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Review Article

Cestode genomics – progress and prospects for advancing basic and applied aspects of flatworm biology

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SUMMARY

Characterization of the first tapeworm genome, Echinococcus multilocularis*, is now nearly complete, and genome assemblies of* E. granulosus*,* Taenia solium *and* Hymenolepis microstoma *are in advanced draft versions. These initiatives herald the beginning of a genomic era in cestodology and underpin a diverse set of research agendas targeting both basic and applied aspects of tapeworm biology. We discuss the progress in the genomics of these species, provide insights into the presence and composition of immunologically relevant gene families, including the antigen B- and EG95/45W families, and discuss chemogenomic approaches toward the development of novel chemotherapeutics against cestode diseases. In addition, we discuss the evolution of tapeworm parasites and introduce the research programmes linked to genome initiatives that are aimed at understanding signalling systems involved in basic host–parasite interactions and morphogenesis.*

Keywords *antigen B, cestode,* Echinococcus*, EG95, genome,* Hymenolepis*, S3Pvac*

INTRODUCTION

Whole-genome sequencing of cestodes began in 2004 and currently includes the aetiological agents of alveolar echinococcosis (AE; *Echinococcus multilocularis*), cystic echinococcosis (CE; *E. granulosus*) and neurocysticercosis (NCC; *Taenia solium*) in addition to the rodent-hosted laboratory model, *Hymenolepis microstoma*. With the genomes of *Echinococcus* spp. near completion, and those of *Taenia* and *Hymenolepis* in advanced drafts, we have only begun to explore their full content, structure and general characteristics. Nevertheless, genomic and transcriptomic data are already advancing research in both basic and applied aspects of tapeworm biology and herald the beginning of a new era in cestodology. Here, we review the progress made in the genomics of tapeworms and provide initial insights into the presence of immunologically relevant molecules and chemogenomic approaches to the development of new vaccines. We begin by discussing their evolution and diversification into homeothermic hosts including humans and finish by introducing the research programmes on signalling systems involved in host–parasite interactions and development that underpin two of the genome initiatives.

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EVOLUTION OF TAPEWORM PARASITES

Tapeworms represent an extreme example in the evolution of parasitism in flatworms (phylum Platyhelminthes), being distinguished from the other parasitic groups by the complete loss of a gut and a highly modified, segmented, body plan. They are almost exclusively enteric parasites of vertebrates as adults, with complex life cycles involving ontogenetically distinct larval stages that first develop in arthropod hosts, although variation in everything from their basic body architecture to their host associations is found among an estimated 6000 species. Like free-living flatworms, tapeworms maintain totipotent stem cells (called neoblasts) throughout their lives (1–5), providing them with an extraordinary degree of developmental plasticity and a theoretical potential for indeterminate growth (6). Although tapeworm infection of humans is less prevalent than that of trematodes such as *Schistosoma* and *Fasciola*, their enormous reproductive output and

potential for metastatic growth can produce severe pathological consequences (7), and cestode diseases remain a significant threat to our health and agriculture.

The notion of flatworms as representing the proto-bilaterian condition promoted throughout most of the 20th century has been difficult to dispel, and they continue to be cited as such today. Wide adoption of the 18S-based 'new animal phylogeny' (Figure 1; 8,9) that showed them to be members of the Lophotrochozoa (a diverse group including annelid worms and molluscs that together with the Ecdysozoa encompasses the spiralian animals) refuted this notion, and their lophotrochozoan affinities have been supported consistently by studies based on increasingly large numbers of genes. Less support has been found for their exact position within the Lophotrochozoa, but they appear to have closer affinities to 'platyzoan' groups including rotifers and bryozoans than to either annelids or molluscs (10). Based on their position, there is no longer any *a priori* reason to assume them to be representative of an early, or 'primitive', bilaterian condition. Moreover, not only are flatworms a more recently evolved animal lineage than previous ideas suggested, but the parasitic flatworms form also a derived clade (i.e. Neodermata; 'new skin') within the phylum, having appeared after the major diversification of their free-living cousins (11). We should expect then that flatworm biology, including their genomes, will reflect both their shared affinities to other lophotrochozoan phyla and their unique, lineage-specific adaptations, such as the maintenance of totipotent stem cells and adoption of parasitism.

Phylogenetic studies (11,12) indicate that obligate parasitism first arose through association (e.g. predation, symbiosis) of free-living or symbiotic flatworms and fishes, most likely early in the host's evolution (13), and still today, bony fish and elasmobranch (sharks, rays and chimaeras) are host to sexual stages of the majority of flatworm parasite families. Although free-living species display a high propensity for symbioses spanning the spectrum from commensalism to parasitism, there is strong evidence that the major parasitic lineages form a monophyletic group, demonstrating that obligate parasitism arose only once during the course of flatworm evolution (11). This was associated with a major developmental shift involving the separation of ontogenetically distinct larval and adult stages, with replacement of the larval epidermis by a syncytial tegument. Within this clade, we now recognize four independent lineages: the cestodes (tapeworms), digeneans (flukes) and monopisthocotylean and polyopisthocotylean 'monogeneans'. Interrelationships of these lineages remain controversial, but have begun to point toward a sister relationship between cestodes and digeneans, and paraphyly of the 'Monogenea' (11,14,15), in contrast to previous hypotheses (and classifications) that considered 'monogeneans' to be both monophyletic and the sister group to tapeworms. The main implications of the molecular-based hypotheses are a common origin of both enteric parasitism and complex life cycles in

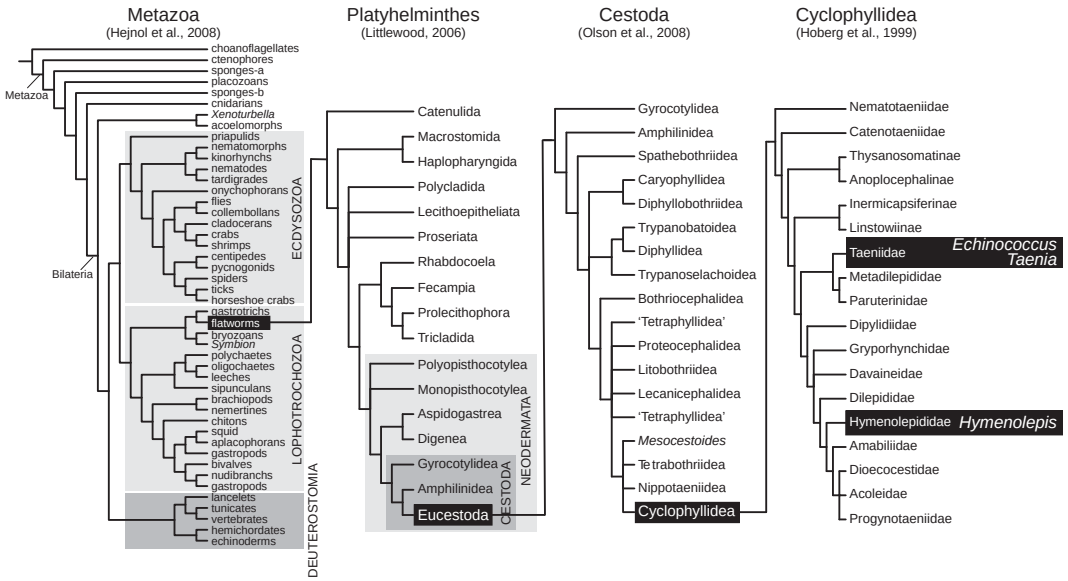

Figure 1 Phylogenetic position of the tapeworm genera *Echinococcus*, *Hymenolepis* and *Taenia*. Note position of the flatworms within the Lophotrochozoa, and the derived positions of both the true tapeworms (Eucestoda) and the Cyclophyllidea, the order to which all currently sequenced tapeworm genomes belong (Topologies from 10,11,21,165).

tapeworms and flukes despite major differences in their life histories, and that the first neodermatan flatworms were nonenteric and direct-developing, as seen in contemporary monopisthocotylean and polyopisthocotylean parasites.

Only in the last two decades has our understanding of tapeworm interrelationships begun to stabilize, thanks to a more concerted effort on the part of cestodologists (16) and the wide application of molecular phylogenetic techniques (14). Circumscription of even the primary tapeworm lineages has required major revisions to reflect new insights into their affinities, resulting in the proposal of three new tapeworm orders since 2008 (17,18). Interrelationships of the 15 or more natural (i.e. monophyletic) groups of tapeworms have yet to be resolved satisfactorily, but it is clear that early branching lineages colonized a wide spectrum of cartilaginous and bony fishes before subsequent diversification led to the colonization of homeothermic hosts (e.g. birds, mammals) (19–21). Among the early branching groups, only the Diphyllobothriidea [n.b. formally classified as a family of Pseudophyllidea (18)] radiated into homeotherms, but retained its association with fishes (which became 2nd intermediate hosts) and transmission via aquatic life cycles (22). There was thus a single primary colonization of homeothermic hosts coincident with the adoption of fully terrestrial life cycles that gave rise to the most speciose contemporary group, the Cyclophyllidea.

The extent to which tapeworm–host associations were shaped by the unique adaptive immunity of the mammalian host is not clear from an evolutionary perspective. However, even enteric infections are subject to immunological defences and nonpermissive hosts readily expel foreign species (23), resulting in a high specificity of tapeworms to their definitive hosts. In general, mammals act as apex predators in tapeworm life cycles, playing host to adult, enteric stages. In the unique case of taeniid cyclophyllideans, in which mammals also act as intermediate hosts (24), they are the primary prey items of larger mammals, such as in the rodent/fox cycles of *Echinococcus*, *Mesocestoides* and some *Taenia* species (25). With regard to human infection with tapeworms, there is at least some evidence that the *Taenia* species infecting humans evolved before the development of agriculture, animal husbandry and the domestication of cattle and swine (24,26), indicating that humans were responsible for introducing *Taenia solium* and *T. saginata* to contemporary agricultural cycles. Moreover, phylogenetic analysis showed that these species evolved in humans independently (26): *T. solium* associated with the tapeworms of hyenas and *T. saginata* with those of lions. This unsettling scenario suggests that in prehistoric times, food webs selected a role for ourselves not only as definitive hosts, but also as intermediate hosts, in transmission cycles including larger carnivores as the apex predators.

