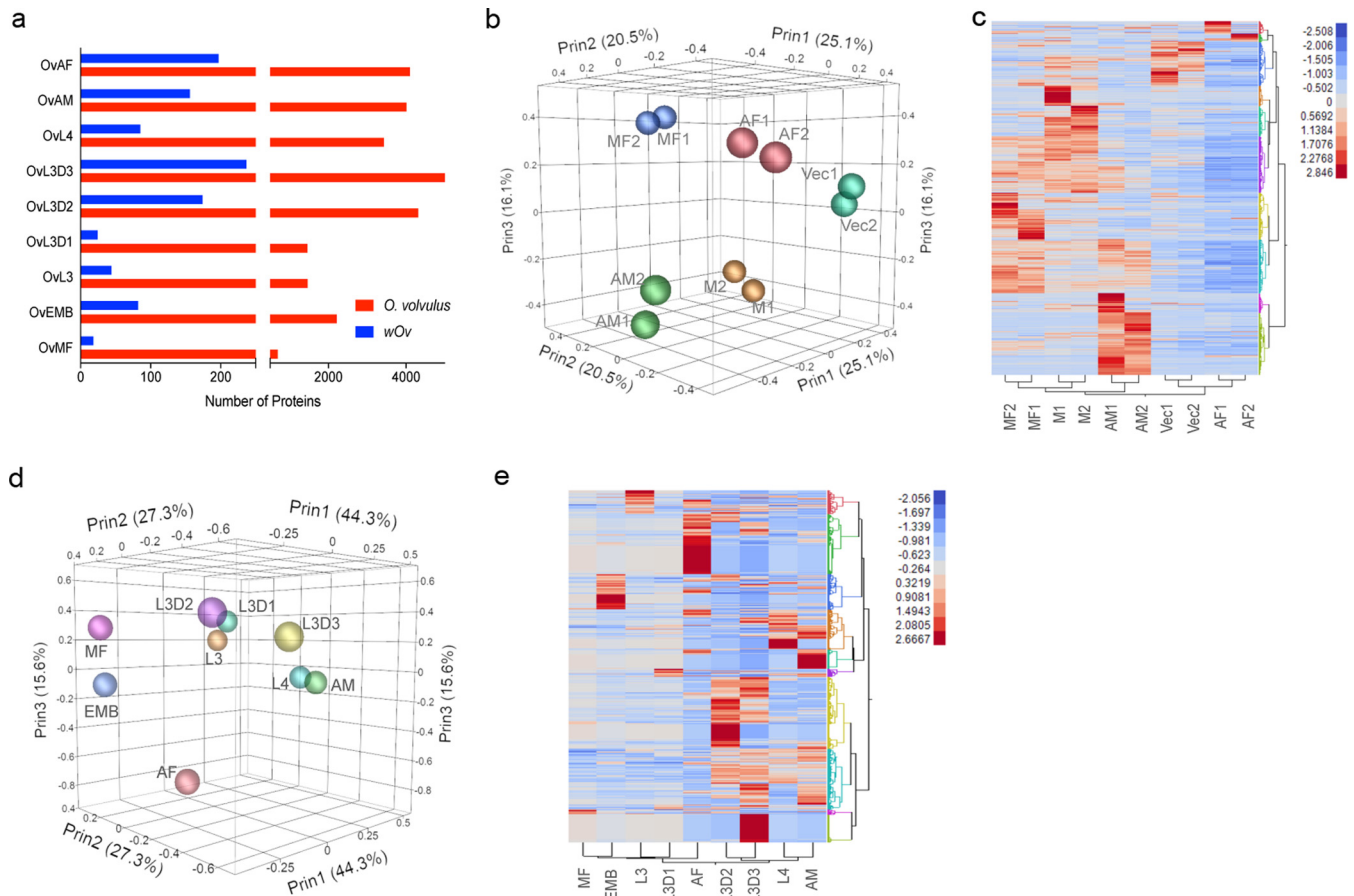
FIG 1 Overview of the transcriptome and proteome of *O. volvulus*. (a) Numbers of *O. volvulus*-specific (red) and *Wolbachia*-specific (blue) proteins identified across the stages by proteomic analyses. (b) Three-dimensional principal-component analysis plots highlighting the transcriptomic signatures of adult males (AM), adult females (AF), mf (MF), vector stages (Vec), and molting (M). (c) Heat map illustrating the hierarchical clustering of the normalized expression of transcripts of the stages in panel b. (d) Three-dimensional principal-component analysis plot highlighting the stage-specific proteomic signatures in adult females (AF), adult males (AM), embryos (EMB), mf, the L2 larval stage, the L3 larval stage, L3 larvae cultured *in vitro* for 1 to 3 days (L3D1, L3D2, and L3D3), and *in vitro*-developed L4 larvae (e). Heat map illustrating the hierarchical clustering of the normalized protein abundance in the stages analyzed in panel d.

biont (*w*Ov), a process made possible by having a high-quality assembly and annotation of the entire *O. volvulus* and *w*Ov genomes (9). Systematic comparisons across the parasite stages and across related nematodes have offered insights into the unique biology of this long-lived filarial parasite, while an immunomic approach has leveraged this information to allow the identification of potential vaccine and diagnostic candidates.

RESULTS

Transcriptome and proteome of *O. volvulus*. Transcriptome data obtained using RNA-seq obtained from most of the vector- and human-derived stages (see Fig. S1 in the supplemental material) of the parasite were not only used to help curate the *O. volvulus* genome annotation (9) but were used in the present study to understand stage-specific mRNA expression. Over 75% of the genes had 100% transcript coverage in all of the stages, with the exception of the adult female worm (http://exon.niaid.nih.gov/transcriptome/O_volvulus/v245/Ov-v245-web.xlsx), most of which, in contrast to the adult male, is composed of uterine tissue containing all of the intrauterine stages that range from early embryos through pretzel-like immature mf to mature mf (10). Because many transcripts with expression levels of <1 RPKM (reads per

kilobase of transcript per million mapped reads) were found to encode proteins whose products were validated in separate proteomic analyses, they also have been included in the analyses (https://exon.niaid.nih.gov/transcriptome/O_volvulus/Additional_file_1.xlsx).