PROGRESS IN CESTODE GENOMICS

Table 1 summarizes the general characteristics of tapeworm genomes as represented by three taeniid and one hymenolepidid cyclophyllidean species. At present, the only published flatworm genomes are those of the human bloodflukes *Schistosoma mansoni* (27) and *S. japonicum* (28), but available draft data for the planarian model *Schmidtea mediterranea* (29) and the 'turbellarian' *Macrostomum lignano* (30) provide important reference genomes of free-living flatworms. By comparing parasitic and free-living species, identification of both loss and expansion of gene families will provide the most comprehensive picture to date of the effects of evolving obligate parasitism, allowing its signature to be compared with that in other animal groups, such as the nematodes (31). Much of this

Table 1 General characteristics of cestode genomes

Species (common name)	Disease	Genome size (Mb)	No. of chromosomes[a]	G/C (%)	N50 (Mb) (no. of scaffolds)	Institutions
Echinococcus multilocularis (fox tapeworm)	Alveolar echinococcosis	113	$2N = 18$ (157–160)	42	4·9 (1816)	WTSI and UW
Echinococcus granulosus (dog/fox tapeworm)	Cystic echinococcosis/ hydatid disease	106	$2N = 18$ (158,161)	42	1·9 (2874)	WTSI and UM
Taenia solium (pork tapeworm)	Neuro-cysticercosis	117	Unknown $2N = 18$? (43,162)	43	0·07 (6284)	UNAM
Hymenolepis microstoma (mouse bile duct tapeworm)	Rodent-hosted laboratory model	147	12 (163,164)	35	0·8 (5387)	WTSI and NHM

NHM, Natural History Museum, London, UK; UM, University of Montevideo, Uruguay; UW, University of Würzburg, Germany; WTSI, Wellcome Trust Sanger Institute, Hinxton, UK.
[a]Numbers in brackets indicate citations from which chromosome estimates were obtained.

signature will surely relate factors evolved to counter host immune defences, and comparative genomics thus hold great promise for advancing the immunology of parasitic flatworms.

Tapeworm genomes are small in size at ~110 Mb, compared with 363 Mb in *Schistosoma* (27), 700 Mb in *Schmidtea* and ~330–1100 Mb in *Macrostomum* (http://www.genomesize.com/index.php). Differences may be due to the fact that tapeworm genomes contain fewer mobile genetic elements and retroposons than trematodes or planarians, in which they are common (32,33). However, it is clear that there has also been significant gene loss. For example, the components for *de novo* synthesis of cholesterol are missing, as is ornithine decarboxylase (a key enzyme in spermidine/putrescine biosynthesis), and these essential components must therefore be acquired from the host. Indeed, the complete loss of a gut has presumably resulted in the loss of many enzymes. Similarly, highly conserved developmental genes families such as Hox and Wnt also show highly reduced numbers of gene classes and orthologs, as discussed later. From a practical standpoint, the small size of tapeworm genomes and minimal amount of repetitive elements make their characterization less problematic than other flatworms and aids in determining the structures and synteny of genes and other genetic elements.

Below, we discuss the history and state of play in ongoing initiatives. Full details of these genomes will be discussed in an article being led by Matt Berriman of the Parasite Genome Group at the Wellcome Trust Sanger Institute (WTSI).

Echinococcus multilocularis Leukart, 1863

An initial meeting to set priorities in pathogen genome sequencing led by Rick Maizels (University of Edinburgh) was held at the WTSI Genome Campus in March 2004. *E. multilocularis*, the causative agent of AE, was chosen as the reference system for all further cestode genome projects (Table 1). Although infections caused by *E. granulosus* or *T. solium* are more prevalent worldwide, *E. multilocularis* was selected primarily because of the availability of better laboratory cultivation techniques. During recent years, several systems for efficient *in vitro* cultivation of the *E. multilocularis* metacestode stage (34,35) as well as a system for complete regeneration of metacestode vesicles from totipotent parasite stem cells (36) have been established, so that the life cycle of this cestode within the intermediate host, from the initial infecting oncosphere to the stage that is passed on to the definitive host, can now be mimicked under controlled laboratory conditions. As a source of genomic DNA, the natural parasite isolate JAVA (37) was used, which is derived from a cynomolgus monkey (*Macaca fascicularis*) that was kept in a breeding enclosure in the German Primate Center (Göttingen) and which was intraperitoneally passaged in laboratory mice for a few months prior to DNA isolation. This step appeared important because of the fact that long-term laboratory 'strains' of larval cestodes (i.e. material that has been passaged for years or decades within the peritoneum of mice) usually undergo morphological and physiological (and most probably also genomic) alterations that no longer reflect the *in vivo* situation (1). To minimize contamination with host DNA, it was further necessary to isolate DNA from protoscoleces that had previously been treated with pepsin at pH 2, leading to almost complete digestion of host material but leaving parasite material intact.

After extensive generation of bacterial artificial chromosomes libraries and determination of the parasite's genome size (36), a first round of conventional Sanger capillary sequencing to ~4-fold coverage was carried out which was complemented by several runs of paired and unpaired 454- and Solexa-sequencing. At the time of most analyses presented here, sequence information representing 140-fold coverage of the genome had been generated which, in version 1 (13 August 2010), had been assembled into 600 supercontigs with an N50 contig size of more than 1·6 Mb (n.b. in the latest assembly, half the genome is contained in only 18 supercontigs; see Table 1). Thus, by combining classical capillary sequencing with next-generation sequencing methodology, a data set has been produced for the *E. multilocularis* genome that is more comprehensive than those of the already published genomes of *S. mansoni*, *S. japonicum* and *B. malayi*, which had not been assembled into versions of <5000 contigs (38,39). Interestingly, although the initial determination of the *E. multilocularis* genome size by flow cytometry on isolated parasite cells yielded values around 300 Mb (36), the assembled sequence data strongly suggest a haploid genome size of ~110 Mb. The reason for this discrepancy is currently unknown, but may represent a case of polyploidy. However, in BLAST analyses of a set of several thousand ESTs that are available for *E. multilocularis* (40,41) and *E. granulosus* (41) against the genome assembly, none could be identified that was not represented on one of the 600 supercontigs. This indicates that at least the protein-encoding portion of the genome is very well covered by the latest assembly version, which is publicly available via http://www.sanger.ac.uk/resources/downloads/helminths/echinococcus-multilocularis.html.

In parallel to genome sequencing and assembly, transcriptomes of different life cycle stages of *E. multilocularis* are currently being characterized using next-generation

sequencing (NGS). Initial data sets are available at the WTSI webpage of the *E. multilocularis* sequencing project for isolated primary cells after one week of regeneration (representing the early oncosphere–metacestode transition; 36), for *in vitro* cultivated metacestode vesicles and for protoscoleces prior to or after activation by low-pH/pepsin treatment, which mimics the transition into the definitive host. Further RNA sequencing is carried out for regenerating primary cells after three weeks of culture (late phase of oncosphere–metacestode transition), for metacestode vesicles with brood capsules (early formation of protoscoleces) and for the adult stage. Thus, transcriptome data that almost completely cover the *E. multilocularis* life cycle will soon be available, although it will still be difficult to obtain material of activated *E. multilocularis* oncospheres in amounts that are sufficient for RNA sequencing.

Using the available transcriptome data as well as a large set of *E. multilocularis* and *E. granulsous* EST information (available under http://www.nematodes.org/NeglectedGenomes/Lopho/LophDB.php, http://fullmal.hgc.jp/em/docs/echinococcus.html and http://www.sanger.ac.uk/resources/downloads/helminths/echinococcus-multilocularis.html), gene prediction and annotation is currently under way. In a first, AUGUSTUS-based analysis of the assembled genome, we identified ~11 000 protein-encoding genes, which is slightly less than the gene number (11 800) that has been predicted for the trematode *Schistosoma mansoni* (27). For 70% of these genes, we could identify clear orthologs in other organisms, whereas the remaining 30% are most probably *Echinococcus*- or cestode-specific genes or gene families.

Echinococcus granulosus (Batsch, 1786)

Mostly for comparative studies with the *Echinococcus multilocularis* reference genome, NGS has very recently also been used for a first characterization of the genome of *E. granulosus*. This project is being carried out by the parasite genomics group of the WTSI led by Matt Berriman in collaboration with Cecilia Fernandez (University of Montevideo). Because of its importance in human infections, the G1 (sheep) strain was chosen for sequencing and, like in the case of *E. multilocularis*, protoscoleces after treatment with low pH/pepsin were used as a source for genomic DNA to minimize host contamination (C. Fernandez, pers. comm.). After a first round of Illumina sequencing, the genome has been assembled into 5200 contigs that, using the *E. multilocularis* genome as a reference framework, have been further assembled into ~2000 scaffolds that are available via http://www.sanger.ac.uk/resources/downloads/helminths/echinococcus-granulosus.html. As expected, the genomes of *E. granulosus* and *E. multilocularis* are highly homologous with overall 96% identity at the nucleotide sequence level within the coding regions of predicted genes, and still around 91% identity in promoter regions. Because the *E. granulosus* contigs have been assembled into supercontigs using *E. multilocularis* as a reference, no valid conclusions concerning genomic rearrangements between the species can been made at present. Direct comparisons of longer contigs of the *E. granulosus* genome assembly with the *E. multilocularis* sequence, however, indicate that there is also a high level of synteny between both species. Differences in gene structure and sequence can mostly be observed in the case of expanded gene families, such as the recently described *hsp*70 family (42) that contains a significant number of pseudogenes. The *E. granulosus* genome assembly is currently awaiting additional Illumina data, and thus, substantial improvement is expected soon.

Taenia solium L., 1758

A third important project on a taeniid cestode addresses the whole genome of *T. solium* (43) and is being carried out by a Mexican consortium directed by Juan-Pedro Laclette (http://bioinformatica.biomedicas.unam.mx/taenia/) located at the Universidad National Autonoma de Mexico. As in the case of the *E. multilocularis* genome, this project has followed a hybrid strategy in which classical capillary sequencing of cloned genome fragments has been combined with NGS. In a first phase of the project, ~20 000 ESTs from adult worms and cysticerci were generated, followed by estimation of the parasite's genome size. Using genomic DNA from cysticerci as a source for analysis, 2× coverage by capillary sequencing and 5× coverage by 454 sequencing have been reached, and the hybrid assembly process has so far yielded ~50 000 contigs (N50 > 5000 bp) that cover ~90% of the EST-based transcriptome profile. Additional 454- and Solid-reads are planned in this project so that a much more comprehensive assembly will soon be available. Furthermore, because EST information and next-generation transcriptome data from *Echinococcus* spp. are informative for identifying genes in *Taenia* spp. (and vice versa), close collaboration of the bioinformatic teams that work on all three ongoing taeniid cestode genome projects has been established that should greatly facilitate the annotation process. Interestingly, as in the case of *E. multilocularis*, the haploid genome size of *T. solium* was first determined to be ~260 Mb using flow cytometry, whereas the NGS assembly so far indicates a genome size of 130 Mb (43). Whether this is, in both cases, associated with genome duplications or polyploidy remains to be elucidated.

Hymenolepis microstoma (Dujardin, 1845)

Hymenolepis microstoma, the mouse bile duct tapeworm, is one of three beetle/rodent-hosted hymenolepidid laboratory models commonly used in research and teaching since they were first domesticated in the 1950s. Although less studied than either the rat tapeworm *H. diminuta* (44) or the dwarf tapeworm *H. nana*, *H. microstoma* has advantages of being small and mouse-hosted unlike *H. diminuta* and is refractory to both human infection and cross-contamination of rodents via a direct life cycle, unlike *H. nana*. Use of *H. microstoma* has thus both practical and regulatory advantages that make a good model for research requiring easy access to both larval and strobilate stages of the tapeworm life cycle. Although we expect the genome of *H. microstoma* to be representative of all three model species, it is worth noting that they are not each other's closest relatives (45) and that there has long been disagreement as to whether or not *Hymenolepis* spp. bearing hooks should be classified in their own genus (i.e. *Rodentolepis*) (see 46). If so, then we expect *H. microstoma* to be better representative of *H. nana* than to *H. diminuta*.

Genome characterization of *H. microstoma* began in 2009 as a pilot project in collaboration with the Sanger Institute after their implementation of NGS allowed for expansion of existing genome sequencing programmes. Although *Hymenolepis* tapeworms are not significant human pathogens, they represent an important laboratory model in cestodology and access to a highly inbred culture made them well suited for *de novo* genome assembly. Genomic and transcriptomic data are based on specimens of a 'Nottingham' strain maintained by the author (PDO) *in vivo* using natural hosts (flour beetles, *Tribolium confusum*, and BALB/c mice). The origin of the strain can be traced back to the original domestication of the species by the C. P. Read laboratory at Texas Rice University in the 1950s (47), making the genome data directly relevant to a large body of previous research based on isolates of this strain. A complete description of the strain and it origins, including a review of its general biology and use as a laboratory model, has been recently published in open access format (46).

The *H. microstoma* genome assembly consists entirely of data generated via NGS technologies and has been assembled and analysed using bioinformatic pipelines developed by the Parasite Genomics Group at the WTSI (48–53) and others (54–57). The current assembly (April 2011) comprises data from six full Roche 454 Titanium runs (three unpaired runs, two paired runs with 3–4-kb inserts, and one with 9-kb inserts) and three Illumina Solexa lanes (76-bp reads, two lanes with 250-bp inserts, and one lane with 3-kb inserts). The combined data resulted in more than 40× coverage of the estimated 147-Mb genome (Table 1). Separate *de novo* assemblies of the two technologies were made using the software NEWBLER 2.5 (58) (for Roche/454) and ABySS 1.2.1 (55) (for Illumina), and contigs then merged using the pipe-line GARM (A. Sanchez, unpubl. data), based on the genome assembler Minimus (59). Remaining gaps were closed with IMAGE (dev. ver.) (48) for 20 iterations with gradually more permissive parameter settings (kmer = 61–30, overlap = 100–200). The final sequences were corrected using five iterations of iCORN (dev. ver.) (49). Genome data are made available from http://www.sanger.ac.uk/resources/downloads/helminths/hymenolepis-microstoma.html.

Transcriptomic data are also being profiled using Illumina technologies for the purposes of RNA-seq analysis and annotation, as well as to address specific questions in adult development. Presently, this includes whole adult cDNA from the mouse gut, and thus profiles all grades of development represented by the strobilate adult worm, as well as cDNA from a combined developmental series of metamorphosing larvae (i.e. 3–7 days PI) from the haemocoel of beetles. Additional cDNA samples representing progressively mature regions of the adult tapeworm strobila are being sequenced by the WTSI, and each sample will be replicated multiple times for statistical support. This will allow us to determine differential expression associated with the process of segmentation in the neck region, the maturing of the reproductive organs in the strobila and the process of embryogenesis occurring in gravid segments.

Unlike *E. multilocularis* and *E. granulosus*, the *H. microstoma* genome assembly has not undergone manual curation or refinement and is thus a good example of the kind of assembly that can be achieved using medium-coverage NGS and bioinformatics alone. For comparative purposes, completeness was assessed using CEGMA 2.0 (60), which looks for a set of 458 'core' genes that are highly conserved in eukaryotes. This method estimated the *H. microstoma* genome assembly to be 90% complete, compared to 87–93% in *Echinococcus* species, and demonstrates that genome projects on a medium scale, with restricted coverage and without manual curation, are feasible and can give excellent estimates of gene content. Moreover, as some percentage of these 'core' genes will have been lost from the reduced genomes of parasitic flatworms, estimating completeness on this basis almost certainly provides an underestimation. Indeed, the very high sequence coverage of the current cestode genome assemblies suggests that tapeworms have simply lost ~7 to 10% of these 'core' genes. The biggest difference between the *H. microstoma* and *E. multilocularis* assemblies is seen in the scaffold-statistics: more than 50% of the *E. multilocularis* genome is contained in 13 scaffolds in the latest assembly (N50; Table 1), whereas *H. microstoma* is

contained in 747 scaffolds. Besides better read depth, the *E. multilocularis* genome has more long-range mapping information and has undergone several rounds of dedicated manual curation to join scaffolds and resolve miss-assemblies resulting from the presence of repeat elements or heterozygosity. The difference in genome coverage is negligible for most research questions, such as those that primarily make use of gene sequence information and expression data, but could be problematic for research requiring long-range mapping information.

TOWARD IMPROVED CHEMOTHERAPY

The drugs most frequently employed in the treatment for cestode infections are praziquantel (PZQ) and benzimidazoles (BZs; e.g. albendazole, mebandazole). PZQ, which is well known for its activity against adult schistosomes, is also a highly potent drug against cestode adult stages and is frequently used to treat taeniasis, or is employed in deworming campaigns against foxes or dogs in endemic areas (61). Although the precise cellular target(s) for PZQ in schistosomes are not yet known, voltage-gated calcium channels are considered very good candidates and have thus already been experimentally addressed using the *Xenopus* oocyte expression system (62). Interestingly, unlike other organisms, schistosomes express two different β subunits of calcium channels, one of which confers PZQ sensitivity in the *Xenopus* system, the other not (63). A major difference between these subunits is the presence or absence of two canonical serine residues in the so-called beta interaction domain (BID) that are typically phosphorylated through protein kinase C (PKC). In the case of the β subunit that conferred PZQ sensitivity, these residues were replaced by amino acids that can no longer be phosphorylated by PKC, and this difference might be the structural reason for the general PZQ sensitivity of schistosomes (63). Recently, Jeziorski and Greenberg (64) also identified calcium channel β subunits in *T. solium* and demonstrated that this cestode, like schistosomes, expresses an unusual subunit in which the PKC target residues were replaced by Asp and Ala, alongside a canonical subunit with Thr/Ser residues at these positions. In the ongoing sequencing projects, this could be verified for all four cestode species under study. Both *Echinococcus* species and *H. microstoma*, like *T. solium*, express two β subunits of calcium channels of which one represents the canonical form and the second a modified version with amino acid replacements at the PKC responsive sites (data not shown). This could, at least in part, explain the PZQ sensitivity of adult cestodes. Although PZQ resistance will most probably never be an issue in the treatment of taeniasis patients, it could become a problem in large scale deworming campaigns against *E. multilocularis*, *E. granulosus* and *Mesocestoides* spp. that have been suggested already for parts of Central Europe and China (25,65,66). Particularly for such projects, genetic information on the cellular targets of PZQ, as available through the genome projects, will be highly valuable in assessing treatment efficacy and the emergence of drug resistance.