The median coverage over the length of any predicted protein by peptides derived from the proteome of each of the stages profiled ranged between ~10 and 15% (see Fig. S2 in the supplemental material). A total of 7,774 *O. volvulus* proteins were identified across all of the stages, resulting in the validation of >64% of the predicted proteins (9). There was maximal proteomic coverage during the development of L3 to L4; adult male and female worms had the second most extensive coverage (Fig. 1a). We were also able to identify/validate the presence 465 of the 785 *Wolbachia* (*w*Ov) proteins predicted by the genomic sequencing of *w*Ov (Fig. 1a and the entire *w*Ov data set at http://exon.niaid.nih.gov/transcriptome/O_volvulus/v245/wOv_web/wOv_Web.xlsx). Overall, there was a high degree of correlation ($P < 0.0001$) between the RPKM (for the transcriptome) and the normalized protein abundance (for the proteome) (see Fig. S2 in the supplemental material), with *r* values that ranged from 0.25 to 0.39, values considered acceptable for global comparisons (11, 12).
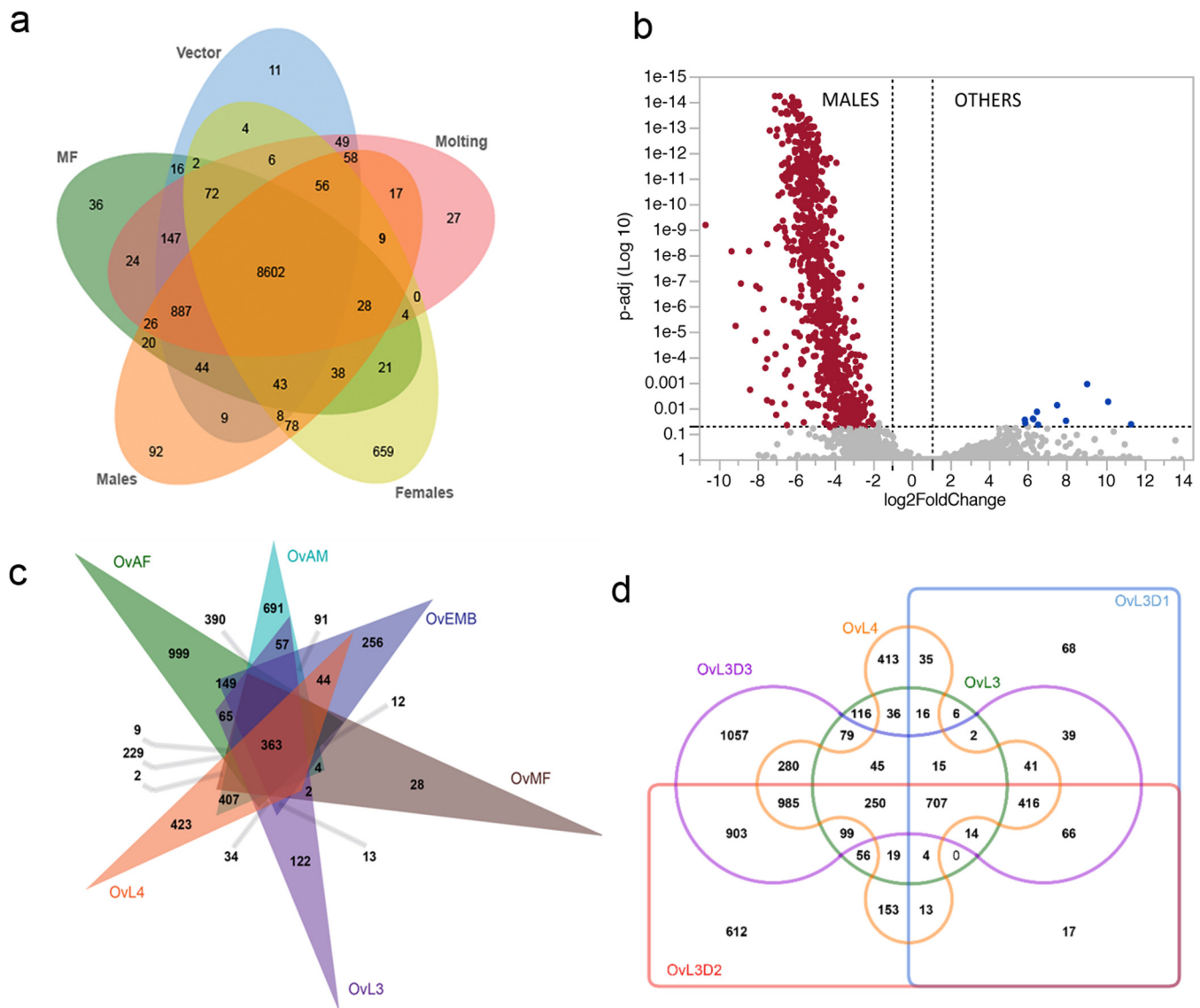
FIG 2 Stage specificity of *O. volvulus* transcriptomes and proteomes. (a) Venn diagram illustrating the numbers of stage-specific transcripts identified in mf, vector-derived stages, molting larvae, adult males, and adult females. (b) Genes differentially expressed in adult males compared to all other stages plotted using $\log_2$-fold changes on the x axis and adjusted P values ($\log_{10}$) on the y axis. (c, d) Venn diagrams illustrating the numbers of stage-specific proteins identified by mass spectrometry in the major somatic stages (c) and during the L3-to-L4 molt (d).

Multivariate analysis using closely related/overlapping larval stages as replicates ($r = >0.9$; see Fig. S3 in the supplemental material) revealed stage-specific transcript profiles that clearly segregated the L2 and L3 vector-derived stages (Vec1, Vec2), the early human developmental stages mimicked *in vitro* (L3 to L4 molting; M1, M2), microfilarial stages (MF1, MF2), adult males (AM1, AM2), and adult females (AF1, AF2) (Fig. 1b and c). Proteomic analyses (Fig. 1d and e) indicate a bias toward male-like expression profiles as the parasites develop from L3 to L4, suggesting a slight selective advantage for the males during early development. As expected from the principal-component analyses, there was a clear stage-specific clustering of the transcriptional and proteomic expression profiles (Fig. 1c and e). Although relatively few transcripts were expressed only in a single stage, except for the females (Fig. 2a), the adult males appeared to have the highest

number of differentially expressed transcripts (Fig. 2b). Comparative analysis based on *Caenorhabditis elegans* germline expression data (13) indicated that a majority (69%) of the male-associated differential gene expression is related to spermatogenesis (see Fig. S3 in the supplemental material and https://exon.niaid.nih.gov/transcriptome/O_volvulus/Additional_file_2.xlsx). Inferences, however, from *C. elegans* also appear to classify 6% of the *O. volvulus* male-enriched transcripts as oogenic. Of note, the mf have significantly increased expression of two hypothetical proteins (OVOC1851 and OVOC1852) that are unique to *O. volvulus*, along with OVOC1611, a member of the serine protease inhibitor (SPI) family (see Fig. S3 in the supplemental material).

Amino acid repeats make up 12 to 14% of most of the proteomes studied to date (14). A total of 1,590 *O. volvulus* proteins were found to harbor single amino acid repeats, tandem re-

peats, or sequence repeat regions (https://exon.niaid.nih.gov/transcriptome/O_volvulus/Additional_file_3.xlsx). In addition to the transcription factors and signaling proteins, uncharacterized proteins had the highest number of amino acid repeats in *O. volvulus* (https://exon.niaid.nih.gov/transcriptome/O_volvulus/Additional_file_4.tif). Proteins containing polyglutamine repeats are common in most organisms, including *O. volvulus* (https://exon.niaid.nih.gov/transcriptome/O_volvulus/Additional_file_4.tif) and other nematodes. While their exact role in *O. volvulus* is not clear, the expression of polyglutamine repeats was shown to be neurotoxic in both *C. elegans* and *Drosophila* and is dynamically regulated throughout the lifetime of an organism, suggesting a link to longevity (15). Interestingly, one of the lead *O. volvulus* vaccine candidates (16), Ov-RAL-2 (OVOC9988), has 11 glutamine residues in its amino terminus. These residues are probably not important for the protective ability of this protein, as the *Brugia malayi* homologue (Bm2001), that is also protective in animals, does not have this repeat (17).

Stage-specific functional enrichment. Although many of the putative proteins were classified into functional categories, ~20% were considered to be uncharacterized and unique (or divergent) (9). Clustering of these unique genes on the basis of transcript and protein abundances indicates not only that distinct subsets are enriched at specific stages (https://exon.niaid.nih.gov/transcriptome/O_volvulus/Additional_file_5.tif) but also that 7% have signatures (SignalP and SecretomeP 2.0) indicative of secretion (secreted-divergent) (see the wOv data set at http://exon.niaid.nih.gov/transcriptome/O_volvulus/v245/wOv_web/wOv_Web.xlsx). Although the functions of these uncharacterized gene products remain largely unknown, identifying the stages in which they are highly expressed may provide a method to infer their functions on the basis of associations with particular developmental processes, such as molting or embryogenesis.

There were no significant differences in the total number of genes or proteins identified per functional category across the various stages (https://exon.niaid.nih.gov/transcriptome/O_volvulus/Additional_file_6.tif). However, genes involved in metabolism, cytoskeletal elements, protein modification, protein export, proteasome, and transcription machinery had significantly lower expression (adjusted $P < 0.001$, analysis of variance [ANOVA]) in adult females than in all of the other stages. Gene set enrichment analyses (GSEA) indicated that selected functional categories were enriched on the basis of the stage (see Fig. S4 in the supplemental material). The adult female stage was associated with pathways linked to detoxification and the extracellular matrix (see Fig. S4). This enriched subset of extracellular-matrix-related genes was primarily composed of collagens and gene products associated with chitin synthesis machinery. Although the mf are an integral part of the fertile adult female, genes corresponding to NADH dehydrogenase activity (gene ontology [GO] category 0008137) and cytochrome *c* oxidase activity (GO category 0004129) were enriched in the adult females compared to all other stages. In contrast to the adult female worms, the mf stages showed significant enrichment of genes associated with protein synthesis (ribosomal proteins) and protein modification, with cyclophilins and chaperones (heat shock proteins) as the major contributors. These are likely part of the machinery required for cellular morphogenesis that occurs after the parasite is ingested by the blackfly vector. The expression of genes representing nuclear regulation was enriched in adult males, an enrichment that could

be attributed to DNA replication and spermatogenesis. In addition, molecular functions of nucleotide binding (GO category 0000166), peptidase activity (GO category 0070011), and phosphoprotein phosphatase activity (GO category 0004721) were the top GO processes enriched in adult male *O. volvulus*.

Insights into development. Analyses of protein abundance in each of the stages identified 363 proteins expressed in all of the somatic stages (Fig. 2c). Genes encoding proteins involved in metabolism, cytoskeletal elements, and protein modification made up more than 50% of these core genes. Proteins common in OvEMB and OvAF are likely to play a role in oogenesis and/or embryogenesis. Similarly, proteins identified exclusively during the L3-to-L4 molt highlight the machinery required during this important first developmental molt after adaption to the human host environment (Fig. 2d). On the basis of *C. elegans* RNA interference data, *O. volvulus* homologues of *C. elegans* that are associated with embryonic lethality (EMB), larval arrest (LVA), larval lethality (LVL), defective molting (MLT), and lethality (LET) phenotypes were observed to be enriched not only in the embryos, mf (and thereby adult females), and L3 larval stages (https://exon.niaid.nih.gov/transcriptome/O_volvulus/Additional_file_7.tif) but also in adult males. Similarly, *O. volvulus* encodes orthologues of the most critical genes essential for molting (on the basis of *C. elegans*; https://exon.niaid.nih.gov/transcriptome/O_volvulus/Additional_file_8.xlsx) during the L3-to-L4 transition.

The analyses also highlight other proteins that have been shown to be essential for molting and/or other developmental processes of filarial parasites. For example, embryogenesis and molting of filarial parasites are dependent on the activity of cathepsin L-like cysteine proteases (CPLs). During molting, CPLs stored in the glandular esophagus are released and help in the breakdown of the old cuticle and possibly support the synthesis of a new cuticle (18, 19). Analyses using EnsemblCompara (20) indicate an expansion of CPL-like enzymes in *O. volvulus* (9). Significantly increased expression of CPL and CPZ molecules (21) was observed in L2 and L3 larvae compared to that in other stages ($P < 0.002$, ANOVA). While their role during L3-to-L4 molting is known (19, 22, 23), their increased expression in L2 larvae suggests that CPLs and CPZ also play a role in the L2-to-L3 molt in the vector, similar to what was described in *B. malayi* (24). Interestingly, the GO categories of GTPase activity (GO category 0003924), oxidoreductase activity (GO category 0016491), procollagen-proline dioxygenase activity (GO categories 0004656 and 0019798), peptidyl-proline dioxygenase activity (GO categories 0031543 and 0031545), and protein disulfide oxidoreductase activity (GO category 0015035) were among the categories represented by the differentially expressed genes during both the L2-to-L3 and L3-to-L4 molting events. GSEA also identified an immunologically important class of molecules as being enriched in L3 larval stages (see Fig. S4 in the supplemental material) and a set of extracellular-matrix-related genes distinct from those extracellular matrix proteins enriched in adult female worms. Some of these include collagens that can be regulated by prolyl-4 hydroxylases, part of an expanded family of genes based on the analysis of the *O. volvulus* genome (9) that also shows differential expression across the various stages (https://exon.niaid.nih.gov/transcriptome/O_volvulus/Additional_file_9.tif).

Gene families—protein kinases, G protein-coupled receptors (GPCRs), and nuclear hormone receptors (NHRs). Approximately 55% of the predicted proteins had significant matches
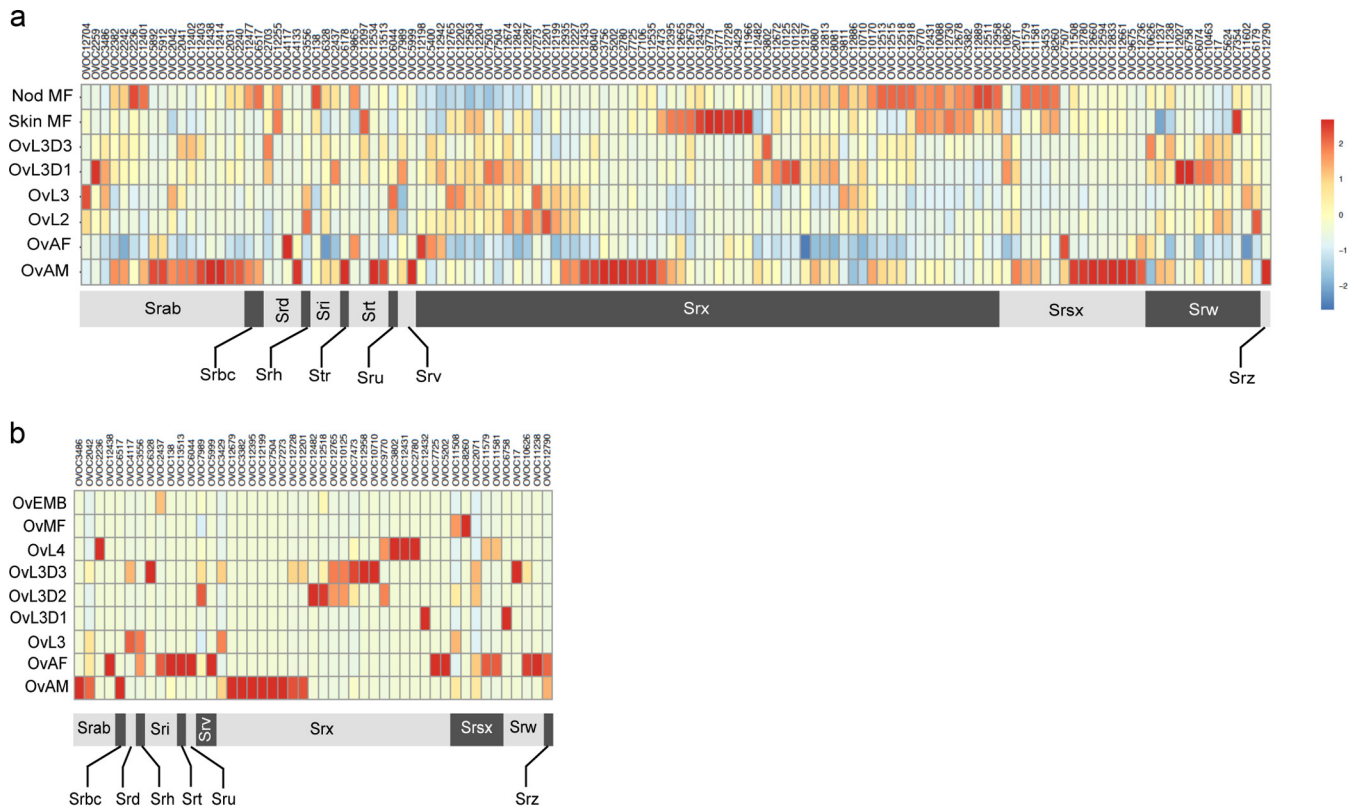
FIG 3 *O. volvulus* stage-specific expression of G protein-coupled receptors (GPCRs). Shown are heat maps depicting the stage-specific expression of GPCRs in *O. volvulus* ordered by family. Values plotted are the *z* scores of RPKM (a) and normalized spectral abundance factors of proteins (b). Red to blue denotes high to low expression, as shown on the scale to the right.

(<1E-05) to Pfam databases, with a protein kinase domain (Pkinase, PF00069), an RNA recognition motif (RRM_1, PF00076), and a seven-transmembrane receptor (7TM_1, PF00001) being the top three represented categories. The Pkinase domain forms the catalytic center of protein kinases that are involved in a wide variety of cellular processes influencing apoptosis, differentiation, and embryonic development. Their increased stage-specific increased expression (see Fig. S5 in the supplemental material) likely reflects their role in germline differentiation (in adult worms) or in larval developmental stages (mf and L3). Comparative analyses of the *O. volvulus* kinome (see Table S1 in the supplemental material) with other filarial and nonfilarial control kinomes resulted in the identification of *O. volvulus* kinases that are unique or belong to nematode-specific families (25) (see Table S2 in the supplemental material). More interestingly, the expression of casein kinase 1 and its subfamily the Tau tubulin kinase-like (CK1/TTBKL) and FER subfamily of tyrosine kinases (TK/FER) was enriched in adult males (https://exon.niaid.nih.gov/transcriptome/O_volvulus/Additional_file_10.xlsx). Both CK1 and Fer kinases have been implicated in mammalian Wnt signaling. The nonreceptor tyrosine kinase of the Fer family was also shown to be important in spermatogenesis (26). These nematode-specific kinases—clearly distinct from human, fruit fly, and yeast kinases—may provide insights into comparative worm biology studies and are very tractable drug targets.

Among the seven-transmembrane receptors, the GPCR gene families are greatly expanded in *O. volvulus* (9). Hierarchical clus-

tering of the transcriptional and proteomic expression data reveals clear patterns of stage-specific expression (Fig. 3). Some members of the Srx family show very high expression in the skin and nodular mf, while other families show increased expression in adult males. These patterns reveal unique chemosensory requirements for certain stages of *O. volvulus*, including migratory adult males and skin mf, compared to the more stationary female worms.