In sharp contrast to its activity on adult cestodes, PZQ has very limited effects on metacestode stages (67). The underlying reason could be that the calcium channel β subunits (or other potential PZQ targets) are expressed in an adult-specific manner, and in the currently available transcriptome profiles for *E. multilocularis* metacestode vesicles, the respective genes are indeed expressed at a marginal level (data not shown). Because of the low efficacy of PZQ treatment, the current drugs of choice in chemotherapy against AE, CE and NCC are BZs that have a high affinity for helminth-specific β-tubulin isoforms, thus inhibiting microtubule polymerization that eventually leads to parasite death. Although prolonged BZ treatment of the intermediate host can be effective in eliminating *E. granulosus* cysts or *T. solium* cysticerci (68,69), its activity against *E. multilocularis* is very limited. In AE, BZ treatment is mostly parasitostatic rather than parasitocidal and, as a consequence, has to be given lifelong (68). Furthermore, in all three types of infection, BZ treatment can be associated with severe side effects that are due to limited bioavailability of the drug at the site of infection and high structural homology of β-tubulin of parasite and host. Three major β-tubulin isoforms that are expressed by *E. multilocularis* have already been characterized several years ago and were shown to be highly homologous (>90% amino acid identity) to β-tubulin of humans (40; Table 2). In the *E. multilocularis* genome assembly, we have identified at least nine β-tubulin encoding loci, although transcriptome profiling clearly shows that the three previously identified isoforms (40) are abundantly expressed in all larval stages, whereas the other six loci are mostly silent or may even represent pseudogenes. Studies on mechanisms of BZ resistance and sensitivity in nematodes previously identified two amino acid residues (Phe200 and Phe167 in BZ-sensitive isoforms) that are particularly important for drug binding to β-tubulin. In BZ-resistant strains of *Haemonchus contortus*, these residues were frequently exchanged by Tyr or His, leading to diminished BZ binding (70). In this context, it is interesting to note that the β-tubulin isoform which, according to transcriptome profiling, shows the highest expression level in the *E. multilocularis* metacestode (i.e. the target of BZ treatment) displays Tyr residues at positions 200 and 167 and might thus represent a potentially BZ-resistant isoform (Table 2). Highly homologous isoforms with Tyr

at these two positions are also encoded by the genomes of *E. granulosus* and *T. solium* (Table 2), and in the respective EST databases, transcripts for this isoform are particularly abundant (data not shown), indicating high expression in the metacestodes of these species as well. Hence, limited bioavailability of the drug at the site of infection, which is particularly an issue for the infiltratively growing *E. multilocularis* metacestode, combined with a potentially reduced affinity of BZs to the major β-tubulin isoform of the metacestode, could be the main reasons for limited efficacy of BZ treatment in AE.

Employing *in vitro* cultivation systems for the *E. multilocularis* metacestode stage and classical approaches of testing selected compounds for anti-parasitic activities, Andrew Hemphill's laboratory and others (71) have recently identified several compounds such as nitazoxanide, isoflavones or amphotericin B that could be used as drugs in AE treatment, mostly in combination with BZs (reviewed in 68). However, compounds that act not only parasitostatic but truly parasitocidal against *E. multilocularis in vivo* have not been discovered to date, indicating that new chemotherapeutic strategies against AE are urgently needed. With the availability of the *E. multilocularis* whole genome together with those of *E. granulosus* and *T. solium*, targeted drug design should be one of the most promising approaches for the development of anti-cestode drugs in the next years. On the one hand, comparative genomics can be employed to identify factors that are unique to cestodes or flatworms and could serve as targets for compound screening. The drawback of this approach is that the function and biochemical properties of parasite-specific factors are usually unknown, which severely hampers the design of efficient inhibitors. Furthermore, many of these parasite-specific proteins have redundant functions and are often not essential. An alternative and much more promising approach should rather concentrate on drug targets that are, to a certain degree, homologous between parasite and host, thus providing information on function and biochemistry, but that display sufficient functional modification between both species to allow the development of parasite-specific inhibitors. A highly promising group of factors in this regard are protein kinases (Table 3) that are crucially involved in the regulation of metazoan development and that mediate cell–cell communication by participating in cellular signalling systems (72). Because of their important role in cancer, the general biochemistry of these proteins is extremely well studied and a plethora of compounds to modify their activities, mostly directed against the well-conserved ATP-binding pocket, is available (73). Protein kinases have thus already been suggested as promising targets in drug design against schistosomiasis (74), and their suitability as targets in cestodes has recently been demonstrated by Gelmedin *et al.* (75) who identified pyridinyl imidazoles, directed against the p38 subfamily of mitogen-activated protein kinases (MAPK), as a novel family of anti-*Echinococcus* compounds. A number of *E. multilocularis* protein kinases such as the Erk- and p38-like MAPKs EmMPK1 (76) and EmMPK2 (75), respectively, the MAPK kinases EmMKK1 and EmMKK2 (77), or the Raf-like MAPK kinase kinase EmRaf (78) have already been characterized on the molecular and biochemical level, and particularly in the case of the two MAPKs, functional biochemical assays have been established that can be used for compound screening (75,76). Of further interest are already characterized receptor kinases of the insulin- (EmIR; 79), the epidermal growth factor- (EmER; 80) and the transforming growth factor-β- (EmTR1; 81) receptor families that are expressed by the *E. multilocularis* metacestode stage and that are involved in host–parasite cross-communication by interacting with the evolutionary conserved cytokine- and hormone-ligands that are abundantly present in the

Table 2 Structural features and expression of β-tubulin isoforms of *Echinococcus multilocularis*, *Echinococcus granulosus* and *Taenia solium*

Gene	Em.167	Em.200	Eg.167	Eg.200	Ts.167	Ts.200	Expr.MC (%)
tub-1	Phe	Phe	Phe	Tyr	Tyr	Tyr	21
tub-2	Tyr	Tyr	Tyr	Tyr	Tyr	Tyr	100
tub-3	Phe	Phe	Phe	Tyr	Tyr	Phe	26

Nomenclature of the main β-tubulin isoforms expressed by cestodes was made according to Brehm *et al.* (40). Amino acid residues encoded at positions 167 and 200 are given for the orthologous genes in *E. multilocularis* (Em), *E. granulosus* (Eg) and *T. solium* (Ts). 'Expr.MC' refers to the relative expression level in the *E. multilocularis* metacestode stage according to RNA-seq data.

Table 3 Numbers of genes present in the *Echinococcus multilocularis* genome that encode members of particularly druggable enzyme families

Enzyme family	No. of genes
Protein kinases	250
Phosphatases	47
Peptidases	113
Ligand-gated ion channels	9
GPCR (Rhodopsin family)	44
GPCR (Secretin family)	3
Nuclear hormone receptors	17
Glycosyl hydrolases	24
Glycosyl transferases	37

GPCR, G-protein-coupled receptors.

intermediate host's liver (1,72). In total, we could thus far identify ~250 protein kinase-encoding genes on the genome assembly versions of *E. multilocularis* (Table 3) and *E. granulosus*, the majority of which displays considerable homologies to orthologous genes in schistosomes, which could be particularly important for the design of compounds that have a broad spectrum of activity not only against cestodes but also against other parasitic flatworms.

An important issue in rational drug design is not only the identification of targets that display structural and functional differences between the respective parasite and host components, thus ensuring that compounds with sufficient parasite specificity can be found, but also the general 'druggability' of the target, i.e., whether it contains structural features that favour interactions with small molecule compounds (82). Apart from protein kinases, several other protein families such as G-protein-coupled receptors (GPCR) or ligand-gated ion channels proved to be particularly druggable in previous compound screens and chemogenomic approaches (83). For a selection of protein families that are particularly suitable as drug targets, Table 3 lists the number of coding genes that we have identified using the current *E. multilocularis* genome assembly. In addition to a large number of protein kinases, several of which are already under study in the *E. multilocularis* system (72), and nuclear hormone receptors, which have been characterized in cestodes very recently (84), the list also contains multiple peptidases, phosphatases, GPCRs and ligand-gated ion channels which have, so far, been characterized to a certain degree in schistosomes (85), but never in tapeworms. Based on the criteria like expression strength, essentiality, involvement in multiple metabolic pathways, assayability and druggability, Crowther *et al.* (86) recently established a highly interesting *in silico* approach to prioritise parasite proteins for targeted drug design and, in the case of *S. mansoni*, presented a list of particularly promising candidates such as Na+/K+-ATPase, transketolase, vacuolar proton ATPases and a number of additional protein and enzyme components. Once gene annotation for *E. multilocularis* is finished and more extensive data on the larval transcriptome are available, similar approaches are also possible for this species and can, by comparative genomics, also be applied to *E. granulosus* and *T. solium*.

Taken together, all technical and methodological prerequisites for targeted drug design against larval cestodes should soon be (or are already) available. Once suitable targets are identified by *in silico* approaches, respective small molecule lead compounds can be tested for anti-parasitic activity using the established *in vitro* cultivation systems for the *E. multilocularis* metacestode (87) and stem cell systems (1). As an important complementary approach, the essentiality of the target components can be tested using RNA interference (RNAi) assays that have been established very recently for regenerating *E. multilocularis* primary cells (88) and protoscoleces (89). On the basis of the identified lead compounds and libraries of related molecules, parasite-specific drugs can subsequently be identified in comparative host- and parasite cell cultivation systems and eventually be tested *in vivo* in well-established animal models for secondary AE. Based on the considerable homologies between all taeniid cestodes, it is highly likely that all identified anti *E. multilocularis* drugs will be also active against *E. granulosus* and *T. solium*.

PARASITE ANTIGENS AND IMMUNOMODULATORY MOLECULES

Larval stages of *E. multilocularis*, *E. granulosus* and *T. solium* induce chronic, long-lasting infections during which the host immune system is modified in various ways through surface components of the metacestode stage (e.g. the acellular 'laminated layer' of *Echinococcus* species) or by excretory/secretory (E/S) products (90,91). For all three species, the induction of Th2-dominated immune responses is observed in intermediate hosts that are highly susceptible to an infection, and a picture is beginning to emerge that, as in helminth infections caused by nematodes and trematodes, regulatory T cells and alternatively activated macrophages might play a crucial role in suppressing antiparasitic immune responses (91,92). Although little is known on the molecular nature of taeniid cestode E/S products with immunomodulatory activities, previous investigations at least identified a number of parasite antigens or laminated layer components that might be involved in deviating or dampening the immune response (reviewed by Gottstein & Hemphill; 93). With the availability of cestode genome sequences, several important questions concerning immunomodulatory factors can now be addressed from a genomic perspective, and in the following, we will present some initial analyses on important antigen families and molecules that are likely to have immunomodulatory properties.