The NHRs are known to play an important role in nematode developmental processes (27). In comparison to *C. elegans* and *B. malayi*, the NHR repertoire is smaller in *O. volvulus* (9) but still contains all five NHR genes that play a role in molting and embryogenesis (28, 29). Indeed, the *O. volvulus* ecdysone receptor (EcR, OVOC9104) and NHR RXR (OVOC2435) show increased expression during the L3-to-L4 molt (https://exon.niaid.nih.gov/transcriptome/O_volvulus/Additional_file_11.tif). Furthermore, GSEA indicate enrichment of other potential NHRs (such as OVOC351 and OVOC353) involved in signal transduction processes of adult female worms ($P < 0.0001$; false discovery rate, <1%). Similarly, orthologues of the *C. elegans* NHRs *nhr-6* (OVOC8200), *nhr-23* (OVOC464), *nhr-25* (OVOC2839), *nhr-41* (OVOC4741), and *nhr-85* (OVOC827), known to be involved in molting and metamorphosis, were detected as transcripts or proteins during the *in vitro* L3-to-L4 molt. In addition, NHRs implicated in neural differentiation (OVOC635, OVOC3708) and sex determination (OVOC5276) had increased expression levels in the molting stages, reflecting their likely role in development. The

distinct expression profile of OVOC2265 (*nhr-32*) predominantly in the proteomes of the nodular mf and embryonic stages (https://exon.niaid.nih.gov/transcriptome/O_volvulus/Additional_file_11.tif) suggests a possible role in embryogenesis. Among other embryo-enriched transcripts and proteins, OVOC11613 (immunodominant or major antigen) (30) and OVOC9384 (Oveg1) (31) were shown to be related to embryogenesis as well. Moreover, though the precise role of *Ce*-LFI-1 (orthologue of OVOC11613) in development is not yet known, it interacts with LIN-5, which is essential for proper spindle positioning and chromosome segregation (32), an essential feature of cell division.

Secretome and host-parasite interactions. Approximately 20% of the genes in the *O. volvulus* genome are predicted to be secreted through classical secretion mechanisms, and about ~42% are predicted to be secreted through nonclassical mechanisms. All filarial worms are known to release excretory/secretory (E/S) products that are critical components in the helminth arsenal of proteins that perform diverse functions (33). These include (i) modulation of the host immune response, (ii) remodeling of host tissue, (iii) alteration of host tissue nutritional status, and (iv) enhancement of larval tissue migration (34). The *O. volvulus* genome contains many of these immunologically relevant genes (9), among which are the L3-enriched or mf-enriched cystatins that have been shown to interfere with antigen processing and presentation to T cells (35), small SPIs (Ov-SPI-1, Ov-SPI-2), and serpins (https://exon.niaid.nih.gov/transcriptome/O_volvulus/Additional_file_12.tif). SPIs also play an important role in controlling the molting process (36) and immune evasion (37). The analysis of the *O. volvulus* genome revealed the presence of 10 additional SPIs beyond the 2 previously identified, Ov-SPI-1 and Ov-SPI-2 (9). Eleven of these 12 are highly expressed during the L3-to-L4 molt, consistent with their potential role in molting or immune invasion during the first days of establishment in the human host (https://exon.niaid.nih.gov/transcriptome/O_volvulus/Additional_file_12.tif). Interestingly, 2 of the 12 SPIs are highly expressed in adult males, consistent with a previous study demonstrating SPIs being localized in sperm within the adult male testis (36). The other immunologically relevant protein enrichment was the *O. volvulus* adult male-enriched expression of indoleamine 2,3-dioxygenase, an interferon-inducible enzyme that suppresses adaptive T-cell immunity, and the enrichment of the homologue of suppressor of cytokine signaling 7 (SOCS7; OVOC678) in L3/L4 developmental stages.

Stage-specific expression of serine, aspartic, cysteine, and metalloproteases reflects their roles in host invasion, molting and migration in nematodes (https://exon.niaid.nih.gov/transcriptome/O_volvulus/Additional_file_13.tif). Scanning of the *O. volvulus* genome, notably, identified the presence of 13 chitinases, 8 of which belong to glycosyl hydrolase family 18. We know that the larva-specific chitinase (Ov-CHI-1) plays an important role in parasite transmission, molting, and remodeling of the L4 cuticle (38), the inhibition of which inhibits larval molting (38, 39). The relatively higher expression of Ov-CHI-1 in the L2 larval stages suggests a probable role in migration through insect tissues (https://exon.niaid.nih.gov/transcriptome/O_volvulus/Additional_file_13.tif). The increased expression of what had been thought to be larva-specific chitinases in the *O. volvulus* adult male—seen in both transcriptomic and proteome data—raises the possibility that these glycosyl hydrolase 18 family molecules have another functional role not previously recognized.

***Wolbachia* proteome.** *Wolbachia*, a genus of *Alphaproteobacteria*, infects an estimated two-thirds of all arthropod species. The members of this genus are probably the most abundantly and vertically transmitted organisms that exist in facultative or obligate endosymbiotic associations (40). In nematodes, their prevalence is restricted to two families of worms, *Onchocercidae* and *Pratylenchidae* (41), with *Onchocercidae* exhibiting widespread obligate mutualism, as evidenced by retarded larval growth (42), embryostasis (43), and death (44) when the *Wolbachia* bacteria are eliminated. Because we performed RNA-seq analyses with poly(A)-enriched transcripts, *w*Ov transcript identification was limited. Proteomic analyses, however, identified 465 *w*Ov proteins, some of which were common to or uniquely expressed in specific stages of *O. volvulus* (Fig. 1a and Fig. 4a). Comparative analyses with the *Wolbachia* symbiont of the bovine parasite *Onchocerca ochengi* (*w*Oo) (45) was limited because of a lack of corresponding stage-specific protein identifications. However, there was a significant correlation with the number of peptides of wolbachial origin identified from adult *O. ochengi* females (OoAF) and OvAF and OvEMB (see Fig. S6 in the supplemental material). Similar to what was observed in the proteomes of *w*Bm (46) and *w*Oo (45), the GroEL (wOv00466), outer membrane protein (wOv00621), outer surface protein (WSP, wOv00566), and molecular chaperone DnaK (wOv00687) were among the most commonly identified abundant proteins (top 25) across most of the stages. Clustering of the normalized protein abundance values resulted in the identification of *w*Ov clusters that were specifically OvAF, OvEMB, OvL3D2, or OvL3D3 abundant (Fig. 4b). Among the clusters overrepresented in OvL3-to-L4 development (Fig. 4b, right), many could be mapped to functional categories, with the top five functions being (i) translation, ribosomal structure, and biogenesis; (ii) posttranslational modification, protein turnover, and chaperone; (iii) energy production and conversion; (iv) coenzyme metabolism and cell envelope biogenesis, and (v) outer membrane proteins (Fig. 4c).

The increase in proteins related to particular functions in certain developmental stages may reflect multiplication of *Wolbachia* bacteria within these defined stages. In these stages, such as the developing L3 and in the adult female (and thus during embryogenesis), when the metabolic needs are the greatest, it is possible that the increased *w*Ov replication seen may reflect the symbiotic needs of the *O. volvulus* parasite during growth and development (47).

Biomarkers—an immunomic approach. Using an immunomic approach (48–50), we profiled host antibody responses to a subset of parasite stage-specific proteins (397 proteins). These selected proteins (https://exon.niaid.nih.gov/transcriptome/O_volvulus/Additional_file_14.xlsx) were printed on a protein array, quality checked, and assessed for isotype-specific antibody responses (IgG1, IgG3, IgG4, and IgE) (Fig. 5b and c) by using serum samples from 52 individuals comprising *O. volvulus*-infected, putatively immune, and control individuals from Ecuador (51), Guatemala (52), and Cameroon (53). After normalization, clusters specific for IgG4, IgG3 and/or IgG1, and IgE reactivities were identified. Further analyses led to the identification of OVOC10819, OVOC5395, OVOC11598, OVOC12235, OVOC8619, and OVOC7083 as potential novel vaccine candidates (Fig. 5d) on the basis of significant IgG1 and/or IgG3 reactivity (with little to no IgE reactivity [54]) or potentially allergenic proteins that induce elevated levels of IgE (OVOC9414, OVOC7138). The identification of proteins that are
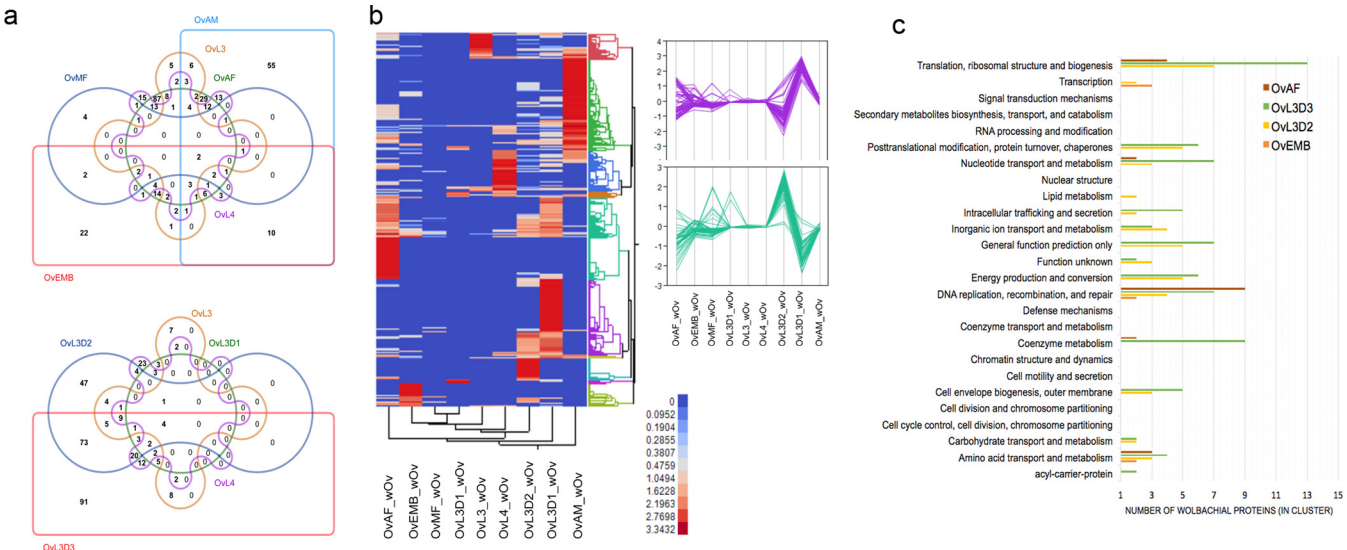
FIG 4 The *w*Ov proteome. (a) Shown are Venn diagrams illustrating the number of stage-specific *w*Ov proteins identified in the somatic proteomes of the major life stages (top) and during the L3-to-L4 molt (bottom). (b) Heat map of *w*Ov stage-specific protein expression with blue to red indicating low to high expression and parallel coordinate plots of protein clusters specifically expressed during the L3-to-L4 molt (right). (c) Number of *w*OV proteins with increased expression during the L3-to-L4 molt (OvL3D2, OvL3D3), in adult females (OvAF), and in embryos (OvEMB) by functional category.

highly expressed by the mf (see Table S3 in the supplemental material and https://exon.niaid.nih.gov/transcriptome/O_volvulus/Additional_file_15.tif) and are specifically recognized by serum samples from protected individuals opens up new possibilities for the development of a safe antitransmission or therapeutic vaccine

(55). Because IgG4 reactivity to filarial antigens provides better serodiagnostic species specificity (56, 57) than other isotypes, the data were also analyzed for IgG4-reactive proteins. On the basis of the IgG4 responses (Fig. 5e), we were able to identify heretofore unrecognized biomarkers of active infection (e.g., OVOC10469,
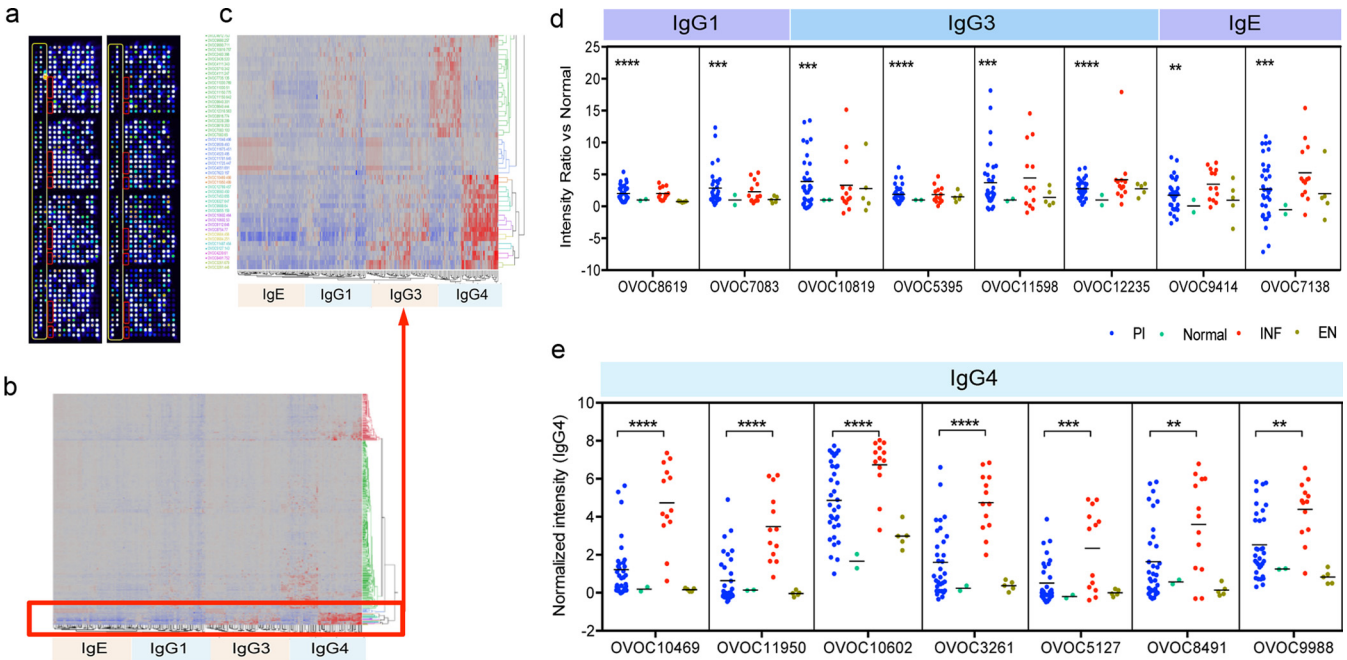

FIG 5 *O. volvulus* protein array. (a) Quality control of *O. volvulus* protein array chips with anti-HA (right) and anti-His (left) antibodies. (b) Heat map depicting the clustered data from all samples for all isotypes with a blowup of the red-boxed image in panel c highlighting the cluster of IgG4-reactive proteins. Red to blue denotes high to low reactivity. (d) Scatter plots of representative proteins with significant IgG1, IgG3, and IgE responses (potentially allergenic) in putatively immune individuals compared to infected individuals, plotted as ratios with respect to normal serum samples. **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P < 0.0001$ (one-way ANOVA corrected for multiple comparisons [Holm-Sidak]). (e) Scatter plot of representative proteins with significant IgG4 reactivity in infected individuals, plotted as normalized intensity. PI, putatively immune; INF, infected individuals; EN, endemic normal individuals; Normal, pools of healthy normal donors.

OVOC10602, OVOC11950, OVOC3261, OVOC5127, OVOC8491, and OVOC9988). Indeed, we have successfully tested and validated OVOC10469 in a luciferase immunoprecipitation system (LIPS) immunoassay that highlights not only the utility of this protein as a biomarker of active *O. volvulus* infection (https://exon.niaid.nih.gov/transcriptome/O_volvulus/Additional_file_16.tif) but also the usefulness of this type of immunomic approach.

DISCUSSION

Using a combination of transcriptomic and proteomic data, we performed stage-specific analyses that are, to our knowledge, the most comprehensive for any of the filarial parasites. Because of the limited availability of *O. volvulus* larval-stage-specific material, we used the transcriptomes of the closely related/overlapping larval stages as biological replicates, since their expression patterns were significantly similar (see Fig. S3 in the supplemental material and https://exon.niaid.nih.gov/transcriptome/O_volvulus/Additional_file_17.xlsx).

Further, for the proteomic analyses, we chose depth of coverage over distribution of the limited stage-specific sample for technical replicates, with the rationale that although a difference in transcript (RNA) and protein recovery from the various stages is expected, normalized data (RPKM and spectral abundance) provide provisional evidence of the relative abundance of any particular gene or protein at a given stage. This data set thus provides an in-depth resource for understanding and analyzing the biological pathways that are critical for the development of the various stages of the parasite in the vector and human hosts, in host-worm interactions, and for the identification of novel biomarkers (e.g., OVOC10469, OVOC3261, and OVOC12838) and targets for interventions. Comparative stage-specific proteomic analyses of the recently available *O. ochengi* (58) proteome and the corresponding *O. volvulus* stages demonstrate a relatively high degree of correlation (https://exon.niaid.nih.gov/transcriptome/O_volvulus/Additional_file_18.tif), albeit a weaker one than that with the more distant species *B. malayi* (46) (https://exon.niaid.nih.gov/transcriptome/O_volvulus/Additional_file_19.tif) that is likely due to differences in coverage depth (58).

Natural immunity to *O. volvulus* can be acquired in affected populations by a few individuals who have been qualified as being putatively immune (52, 59, 60). The hypothesis is that they are protected because they are able to mount an appropriate immune response against the L3 larvae; surface proteins of L3 larvae and/or the E/S products released by molting larvae are considered to be an important source of such protective antigens (59). The identification of *O. volvulus* unique proteins that are larva-specific or the identification of adult and/or mf stage-specific biomarkers suggests that tools are already available to help achieve the lofty goal of onchocerciasis elimination in the coming decades.