The *Echinococcus* antigen B gene family

Undoubtedly, the most studied factor in *Echinococcus* is the so-called antigen B (AgB), a highly immunogenic lipoprotein and major component of hydatid cyst fluid (94). Although there are several reports on immunomodulatory properties of AgB *in vitro* (94), and biochemical investigations that demonstrate binding of different hydrophobic ligands to AgB (95), the precise function of this protein in

the biology of *Echinococcus* or in the immune response during echinococcosis is still unknown. Originally described as a 160 kDa lipoprotein, AgB was later shown to be built up of several 8 kDa monomers that are encoded by a gene family (96), and since the first full description of an AgB-encoding gene by Frosch *et al.* (97), there has been constant debate on how many of these genes are actually expressed in these parasites. By studies of Fernandez *et al.* (98), Chemale *et al.* (99), Arend *et al.* (100) and Mamuti *et al.* (101), the number of AgB subunit genes had grown to five in 2007 (named EmAgB1-EmAgB5 in *E. multilocularis* and EgAgB1-EgAgB5 in *E. granulosus*), whereas genomic Southern blot analyses indicated that there are at least seven loci (102). Studies by Haag *et al.* (103) and Arend *et al.* (100) even suggested the presence of further AgB genes (up to 10 in *E. granulosus* and up to 110 copies in the related *E. ortleppi*) as well as a high degree of genetic polymorphism among those genes (even within protoscoleces that derived from one single cyst). These authors proposed that numerous AgB copies might be involved in gene conversion mechanisms through recombination processes and DNA rearrangements similar to the situation in protozoans such as *Plasmodium* sp. or trypanosomes (103). This theory was recently contradicted by Zhang *et al.* (104) who characterized AgB genes in *E. granulosus* isolates from different geographic origins and proposed the presence of 10 unique genes (or alleles) that are, however, highly homologous between these isolates and did not show gross polymorphisms. To shed more light on the situation, we have analysed the presence and location of AgB genes in the current assemblies of the *E. multilocularis* and *E. granulosus* genomes. As described by Brehm (72), using the first assembly version of the *E. multilocularis* genome (19 000 contigs), a total of seven AgB loci appears to form a cluster on a distinct region of the genome. In the latest genome version (600 supercontigs), all these copies are now assembled into one continuous sequence fragment of 57 kbp that is present on scaffold_29 (Figures 2 and 3). The antigen B cluster is flanked by two genes, Em*LDLR* and Em*MTA*, which are highly conserved among cestodes. The gene product of one of these, EmLDLR, displays significant homologies to low-density lipoprotein (LDL) receptors from other species and contains one single class A LDL receptor domain. The second encodes a factor with considerable homologies (50% identical, 66% similar residues) to the human 'metastasis-associated-protein' MTA3 which is a component of the nucleosome-remodelling and histone-deacetylase complex (105) and, like the human protein, contains one BAH (bromo-adjacent homology) domain, one GATA-type zinc finger domain and one classical zinc finger domain (data not shown). As previously suggested (72), the antigen B cluster is formed of one copy each of AgB1, AgB2, AgB4 and AgB5, two identical genes encoding AgB3 and one slightly altered AgB3 gene (AgB3'). The only difference to the previously suggested cluster organization (72) is that in the newest assembly version the AgB5 locus and one AgB3 locus have changed position (Figure 2). All genes of the cluster display the typical organization (103) of two exons, with a signal peptide encoded by exon 1, separated by a small intron. Transcriptome analyses on *in vitro* cultivated metacestode vesicles further indicate that AgB1 is, by far, the most abundantly expressed isoform, followed by AgB3' (20% of the expression level of AgB1) and AgB3 (10%). Only marginal expression could be detected for AgB2, AgB4 and AgB5 in the metacestode, and likewise, almost no expression was measured for any AgB isoform in the protoscolex (data not shown).

In *E. granulosus*, the situation appears to be highly similar to *E. multilocularis* (Figure 2). Within a region of approximately the same size as in *E. multilocularis*, close orthologs of Em*LDLR* (Eg*LDLR*) and Em*MTA* (Eg*MTA*) are present and are flanking a cluster of seven loci with one copy each of AgB1, AgB2, AgB4 and AgB5, as well as three slightly differing copies of AgB3 (AgB3-1, AgB3-2,

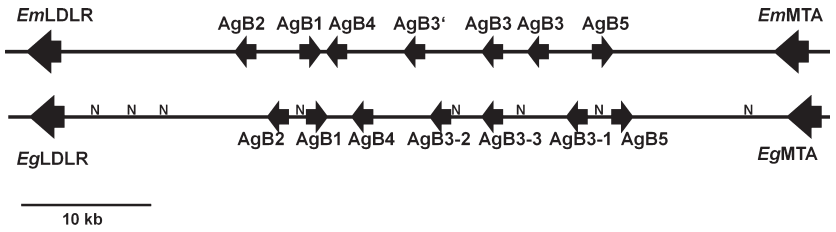

Figure 2 The *Echinococcus multilocularis* and *Echinococcus granulosus* antigen B clusters. Displayed is the genomic organization on scaffold_29 (positions 624.079–681.301) of the *E. multilocularis* genome (above) and supercontig_30740 (680.925–741.961) of the current *E. granulosus* assembly (below). Arrows indicate the location and direction of transcription of AgB isoforms and *Echinococcus* orthologs of the conserved genes *LDLR* and *MTA*. Regions of the *E. granulosus* assembly that are not yet covered by sequencing data are marked by 'N'.

AgB3-3). Although care has to be taken in suggesting complete synteny between both species in this region, because the single *E. granulosus* contigs (flanked by 'N' in Figure 2) have been assembled into supercontigs using the *E. multilocularis* sequence as a reference, at least the *E. granulosus* copies of AgB1, AgB4 and AgB3-2 are clearly assembled into one contig and display the same gene order and transcriptional orientation as in *E. multilocularis* (Figure 2). This makes it highly likely that the genome arrangement as suggested for *E. granulosus* in Figure 2 reflects the true situation. Apart from the AgB cluster, we could not detect any AgB-related sequences elsewhere in the genomes of *E. multilocularis* and *E. granulosus*, with one notable exception of an AgB-like gene on *E. multilocularis* scaffold_7, that is, however, not represented in EST databases, does not show a detectable transcription profile in RNA-seq data, contains inactivating mutations within the reading frame (data not shown), and thus most likely represents a pseudogene.

Taken together, the apparently high level of homology and synteny within the AgB clusters of *E. multilocularis* and *E. granulosus*, and the absence of functional AgB copies outside these clusters, does not support the theory that this region is a hot spot for genomic rearrangements. Furthermore, the structure as depicted in Figure 2 clearly supports previous data on the occurrence of just five distinct subfamilies of AgB genes (101) and the presence of seven distinct bands in Southern blot analyses under low-stringency conditions (102). The gross discrepancies between the genomic situation around the AgB clusters of *E. granulosus* and *E. multilocularis* and previous reports on very high copy numbers of the AgB genes in *Echinococcus* protoscoleces (100,103) are difficult to explain at present. On the one hand, Arend *et al.* (100) and Haag *et al.* (103) exclusively relied on PCR-based methodology to estimate the numbers of AgB genes in isolated parasite material which, because of the amplification process, might be prone to significant errors. On the other hand, involving an as yet unknown mechanism, these genes could be amplified as extra-chromosomal DNA aggregates that might have slipped the genome assembly process. Finally, since the highest number of AgB copies was detected in laboratory material of *E. ortleppi* (103), this species might significantly differ from *E. multilocularis* and *E. granulosus* concerning the AgB cluster. In future studies, it might thus be worthwhile to also characterize the *E.ortleppi* AgB cluster and the surrounding genomic regions.

Interestingly, when analysing the current *Hymenolepis* genome assembly, we also identified four AgB-related genes (on contigs 10534, 20275, 23242 and 25502) with a typical exon–intron structure (Figure 3), suggesting that the AgB family is not taeniid cestode specific but occurs in a wide variety (if not all) cestodes. Unfortunately, the *H. microstoma* assembly used at the time of analysis was too fragmented to determine whether the AgB genes are also clustered in this species. However, the most recent version of its genome, and targeted analyses of additional cestode genomes using sequence information of the conserved *LDLR* and *MTA* genes, should provide valuable information to further dissect the evolution of the *Echinococcus* AgB cluster.