MATERIALS AND METHODS

Detailed materials and methods are available in Text S1 in the supplemental material.

Parasite samples. Parasite material used for RNA-seq and proteomic analyses was collected at the research facility at the Tropical Research Station, Kumba, Cameroon (53), or in Ecuador (51). Adult worm samples were obtained from nodules excised during nodulectomies. L3 larvae were obtained from *Simulium damnosum* flies 7 to 8 days after infection with skin mf as described previously (61). Fresh L3 larvae were also cultured *in vitro* at 37°C for 1 to 3 days (L3D1, L3D2, and L3D3) or for 6 days when they molt and L4 were then collected. Nodular and skin mf were purified

as described previously (62, 63). Embryonic stages were purified from mf and eggs that were extruded into the medium during the process of cleaning female adult worms.

Human serum samples. All human serum samples were obtained by using protocols approved by the Institutional Review Board of either the National Institute of Allergy and Infectious Diseases (Guatemala or Ecuador) or by the New York Blood Center and the Tropical Research Station, Kumba, Cameroon. All infected people were microfilaria positive on the basis of skin snip analyses.

RNA-seq, assembly, and analyses. RNA-seq libraries were prepared from parasite stages in accordance with the RNA-seq protocols of the Illumina mRNA-Seq Sample Prep kit and the Illumina TruSeq kit. The reads were analyzed as previously described (64). Genes that had blast scores of <30% of the maximum possible score (self-blast) in other nematodes with E values of >1E-05 were considered unique. The normalized transcriptome data were analyzed in JMP Genomics (SAS Inc., Cary, NC) for general assessment of distribution analyses, correlations, principal-component analyses, ANOVA, hierarchical clustering and heat map generation, and parallel coordinate plots.

Protein isolation. For proteomic analyses, additional stages of embryos (OvEMB) and L3D2 (OvL3D2) and L4 (OvL4) larvae were also analyzed. Total soluble proteins were extracted from all of the stages, dialyzed, desalted, and digested with trypsin.

Liquid chromatography-tandem mass spectrometry. Liquid chromatography was performed with the Easy-nLC 1000 UHPLC system (Thermo). The spectra were searched by using a combined database of *O. volvulus*, *w*Ov, and human proteins. Proteins were required to have one or more unique peptides across the samples analyzed with E values of ≤0.0001.

Protein arrays. The genes of interest were cloned into T7 expression vector pXT7. Proteins were expressed with a coupled *in vitro* transcription and translation system, the *Escherichia coli*-based cell-free Rapid Translation System 100 High Yield kit (5 Prime), as 10×His-tagged or hemagglutinin (HA)-tagged fusion proteins and printed onto arrays. The arrays were probed with serum samples, and isotype reactivity was detected with Cy5-labeled antibodies. The slides were scanned, and reactivity was quantified. The quantified data were normalized and analyzed for significant proteins.

LIPS assay. For evaluation of antibody titers, a standard antibody-based LIPS assay (65) was performed.

Accession number(s). The entire *O. volvulus* data set analyzed is available as a hyperlinked Excel workbook at http://exon.niaid.nih.gov/transcriptome/O_volvulus/v245/Ov-v245-web.xlsx; the *w*Ov data set is available as a hyperlinked Excel workbook at http://exon.niaid.nih.gov/transcriptome/O_volvulus/v245/wOv_web/wOv_Web.xlsx. The RNA-seq data are available under BioProject PRJEB2965 (*O. volvulus*) under the accession numbers shown in the supplemental material. The mass spectrometry proteomic data have been deposited in the ProteomeXchange Consortium via the PRIDE partner repository under the data set identifier PXD003585.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.02028-16/-/DCSupplemental.

Text S1, DOCX file, 0.1 MB.
Figure S1, TIF file, 12.5 MB.
Figure S2, TIF file, 14.6 MB.
Figure S3, TIF file, 17.7 MB.
Figure S4, TIF file, 10.9 MB.
Figure S5, TIF file, 9.8 MB.
Figure S6, TIF file, 13 MB.
Table S1, XLSX file, 0.1 MB.
Table S2, XLSX file, 0.1 MB.
Table S3, XLSX file, 0.2 MB.

REFERENCES

1. Hotez PJ, Alvarado M, Basáñez MG, Bolliger I, Bourne R, Boussinesq M, Brooker SJ, Brown AS, Buckle G, Budke CM, Carabin H, Coffeng LE, Fèvre EM, Fürst T, Halasa YA, Jasrasaria R, Johns NE, Keiser J, King CH, Lozano R, Murdoch ME, O'Hanlon S, Pion SD, Pullan RL, Ramaiah KD, Roberts T, Shepard DS, Smith JL, Stolk WA, Undurraga EA, Utzinger J, Wang M, Murray CJ, Naghavi M. 2014. The global burden of disease study 2010: interpretation and implications for the neglected tropical diseases. PLoS Negl Trop Dis 8:e2865. http://dx.doi.org/10.1371/journal.pntd.0002865.

2. WHO. 2016. Investing to overcome the global impact of neglected tropical diseases: third WHO report on negelected tropical diseases. World Health Organization, Geneva, Switzerland.

3. Centers for Disease Control and Prevention (CDC). 2013. Progress toward elimination of onchocerciasis in the Americas—1993–2012. MMWR Morb Mortal Wkly Rep 62:405–408. https://www.cdc.gov/mmwr/preview/mmwrhtml/mm6220a4.htm.

4. WHO. 2014. African programme for onchocerciasis control: progress report, 2013–2014. Wkly Epidemiol Rec 89:551–560.

5. Keenan JD, Hotez PJ, Amza A, Stoller NE, Gaynor BD, Porco TC, Lietman TM. 2013. Elimination and eradication of neglected tropical diseases with mass drug administrations: a survey of experts. PLoS Negl Trop Dis 7:e2562. http://dx.doi.org/10.1371/journal.pntd.0002562.

6. Kim YE, Remme JH, Steinmann P, Stolk WA, Roungou JB, Tediosi F. 2015. Control, elimination, and eradication of river blindness: scenarios, timelines, and ivermectin treatment needs in Africa. PLoS Negl Trop Dis 9:e0003664. http://dx.doi.org/10.1371/journal.pntd.0003664.

7. Gardon J, Gardon-Wendel N, Demanga-Ngangue N, Kamgno J, Chippaux JP, Boussinesq M. 1997. Serious reactions after mass treatment of onchocerciasis with ivermectin in an area endemic for Loa loa infection. Lancet 350:18–22. http://dx.doi.org/10.1016/S0140-6736(96)11094-1.

8. Lustigman S, McCarter JP. 2007. Ivermectin resistance in Onchocerca volvulus: toward a genetic basis. PLoS Negl Trop Dis 1:e76. http://dx.doi.org/10.1371/journal.pntd.0000076.

9. Cotton JA, Bennuru S, Grote A, Harsha B, Tracey A, Beech R, Doyle SR, Dunn M, Hotopp JCD, Holroyd N, Kikuchi T, Lambert O, Mhashilkar AS, Mutowo P, Nursimulu N, Ribeiro JM, Rogers MB, Stanley E, Swapna LS, Tsai IJ, Unnasch TR, Voronin D, Parkinson J, Nutman TB, Ghedin E, Berriman M, Lustigman S. The genome of Onchocerca volvulus, agent of river blindness. Nat Microbiol, in press.

10. Neafie RC. 1972. Morphology of Onchocerca volvulus. Am J Clin Pathol 57:574–586. http://dx.doi.org/10.1093/ajcp/57.5.574.

11. Ning K, Fermin D, Nesvizhskii AI. 2012. Comparative analysis of different label-free mass spectrometry based protein abundance estimates and their correlation with RNA-Seq gene expression data. J Proteome Res 11:2261–2271. http://dx.doi.org/10.1021/pr201052x.

12. Vogel C, Marcotte EM. 2012. Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. Nat Rev Genet 13:227–232. http://dx.doi.org/10.1038/nrg3185.

13. Ortiz MA, Noble D, Sorokin EP, Kimble J. 2014. A new dataset of spermatogenic vs. oogenic transcriptomes in the nematode Caenorhabditis elegans. G3 (Bethesda) 4:1765–1772. http://dx.doi.org/10.1534/g3.114.012351.

14. Depledge DP, Lower RP, Smith DF. 2007. RepSeq—a database of amino acid repeats present in lower eukaryotic pathogens. BMC Bioinformatics 8:122. http://dx.doi.org/10.1186/1471-2105-8-122.

15. Brignull HR, Morley JF, Garcia SM, Morimoto RI. 2006. Modeling polyglutamine pathogenesis in C. elegans. Methods Enzymol 412:256–282. http://dx.doi.org/10.1016/S0076-6879(06)12016-9.

16. Hess JA, Zhan B, Bonne-Année S, Deckman JM, Bottazzi ME, Hotez PJ, Klei TR, Lustigman S, Abraham D. 2014. Vaccines to combat river blindness: expression, selection and formulation of vaccines against infection with Onchocerca volvulus in a mouse model. Int J Parasitol 44:637–646. http://dx.doi.org/10.1016/j.ijpara.2014.04.006.

17. Arumugam S, Wei J, Liu Z, Abraham D, Bell A, Bottazzi ME, Hotez PJ, Zhan B, Lustigman S, Klei TR. 2016. Vaccination of gerbils with Bm-103 and Bm-RAL-2 concurrently or as a fusion protein confers consistent and improved protection against Brugia malayi infection. PLoS Negl Trop Dis 10:e0004586. http://dx.doi.org/10.1371/journal.pntd.0004586.

18. Lustigman S, Zhang J, Liu J, Oksov Y, Hashmi S. 2004. RNA interference targeting cathepsin L and Z-like cysteine proteases of Onchocerca volvulus confirmed their essential function during L3 molting. Mol Biochem Parasitol 138:165–170. http://dx.doi.org/10.1016/j.molbiopara.2004.08.003.

19. Lustigman S, McKerrow JH, Shah K, Lui J, Huima T, Hough M, Brotman B. 1996. Cloning of a cysteine protease required for the molting of Onchocerca volvulus third stage larvae. J Biol Chem 271:30181–30189. http://dx.doi.org/10.1074/jbc.271.47.30181.

20. Vilella AJ, Severin J, Ureta-Vidal A, Heng L, Durbin R, Birney E. 2009. EnsemblCompara GeneTrees: complete, duplication-aware phylogenetic trees in vertebrates. Genome Res 19:327–335. http://dx.doi.org/10.1101/gr.073585.107.

21. Wu Y, Egerton G, Underwood AP, Sakuda S, Bianco AE. 2001. Expression and secretion of a larval-specific chitinase (family 18 glycosyl hydrolase) by the infective stages of the parasitic nematode, Onchocerca volvulus. J Biol Chem 276:42557–42564. http://dx.doi.org/10.1074/jbc.M103479200.

22. Guiliano DB, Hong X, McKerrow JH, Blaxter ML, Oksov Y, Liu J, Ghedin E, Lustigman S. 2004. A gene family of cathepsin L-like proteases of filarial nematodes are associated with larval molting and cuticle and eggshell remodeling. Mol Biochem Parasitol 136:227–242. http://dx.doi.org/10.1016/j.molbiopara.2004.03.015.

23. Ford L, Zhang J, Liu J, Hashmi S, Fuhrman JA, Oksov Y, Lustigman S. 2009. Functional analysis of the cathepsin-like cysteine protease genes in adult Brugia malayi using RNA interference. PLoS Negl Trop Dis 3:e377. http://dx.doi.org/10.1371/journal.pntd.0000377.

24. Song C, Gallup JM, Day TA, Bartholomay LC, Kimber MJ. 2010. Development of an in vivo RNAi protocol to investigate gene function in

the filarial nematode, Brugia malayi. PLoS Pathog **6:**e1001239. http://dx.doi.org/10.1371/journal.ppat.1001239.

25. **Taylor CM, Martin J, Rao RU, Powell K, Abubucker S, Mitreva M.** 2013. Using existing drugs as leads for broad spectrum anthelmintics targeting protein kinases. PLoS Pathog **9:**e1003149. http://dx.doi.org/10.1371/journal.ppat.1003149.

26. **Manning G.** 2005. Genomic overview of protein kinases. WormBook **13:** 1–19. http://dx.doi.org/10.1895/wormbook.1.60.1.

27. **Sluder AE, Maina CV.** 2001. Nuclear receptors in nematodes: themes and variations. Trends Genet **17:**206–213. http://dx.doi.org/10.1016/S0168-9525(01)02242-9.

28. **Tzertzinis G, Egaña AL, Palli SR, Robinson-Rechavi M, Gissendanner CR, Liu C, Unnasch TR, Maina CV.** 2010. Molecular evidence for a functional ecdysone signaling system in Brugia malayi. PLoS Negl Trop Dis **4:**e625. http://dx.doi.org/10.1371/journal.pntd.0000625.

29. **Mhashilkar AS, Adapa SR, Jiang RH, Williams SA, Zaky W, Slatko BE, Luck AN, Moorhead AR, Unnasch TR.** 2016. Phenotypic and molecular analysis of the effect of 20-hydroxyecdysone on the human filarial parasite Brugia malayi. Int J Parasitol **46:**333–341 http://dx.doi.org/10.1016/j.ijpara.2016.01.005.

30. **Triteeraprapab S, Richie TL, Tuan RS, Shepley KJ, Dinman JD, Neubert TA, Scott AL.** 1995. Molecular cloning of a gene expressed during early embryonic development in Onchocerca volvulus. Mol Biochem Parasitol **69:**161–171. http://dx.doi.org/10.1016/0166-6851(94)00187-R.

31. **Tume C, Sun E, Titanji V, Bathurst I, Barr P, Hough M, Weiss N, Chakravarti B, Wahi M, Lackey A, Staunton C, Lustigman S, McKerrow JH.** 1995. Onchocerca volvulus: identification and characterization of an immunogenic eggshell protein (Oveg1). Exp Parasitol **81:**445–452. http://dx.doi.org/10.1006/expr.1995.1137.

32. **Fisk Green R, Lorson M, Walhout AJ, Vidal M, van den Heuvel S.** 2004. Identification of critical domains and putative partners for the Caenorhabditis elegans spindle component LIN-5. Mol Genet Genomics **271:** 532–544. http://dx.doi.org/10.1007/s00438-004-1012-x.

33. **Harnett W.** 2014. Secretory products of helminth parasites as immunomodulators. Mol Biochem Parasitol **195:**130–136. http://dx.doi.org/10.1016/j.molbiopara.2014.03.007.

34. **McKerrow JH, Caffrey C, Kelly B, Loke P, Sajid M.** 2006. Proteases in parasitic diseases. Annu Rev Pathol **1:**497–536. http://dx.doi.org/10.1146/annurev.pathol.1.110304.100151.

35. **Hartmann S, Lucius R.** 2003. Modulation of host immune responses by nematode cystatins. Int J Parasitol **33:**1291–1302. http://dx.doi.org/10.1016/S0020-7519(03)00163-2.

36. **Ford L, Guiliano DB, Oksov Y, Debnath AK, Liu J, Williams SA, Blaxter ML, Lustigman S.** 2005. Characterization of a novel filarial serine protease inhibitor, Ov-SPI-1, from Onchocerca volvulus, with potential multifunctional roles during development of the parasite. J Biol Chem **280:** 40845–40856. http://dx.doi.org/10.1074/jbc.M504434200.

37. **Maizels RM, Gomez-Escobar N, Gregory WF, Murray J, Zang X.** 2001. Immune evasion genes from filarial nematodes. Int J Parasitol **31:** 889–898. http://dx.doi.org/10.1016/S0020-7519(01)00213-2.

38. **Gooyit M, Tricoche N, Lustigman S, Janda KD.** 2014. Dual protonophore-chitinase inhibitors dramatically affect O. volvulus molting. J Med Chem **57:**5792–5799. http://dx.doi.org/10.1021/jm5006435.

39. **Gloeckner C, Garner AL, Mersha F, Oksov Y, Tricoche N, Eubanks LM, Lustigman S, Kaufmann GF, Janda KD.** 2010. Repositioning of an existing drug for the neglected tropical disease onchocerciasis. Proc Natl Acad Sci U S A **107:**3424–3429. http://dx.doi.org/10.1073/pnas.0915125107.

40. **Gill AC, Darby AC, Makepeace BL.** 2014. Iron necessity: the secret of Wolbachia's success? PLoS Negl Trop Dis **8:**e3224. http://dx.doi.org/10.1371/journal.pntd.0003224.

41. **Ferri E, Bain O, Barbuto M, Martin C, Lo N, Uni S, Landmann F, Baccei SG, Guerrero R, de Souza Lima S, Bandi C, Wanji S, Diagne M, Casiraghi M.** 2011. New insights into the evolution of Wolbachia infections in filarial nematodes inferred from a large range of screened species. PLoS One **6:**e20843. http://dx.doi.org/10.1371/journal.pone.0020843.