The host protective oncosphere antigens

The prototype of another highly interesting taeniid cestode gene family encodes the oncospheral antigen EG95 which has been successfully used in vaccination trials against CE

Figure 3 Antigen B isoforms encoded by the genomes of *Echinococcus multilocularis*, *Echinococcus granulosus* and *Hymenolepis microstoma*. Displayed is a CLUSTALW alignment of amino acid sequences encoded by exon 1 (signal peptide) and exon 2 of AgB isoforms of *E. multilocularis* (EmAgB1–EmAgB5), *E. granulosus* (EgAgB1–EgAgB5), and four AgB-like proteins of *H. microstoma* encoded on contigs 10534, 20275, 23242 and 25502 of the current genome assembly version. Highly conserved residues are printed in white on black background, residues with similar biochemical function and printed in black on grey background. The signal peptide region is indicated by asterisks below the alignment. Note that the signal peptides of EgAgB3-2, Hm20275 and Hm25502 could not yet be identified on the genome sequence or are not contained in the current assembly versions of the genomes.

in sheep (reviewed by Lightowlers; 106). The EG95 gene has been demonstrated to belong to a gene family that consists of six functional genes in *E. granulosus* of which four encode a protein identical to the original isolate (now named EG95-1; 107). The EG95 gene family is structurally homologous to the 45W gene family and the 16K and 18K groups of antigens that are expressed in various *Taenia* species (108). Like in the case of *E. granulosus*, recombinant antigens of this family were already successfully employed for the development of vaccines against larval *Taenia* infections (106). The biological function of the EG95/45W proteins is largely unknown. However, they all share a common domain structure of a signal peptide, followed by one single fibronectin III (Fn3) domain and a hydrophobic transmembrane region close to the C-terminus (107). Very interesting recent work on different *Taenia* species (109,110) and *E. granulosus* (111) also demonstrated that these proteins are primarily located in the penetration glands of the nonactivated oncosphere and are distributed over the oncospheral parenchyma upon activation with low-pH/pepsin treatment (mimicking the transition to the intermediate host). Because Fn3 domains are typically found in extracellular matrix-associated proteins, it is conceivable that the EG95/45W proteins play a role in providing or organizing a primary matrix framework to which totipotent parasite stem cells (delivered by the oncosphere) can attach to undergo the early oncosphere–metacestode transition, although experimental evidence supporting this theory is still lacking. A close ortholog to EG95 has also already been identified in *E. multilocularis* (named EM95), and the respective recombinant protein was effective in protecting mice against challenge infection with *E. multilocularis* oncospheres (112). Because this was, so far, the only report on these genes in *E. multilocularis* and because the overall genomic organization of the EG95/45W encoding genes had not been determined in the other cestode species, we carried out respective analyses on the assembled *E. multilocularis* genome. When the EM95, EG95 and 45W sequences were used in tBLAST analyses, we could indeed identify a relatively large number (up to 15) of related genes dispersed over the genome, most of which were, however, transcriptionally silent according to RNA-seq data and many contained inactivating mutations in their reading frames. Only five of the genes showed significant levels of transcription and only two of those, located on scaffold_159 (Em95; position 5963–4694) and scaffold_125 (Em95-2; 15880–14568) were closely related to the previously identified EM95 (112) and displayed the same conserved exon–intron structure (Figure 4). Intriguingly, in the RNA-seq transcription profiles, these oncosphere-specific genes displayed considerable levels of expression in regenerating primary cells but not in metacestode or protoscolex (Figure 5) which underscores the suitability of the *E. multilocularis* stem cell cultivation system to mimic the oncosphere–metacestode transition not only morphologically (36), but also concerning gene expression profiling. Two additional EM95-like genes that we identified, located on scaffold_104 (Emy162a; position 44001–45896) and scaffold_7 (Emy162b; 35094–33349) showed considerable homologies to the recently identified EMY162 antigen (113). Unlike EM95, this antigen lacks the C-terminal hydrophobic transmembrane domain (but contains the signal peptide and the Fn3 domain) and is not strictly expressed in the oncosphere but also in other developmental stages (113), which is clearly supported by our RNA-seq transcriptome data (Figure 5). The fifth gene, located on scaffold_45 (Em*oal* for oncosphere-antigen-like; position 4212–3089) represents a novel, distantly related member of the EG95/45W family that has not yet been described in studies on vaccine development (Figure 4).

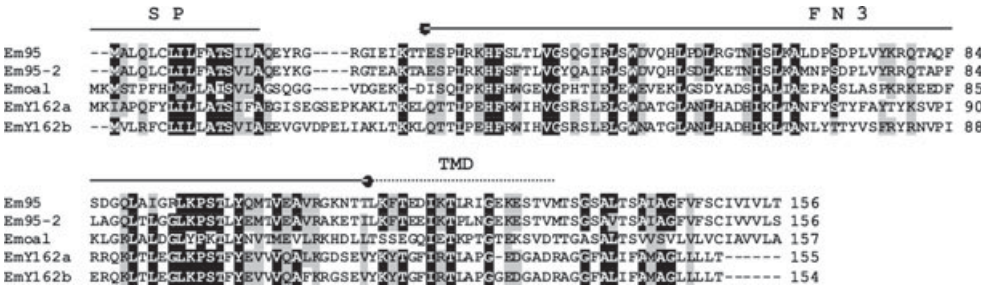

Figure 4 Members of the EG95/45W protein family encoded by the *Echinococcus multilocularis* genome. Displayed is a CLUSTALW alignment of the amino acid sequences of two Em95 isoforms (Em95, Em95-2; 107), two isoforms of EmY162 (EmY162a/b; 113) and an additional isoform, newly identified in this study (Emoal). Highly conserved residues are printed in white on black background, residues with similar function in black on grey background. The location of signal peptides (SP), Fn3 domains (FN3) and transmembrane domains (TMD) is indicated above the alignment. Note that no TMDs are predicted for the two EmY162 isoforms. Sequence- and primary cell-specific expression of the newly identified Em*oal* cDNA was verified by PCR amplification, cloning and sequencing. The Em*oal* cDNA sequence is available in the EMBL, GenBank and DDJB databases under the accession no. FR848832.

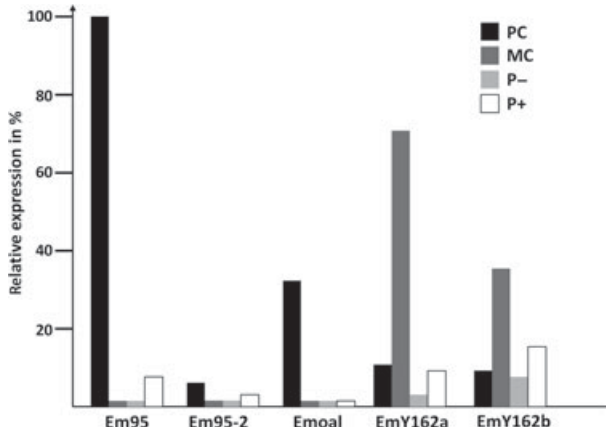

Figure 5 Expression of EG95/45W genes in *Echinococcus multilocularis* larval stages. Displayed is the relative expression level of the two identified Em95 isoforms (Em95, Em95-2), the two EmY162 isoforms (EmY162a/b), and the newly identified Em*oal* in primary cells (PC; black; representing the oncosphere–metacestode transition), in the metacestode stage (MC; dark grey), as well as in protoscoleces prior to (P−; light grey) and after (P+; white) activation by low-pH/pepsin treatment. The expression level of Em95 in primary cells has been set to 100%. Note that the Em95 and Em*oal* isoforms are expressed in a PC-specific manner, whereas the EmY162 isoforms display stronger expression in the metacestode. Data were obtained by RNA-seq analyses on *in vitro* cultivated parasite larval stages.

Very much like EM95, Em*oal* is specifically expressed in regenerating primary cells; it displays an exon–intron structure that is typical for the EG95 gene family, and its gene product comprises a signal peptide, one Fn3 domain and a C-terminal transmembrane domain, suggesting that it has a similar function as the EG95/45W proteins described so far. A close ortholog to Em*oal*, Eg*oal*, is also present on the genome of *E. granulosus* (contig_32513; position 4699–3576), which could prove important for the further development and improvement of vaccine formulations against CE. Interestingly, and in contrast to the AgB family, the genome of *H. microstoma* is absolutely free of EG95/45W-like sequences, which supports the idea that this gene family is indeed highly specific to taeniid tapeworms.

Additional antigens and immunomodulators

In addition to the TSOL18 and TSOL45 antigens of *T. solium*, extensive vaccination trials against porcine cysticercosis have already been undertaken using the so-called S3Pvac vaccine (114,115). S3Pvac consists of three synthetic peptides (named KETc12, KETc1, GK1) that had been identified by immune-screenings against *T. crassiceps* cDNA libraries and when tested under field conditions, SP3vac could reduce the number of *T. solium* infected pigs by 50% and lowered parasite load by >90% (90). Interestingly, in spite of the fact that a considerable amount of information has already been published on S3Pvac (90), including a recent report on the presence of similar sequences in other cestodes (116), the proteins and genes which correspond to the synthetic peptides have never been characterized so far. We therefore analysed the situation for *E. multilocularis* using the published KETc1 and GK1 sequences as well as *E. multilocularis* genome and transcriptome data. The GK1 peptide clearly maps to the amino acid sequence encoded by a predicted gene on scaffold_13 (position 1.570.711–1.568.292). The encoded protein (264 amino acids; 29 kDa; Figure 6) contains one Glucosyltransferase/Rab-like GTPase activators/Myotubularin domain (GRAM domain), which is thought to be an intracellular protein-binding or lipid-binding signalling domain, and one WWbp domain which is characterized by several short PY- and PT-motifs and which presumably mediates tyrosine phosphorylation in WW domain–ligand interactions (Figure 6). At least within the WWbp domain, this protein displays significant homologies (47% identical, 68% similar residues) to a predicted *S. mansoni* protein, WW domain-binding protein 2 (accession no. FN313948), of unknown function. The KETc1 peptide also clearly maps to a genomic region that encodes a 67 kDa protein with significant homologies (46%, 62%) to a hypothetical protein of *S. mansoni* (accession no. FN357512). Interestingly, however, the KETc1 encoding region is out of frame of the actual protein-encoding sequence and should, actually, not be present in *E. multilocularis* (and most probably all