42. **Hoerauf A, Nissen-Pähle K, Schmetz C, Henkle-Dührsen K, Blaxter ML, Büttner DW, Gallin MY, Al-Qaoud KM, Lucius R, Fleischer B.** 1999. Tetracycline therapy targets intracellular bacteria in the filarial nematode Litomosoides sigmodontis and results in filarial infertility. J Clin Invest **103:**11–18. http://dx.doi.org/10.1172/JCI4768.

43. **Bandi C, McCall JW, Genchi C, Corona S, Venco L, Sacchi L.** 1999. Effects of tetracycline on the filarial worms Brugia pahangi and Dirofilaria

44. immitis and their bacterial endosymbionts Wolbachia. Int J Parasitol **29:** 357–364. http://dx.doi.org/10.1016/S0020-7519(98)00200-8.

44. **Langworthy NG, Renz A, Mackenstedt U, Henkle-Dührsen K, de Bronsvoort MB, Tanya VN, Donnelly MJ, Trees AJ.** 2000. Macrofilaricidal activity of tetracycline against the filarial nematode Onchocerca ochengi: elimination of Wolbachia precedes worm death and suggests a dependent relationship. Proc Biol Sci **267:**1063–1069. http://dx.doi.org/10.1098/rspb.2000.1110.

45. **Darby AC, Armstrong SD, Bah GS, Kaur G, Hughes MA, Kay SM, Koldkjær P, Rainbow L, Radford AD, Blaxter ML, Tanya VN, Trees AJ, Cordaux R, Wastling JM, Makepeace BL.** 2012. Analysis of gene expression from the Wolbachia genome of a filarial nematode supports both metabolic and defensive roles within the symbiosis. Genome Res **22:** 2467–2477. http://dx.doi.org/10.1101/gr.138420.112.

46. **Bennuru S, Meng Z, Ribeiro JM, Semnani RT, Ghedin E, Chan K, Lucas DA, Veenstra TD, Nutman TB.** 2011. Stage-specific proteomic expression patterns of the human filarial parasite Brugia malayi and its endosymbiont Wolbachia. Proc Natl Acad Sci U S A **108:**9649–9654. http://dx.doi.org/10.1073/pnas.1011481108.

47. **Slatko BE, Luck AN, Dobson SL, Foster JM.** 2014. Wolbachia endosymbionts and human disease control. Mol Biochem Parasitol **195:**88–95. http://dx.doi.org/10.1016/j.molbiopara.2014.07.004.

48. **Driguez P, Doolan DL, Loukas A, Felgner PL, McManus DP.** 2010. Schistosomiasis vaccine discovery using immunomics. Parasit Vectors **3:**4. http://dx.doi.org/10.1186/1756-3305-3-4.

49. **Gaze S, Driguez P, Pearson MS, Mendes T, Doolan DL, Trieu A, McManus DP, Gobert GN, Periago MV, Correa Oliveira R, Cardoso FC, Oliveira G, Nakajima R, Jasinskas A, Hung C, Liang L, Pablo J, Bethony JM, Felgner PL, Loukas A.** 2014. An immunomics approach to schistosome antigen discovery: antibody signatures of naturally resistant and chronically infected individuals from endemic areas. PLoS Pathog **10:**e1004033. http://dx.doi.org/10.1371/journal.ppat.1004033.

50. **Tang YT, Gao X, Rosa BA, Abubucker S, Hallsworth-Pepin K, Martin J, Tyagi R, Heizer E, Zhang X, Bhonagiri-Palsikar V, Minx P, Warren WC, Wang Q, Zhan B, Hotez PJ, Sternberg PW, Dougall A, Gaze ST, Mulvenna J, Sotillo J, Ranganathan S, Rabelo EM, Wilson RK, Felgner PL, Bethony J, Hawdon JM, Gasser RB, Loukas A, Mitreva M.** 2014. Genome of the human hookworm Necator americanus. Nat Genet **46:** 261–269. http://dx.doi.org/10.1038/ng.2875.

51. **Elson LH, Guderian RH, Araujo E, Bradley JE, Days A, Nutman TB.** 1994. Immunity to onchocerciasis: identification of a putatively immune population in a hyperendemic area of Ecuador. J Infect Dis **169:**588–594. http://dx.doi.org/10.1093/infdis/169.3.588.

52. **Ward DJ, Nutman TB, Zea-Flores G, Portocarrero C, Lujan A, Ottesen EA.** 1988. Onchocerciasis and immunity in humans: enhanced T cell responsiveness to parasite antigen in putatively immune individuals. J Infect Dis **157:**536–543. http://dx.doi.org/10.1093/infdis/157.3.536.

53. **MacDonald AJ, Turaga PS, Harmon-Brown C, Tierney TJ, Bennett KE, McCarthy MC, Simonek SC, Enyong PA, Moukatte DW, Lustigman S.** 2002. Differential cytokine and antibody responses to adult and larval stages of Onchocerca volvulus consistent with the development of concomitant immunity. Infect Immun **70:**2796–2804. http://dx.doi.org/10.1128/IAI.70.6.2796-2804.2002.

54. **Hotez PJ, Bethony JM, Diemert DJ, Pearson M, Loukas A.** 2010. Developing vaccines to combat hookworm infection and intestinal schistosomiasis. Nat Rev Microbiol **8:**814–826. http://dx.doi.org/10.1038/nrmicro2438.

55. **Makepeace BL, Babayan SA, Lustigman S, Taylor DW.** 2015. The case for vaccine development in the strategy to eradicate river blindness (onchocerciasis) from Africa. Expert Rev Vaccines **14:**1163–1165. http://dx.doi.org/10.1586/14760584.2015.1059281.

56. **Weil GJ, Ogunrinade AF, Chandrashekar R, Kale OO.** 1990. IgG4 subclass antibody serology for onchocerciasis. J Infect Dis **161:**549–554. http://dx.doi.org/10.1093/infdis/161.3.549.

57. **Kurniawan-Atmadja A, Sartono E, Partono F, Yazdanbakhsh M, Maizels R.** 1998. Specificity of predominant IgG4 antibodies to adult and microfilarial stages of Brugia malayi. Parasite Immunol **20:**155–162.

58. **Armstrong SD, Xia D, Bah GS, Krishna R, Ngangyung HF, LaCourse EJ, McSorley HJ, Kengne-Ouafo JA, Chounna-Ndongmo PW, Wanji S, Enyong PA, Taylor DW, Blaxter ML, Wastling JM, Tanya VN, Makepeace BL.** 2016. Stage-specific proteomes from Onchocerca ochengi, sister species of the human river blindness parasite, uncover adaptations to a

nodular lifestyle. Mol Cell Proteomics **15:**2554–2575 http://dx.doi.org/10.1074/mcp.M115.055640.

59. **Lustigman S, MacDonald AJ, Abraham D**. 2003. CD4⁺-dependent immunity to Onchocerca volvulus third-stage larvae in humans and the mouse vaccination model: common ground and distinctions. Int J Parasitol **33:**1161–1171. http://dx.doi.org/10.1016/S0020-7519(03)00170-X.

60. **Turaga PS, Tierney TJ, Bennett KE, McCarthy MC, Simonek SC, Enyong PA, Moukatte DW, Lustigman S**. 2000. Immunity to onchocerciasis: cells from putatively immune individuals produce enhanced levels of interleukin-5, gamma interferon, and granulocyte-macrophage colony-stimulating factor in response to *Onchocerca volvulus* larval and male worm antigens. Infect Immun **68:**1905–1911. http://dx.doi.org/10.1128/IAI.68.4.1905-1911.2000.

61. **Lustigman S, Huima T, Brotman B, Miller K, Prince AM**. 1990. Onchocerca volvulus: biochemical and morphological characteristics of the surface of third- and fourth-stage larvae. Exp Parasitol **71:**489–495. http://dx.doi.org/10.1016/0014-4894(90)90075-N.

62. **Taylor DW, Goddard JM, McMahon JE**. 1984. Isolation and purification of microfilariae from nodules of Onchocerca volvulus. Trans R Soc Trop Med Hyg **78:**707–708. http://dx.doi.org/10.1016/0035-9203(84)90255-4.

63. **Medina-De la Garza CE, Brattig NW, Tischendorf FW**. 1987. Rapid method for the purification of viable microfilariae from nodules of Onchocerca volvulus by Percoll gradient centrifugation. Trop Med Parasitol **38:**53–54.

64. **Ribeiro JM, Schwarz A, Francischetti IM**. 2015. A deep insight into the sialotranscriptome of the Chagas disease vector, Panstrongylus megistus (Hemiptera: Heteroptera). J Med Entomol **52:**351–358. http://dx.doi.org/10.1093/jme/tjv023.

65. **Burbelo PD, Ching KH, Klimavicz CM, Iadarola MJ**. 2009. Antibody profiling by luciferase immunoprecipitation systems (LIPS). J Vis Exp http://dx.doi.org/10.3791/1549.