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MSINTAHTQDGLGVVLFYGERLLITYDGCKLTLSGSGQQPNGRYSGTAYLTSHRVIFLSK   60
DKSPALNSLSMAFIYMRRVAIKQPTFGPNHIEGFVLSESGQWAGEMPFKLAFNHGGAIEF  120
GKSLLELGTRASKLQNSYKTPVAPPLCEIYACPPPAYTPFVNDPYYNSFMQVHPSFSPPP  180
VEFLYQTNSPPPYPGAVPPPYTPNPGPPPPYTATAASPMPPYPAGGPPFVNTGYYYPSDP  240
NTFYAPPYSQASAPPMEPEDKKNL                                       264
```

Figure 6 The *Echinococcus multilocularis* WBP-2 protein. Displayed is the deduced amino acid sequence of the GK1-peptide-containing *E. multilocularis* WBP-2 protein. Identified GRAM- and WWbp-domains are indicated below the sequence by full and dashed lines, respectively. The GK1 peptide which has been used in *Taenia* vaccination trials (90) is printed in white on black background.

other cestodes). As briefly discussed by Rassy *et al.* (116), the initial identification of KETc1 might have resulted from a reading frame error of the employed λZAP vector which, nevertheless, does not explain why this peptide induces high levels of protection when used as an immunogen against cysticercosis (90).

Apart from the characterization of parasite-specific antigen families, the available genome information should also facilitate the identification of parasite orthologs with homologies to immunomodulatory host proteins or cestode orthologs of trematode proteins with such activities. As already outlined, for cell–cell communication, cestodes utilize evolutionarily conserved signalling systems of the insulin-, the epidermal growth factor-, and the transforming growth factor-β (TGF-β)-pathways and respective parasite receptors that are able to functionally interact with corresponding host hormones and cytokines have already been identified (72). This makes it likely that cestodes also express cognate ligands of these signalling systems which, provided that they are secreted, could activate the corresponding host receptors to affect host physiology or the immune response. In fact, in preliminary analyses, we could already identify several genes on the genome of *E. multilocularis* that encode insulin-like peptides and cytokines with significant homologies to members of the TGF-β/BMP families (72). Particularly, regarding the prominent role of TGF-β in inducing anti-inflammatory immune responses (117), the parasite cytokines of the TGF-β/BMP family are of considerable interest and are currently under study in our laboratories concerning influences on immune effector cells such as dendritic cells and T cells.

Prominent examples of immunomodulatory factors from schistosome eggs are the 'interleukin 4 (IL-4)-inducing principle' IPSE, which stimulates basophils to express and secrete the Th2-associated cytokines IL-4 and IL-13 (118), as well as the Omega-1 component of schistosome egg antigen, which drives Th2 immune responses in mice (119). Although *E. multilocularis* extract contains a component with similar activities as IPSE (120), we could so far not identify any cestode gene that encodes an IPSE-like peptide, indicating that the IL-4 inducing activity is caused by another component in these organisms. An ortholog to Omega-1, on the other hand, is clearly encoded by the *E. multilocularis* and *E. granulosus* genomes and could, like its schistosome counterpart, be involved in driving Th2 responses during AE and CE, respectively. Another family of proteins that are of tremendous interest concerning immunomodulation by helminths are the so-called cystatins, which are cysteine protease inhibitors that are secreted by nematodes and interfere with host cell antigen processing and presentation (121). Very recently, one of these molecules has been demonstrated to exploit activation and deactivation pathways of MAPKs to induce regulatory macrophages in filarial infections (122). Interestingly, the *E. multilocularis* genome encodes at least one cystatin with homologies to those of nematode parasites, and transcriptome data show that this factor is specifically (and highly) expressed in the metacestode stage that is representative for the chronic phase of AE (data not shown). Because macrophages from *E. multilocularis* infected mice are impaired in their ability to present antigen to lymph node T cells (123), respective activities of the *E. multilocularis* cystatin would be of particular interest and are currently addressed in our (KB) laboratory. Hence, not only for investigations on cestode evolution and development, or for the design of effective chemotherapeutics, but also for novel approaches into the immunology of cestode infections, the currently ongoing genome projects hold great potential.

DEVELOPMENTAL SIGNALLING SYSTEMS IN FLATWORMS

Our laboratory (PDO) began developing the *H. microstoma* model to investigate the roles of developmental regulatory genes in cestodes, with the aim of understanding the complex life histories of parasitic flatworms from a comparative evolutionary context. It has become clear that metazoans share a surprisingly small number of signalling systems used to pattern their bodies (e.g. Notch, Hedgehog, Wnt, TGF-β and Receptor Tyrosine Kinase) and the presence of most of these systems in the earliest branching metazoans suggests that complexity in contemporary animal form has not arisen through invention of new systems, but through modification of ancient, highly conserved genetic programmes (124). Current knowledge of the signalling systems that underpin flatworm morphogenesis is based primarily on the study of planarians, for which availability of a draft genome of *S. mediterranea* has greatly accelerated research on planarian regeneration and stem cells and has helped to re-establish them as a powerful model in developmental biology (29,125,126). In particular, investigations of highly conserved signalling systems such as the Wnt/β-catenin pathway have yielded several important discoveries in recent years regarding the cellular decision making used to pattern their bodies during growth and regeneration (127). By contrast, the developmental biology of parasitic flatworms, and of parasitic organisms generally, has been largely ignored in preference to research relating to disease processes (128). Consequently, little is known about the genetic basis of their morphogenesis or the extent to which they share the same compliment of developmental systems and genes found in free-living animals (124). Thanks to new genome

data, however, we are now in a position to begin cataloguing developmentally relevant genes and investigating their roles in their complex life histories. Although we do not focus here on immunology or a medically important model species, elucidating signalling systems that regulate basic developmental processes in parasitic flatworms has obvious relevance to the design and evaluation of chemotherapeutic targets.

The segmented, or strobilate, condition that is the hallmark of tapeworms is a derived trait that evolved as an adaptation to reproduction, as opposed to locomotion, and has been considered an evolutionary novelty by most developmental biologists, suggesting it lacks homology with known mechanisms in, e.g., annelid worms, flies or mice (129,130). Using *Hymenolepis* as a classical model for studying adult development in tapeworms, we have initiated investigations on the mechanisms of axial patterning through investigation of Hox and Wnt regulatory genes (128,131). Hox genes encode transcription factors that establish anteroposterior (AP) polarity, regional differentiation and axial elaboration by regulating gene expression in spatially and temporally specific patterns, whereas Wnt genes encode ligands involved in cell–cell communication and have been hypothesized as the ancestral metazoan patterning system (132) that evolved to work in concert with Hox genes during embryogenesis (133). Together, these gene families and their interacting partners are the most important known regulators of axial patterning in metazoans (133). Elucidating their roles in tapeworms will provide a common means by which the mechanisms of segmentation and larval metamorphosis can be compared with other parasitic and free-living flatworms, and to more distantly related animal groups.

Hox genes in flatworms

The Hox genes and their evolutionary cousins the ParaHox genes (134,135) are notable not only for their universality in regulating axial patterning in animals, but for their 'colinear' architecture, by which the order in which they are arrayed in the genome corresponds to their spatial domains of expression, anterior to posterior (136). Three paralogy groups (anterior, central and posterior) are recognized corresponding to these domains, and a total of 11 genes has been hypothesized to be the ancestral state in lophotrochozoans, including duplication of their ancestral posterior Hox ortholog, giving rise to the lophotrochozoan-specific *Post-1* and *Post-2* genes (137). Although the presence of Hox genes in flatworms has been known since some of the first searches for Hox orthologs outside flies and mice (138), the first investigation to focus specifically on Hox genes in a parasitic flatworm was in 2005 by Pierce *et al.* (139) who examined *S. mansoni*. Their work indicated that flatworms had both a reduced and a dispersed complement of Hox genes, and subsequent empirical and *in silico* investigations of the tapeworms *H. microstoma*, *Mesocestoides corti* and *E. multilocularis*, the polyopisthocotylean 'monogenean' *Polystoma* spp. and additional work on *S. mansoni* have now confirmed this to be true in each of the major parasitic groups (128,140,141).

Table 4 shows the presence of genes encoding Hox orthologs in the genomes of *Hymenolepis* and *Echinococcus* spp., *S. mansoni*, polyopisthocotylean 'monogeneans', and the planarian *S. mediterranea*. From these representatives, it appears that flatworms have a core set of one anterior gene (*Hox1/Lab*) and three central genes (*Hox3*, *Hox4/Dfd*, *Lox4/Abd-A*). In addition, both characteristic lophotrochozoan posterior Hox genes (Post-1/2) are found, although those were initially thought to be missing from flatworms (128,142). Planarians also show the presence of Hox5 orthologs and larger numbers of central and posterior paralogs than found in parasitic flatworms, although it must be noted that whereas some of the homeobox sequences (e.g. *Hox1*, *Hox4/Dfd* and *Hox8/-Abda*) show high levels of similarity to cognates outside the group, other flatworm homeoboxes are divergent and difficult to classify. Nevertheless, compared with other major lophotrochozoan groups such as annelids and molluscs, both free-living and parasitic flatworms show reductions in the numbers of Hox gene classes, and this may relate to their lack of axial elaboration. *Hymenolepis* is also oddly missing an ortholog of the central Hox3 gene found in all other flatworms examined.

Table 4 Hox transcription factors in the genomes of parasitic and free-living flatworms

	Cestoda			Trematoda	'Monogenea'	Planarian
Gene	H.m.	E.m.	E.g.	S.m.	Pol.	Scm.m.
Hox1/Lab	1	1	1	1	1	1
Hox3/Zen		1	1	1	1	1
Hox4/Dfd	1	1	1	1	1	1
Hox5						1
(Lox4)/AbdA	1	1	1	1	1	3
Hox9-14 (Post-1/2)	2	2	2	2	?	5
Total	5	6	6	6	5+	12

Hox nomenclature based on vertebrates (lophotrochozoans)*/Drosophila*. Data based on searches of publically available genomes and previous reports including empirical and *in silico* analyses (128,139–141).

H.m., *Hymenolepis microstoma*; E.m., *Echinococcus multilocularis*; E.g., *Echinococcus granulosus*; Pol., species of polyopisthocotylean 'monogeneans' (140); S.m., *Schistosoma mansoni*; Scm.m. *Schmidtea mediterranea*.

In all cases, flatworm Hox genes are found to be widely dispersed in the genome and have been shown previously to reside on at least two different chromosomes in *S. mansoni* (139). RNA-seq data indicate the presence of multiple non-Hox coding regions flanking the Hox genes in the *Hymenolepis* genome and thus further confirm the complete lack of clustering of flatworm Hox genes. The genomic structure of *Hymenolepis* orthologs appears normal, and full-length transcripts range in size between ~1500 (*HmHox1*)–2600 (*HmPost-2*) bp and are made up of 2–4 exons separated by introns 81–8946 bp in length. The *abdominal-B* ortholog *HmPost-2* shows a characteristic intron interrupting the homeobox region. In contrast, typically structured Post-1 orthologs have not been described in flatworms, and the one (possibly two) *Hymenolepis* Post-1 orthologs appear as pseudogenes, and full-length exons cannot be deduced from present data.

Expression of Hox genes in parasitic flatworms is so far known only from quantitative PCR and RNA-seq data that indicate dynamic patterns throughout their complex life cycles. Stage-specific expression has been demonstrated in *S. mansoni* (139), the 'monogenean' *Polystoma gallieni* (143), and in *Hymenolepis* and RNA-seq data in *Hymenolepis* also indicate at least minimal expression levels during both adult and larval development, with peaks of expression seen in central and posterior genes. How the dispersed structure of their genes affects the principal of colinearity is not known, and only a few studies of Hox spatial expression have been conducted in free-living flatworms, with somewhat inconsistent results (144), and none in parasitic flatworms. Ongoing investigations of Hox spatial expression patterns throughout the life cycle of *Hymenolepis* do show regionalized, as well as segmental expression patterns that suggest collinear expression is still maintained in the absence of clustering, as has been found in other animals (145). Spatial expression studies in *S. mansoni* and *Protopolystoma xenopodi* (the latter in collaboration with M. Badet) have been recently initiated and will provide valuable comparative data for understanding how Hox expression relates to their disparate life history strategies and body plans.

Wnt genes in flatworms

Wnt genes encode secreted glycoproteins, typically between 350 and 400 amino acids in length, characterized by the presence of 23–25 conserved cysteine residues and by homology to the *Drosophila* gene *wingless* (*wg*) and murine *Int1* (146). Wnts function as extracellular ligands involved in highly conserved cell–cell signalling pathways that regulate a wide array of basic cellular processes, from differentiation to apoptosis (146). In addition, Wnt signalling is known to work in concert with Hox genes to pattern the anteroposterior (AP) axis during embryogenesis (133). Recognizable orthologs of Wnts and other core components of Wnt pathways have been found in the earliest branches of Metazoa, such as sponges and placozoans, but not in the unicellular choanoflagellates (147), indicating that Wnt signalling was essential to the evolution of multicellularity (124,148). Arising earlier in the evolution of Metazoa than the Hox genes, Wnt signalling is also thought to represent the ancestral mechanism of axial patterning in animals.

Wnt signalling is typically described as acting in three discrete pathways: the canonical Wnt/β-catenin, and non-canonical Wnt/planar cell polarity and Wnt/Ca^{2+}-dependent pathways. In planarians, the canonical β-catenin pathway is essential for the maintenance of AP identity, and many aspects of Wnt signalling have now been elucidated in *S. mediterranea*. (149). Only one paper has been published regarding Wnt genes in a parasitic flatworm, which characterized a gene encoding Wnt4 in *S. japonicum* and demonstrated its involvement in the canonical pathway (150). More recently, Riddiford and Olson (131) used genomic data of both free-living and parasitic species to produce a comprehensive listing of Wnt ligand and Wnt pathway components, summarized here in Table 5. Similar to the Hox genes, the diversity of Wnt ligands is greatly reduced in flatworms and indicates a total loss of seven Wnt subclasses. Whether this loss was specific to flatworms or was inherited from the common ancestor of the platyzoans cannot be inferred before Wnts are known from more closely related lophotrochozoans. The core set of ligands in flatworms thus consists of single orthologs of Wnt1, Wnt2, Wnt4, Wnt5 and two of Wnt11, with no differences found between free-living and parasitic species, save the presence of additional Wnt4 paralogs in *Schmidtea* (Table 5). Despite this reduction, one or more orthologs of a full complement of interacting partners are present in their genomes, and they show an especially high diversity of Frizzled receptors. Functional domains of all component genes were found to be intact in the *Hymnenolepis* genome, and RNA-seq data indicate the genes are expressed throughout both phases of the life cycle, suggesting all three pathways are functional in parasitic flatworms (131). RNA-seq data also show Wnt1 to be differentially expressed in adult worms, consistent with its role as a segment polarity gene in some organisms (e.g. Drosophila).

ParaHox genes and segmentation

Although a few ParaHox orthologs have been characterized in free-living flatworms (151), none of the three genes (*Gsh*, *Xlox*, *Cdx*) is found in parasitic flatworms (128,141). They thus lack entirely the additional anterior, central,

Table 5 Wnt ligands and Wnt pathway components in the genomes of parasitic and free-living flatworms

	Cestoda			Trematoda	Planarian
Gene	H.m.	E.m.	E.g.	S.m.	Scm.m.
Wnt ligands					
Wnt1	1	1	1	1	1
Wnt2	1	1	1	1	1
Wnt4	1	1	1	1	3
Wnt5	1	1	1	1	1
Wnt11	2	2	2	1	2
Unclassified					1
Total	6	6	6	5	9
Wnt/β-catenin pathway					
Frizzled	8	7	8	9	13
Dishevelled	✔	✔	✔	✔	✔
GSK3	✔	✔	✔	✔	✔
APC	✔	✔	✔	✔	✔
Axin	✔	✔	✔	✔	✔
β-catenin	✔	✔	✔	✔	✔
LEF/TCF	✔	✔	✔	✔	✔
Ca^{2+}-dependent pathway					
Phospholipase C	✔	✔	✔	✔	✔
CaMKII	✔	✔	✔	✔	✔
Planar cell polarity pathway					
Rho GTPase	✔	✔	✔	✔	✔
JNK	✔	✔	✔	✔	✔
Wnt antagonists					
Dickkopf					
WIF	✔	✔	✔	✔	✔
Cerberus					
SFRP	✔	✔	✔	✔	✔

Numbers of paralogs shown were investigated; checkmarks denote the presence of one or more paralogs. Wnt ligands and Wnt pathway genes reported from (131).
H.m., *Hymenolepis microstoma*; E.m., *Echinococcus multilocularis*; E.g., *Echinococcus granulosus*; S.m., *Schistosoma mansoni*; Scm.m., *Schmidtea mediterranea*.

and posterior regionalizing morphogens found in most Metazoa, and this may again reflect their lack of overt axial differentiation as compared to other animals groups. Moreover, the posterior ParaHox gene is a downstream target of Wnt signalling in the segmentation mechanisms of flies and mice (152), and thus, if the Wnt pathway is also involved in tapeworm segmentation, their lack of ParaHox orthologs makes it clear that the mechanism is modified, if not in fact distinct, from the canonical bilaterian mechanism of segmentation. Additional cDNA samples currently being characterized at the WTSI for RNA-seq analyses will enable comparisons to be made regarding differences in expression along the progressively maturing length of the adult tapeworm body. In this way, we can efficiently characterize the entire transcriptomes associated with the segmenting neck region, maturing strobila and gravid proglottides, and examine differences in gene expression *in silico* via RNA-seq. Data will enable a comprehensive examination of the gene systems active during different phases of their development, including those regulating the process of segmentation, for which we have little information at present (e.g. 153).

CONCLUSIONS AND OUTLOOK

Cestodology has entered the era of nuclear genomics and transcriptomics. With the *E. multilocularis* genome almost finished and those of *E. granulosus*, *T. solium* and *H. microstoma* in advanced draft versions, a significant body of cestode genome information is now publicly available. Although annotation is still ongoing, we can already state that there is a wealth of information on potential immunomodulatory factors, promising targets for the development of improved chemotherapeutics, and signalling pathways involved in host-dependent development and morphogenesis in cestodes. Comparisons with trematodes and free-living flatworms will yield valuable information concerning genomic rearrangements and gene gain/loss associated with the evolution of parasitism, allowing us to identify common factors involved in host immunity. The projects also demonstrate that genome characterization in tapeworms is manageable thanks to their comparatively small size and low amount of repetitive and mobile genetic elements. These characteristics greatly facilitate assembly and annotation of NGS data and provide good prospects to future projects on other cestode species. In particular, the laboratory model *Mesocestoides corti*, and the pathogens *Diphyllobothrium*, *Spirometra*, and *T. crassiceps* should be considered priorities for future sequencing, as should the other *Hymenolepis* models, *H. diminuta* and *H. nana*, which would enable fine-scale differences in tapeworm genomes to be investigated.

Given that the genomes of the major flatworm pathogens will be soon fully sequenced, more comprehensive approaches concerning comparative chemogenomics and immunomodulatory factors can be launched in the near future. Similarly, the efficiency of NGS technologies for the characterization of genomes the size of tapeworms means that basic aspects of their biology can be addressed by sequencing species that, for example, show different forms of body organization. Beyond such comparative approaches, targeted genetic manipulation is required to address functional hypotheses. Despite progress (89,154), these techniques are still in their infancy and hold great potential for improvement. Success in this area will also depend on better cultivation systems for cestode laboratory models other than *E. multilocularis*. Apart from

RNAi by which knock-down can be achieved, albeit often with limited success depending on organism and gene, it will be most important to achieve stable expression of trans-genes in stem cells either by retroviral expression systems as has been performed in bloodflukes and planarians (155,156), or by exploiting (the few) mobile genetic elements that we have found in cestodes. Respective studies are currently underway in our laboratories and others and will, in combination with additional genome information (e.g. promoter structures; microRNA targets), open the door to a new understanding of cestode biology.

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