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Research paper

Gene expression changes with tumor disease and leech parasitism in the juvenile green sea turtle skin transcriptome

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ABSTRACT

Emerging infectious diseases are a major threat to biodiversity in the 21st century. Fibropapillomatosis (FP) is an epithelial tumor disease that affects immature and adult marine turtles worldwide, particularly green turtles (*Chelonia mydas*). We know little about the host factors contributing to FP susceptibility, in part because transcriptomic studies that compare transcript expression in turtles with and without FP are lacking. Here, we performed RNA-Seq on healthy skin tissue from immature *C. mydas* in the Indian River Lagoon, Florida, USA, comparing turtles (1) with and without FP and (2) with and without leech parasites, a putative vector of FP. We assembled a *de novo C. mydas* skin transcriptome to identify transcripts with significant differential expression (DE) across FP and leech categories. Significant DE transcripts were found across FP and leech comparisons, including 10 of the same transcripts with DE across both comparisons. Leech-positive individuals significantly upregulated different immune and viral interaction transcripts than did leech-negative individuals, including viral interaction transcripts associated with herpesvirus interactions. This finding strengthens the role of marine leeches as mechanical vectors of Chelonid herpesvirus 5 (ChHV5) which has been implicated as a causative agent of FP. FP-positive turtles upregulated several tumor progression and suppression transcripts relative to FP-negative turtles, which had no significant DE tumor progression transcripts. FP-positive turtles also upregulated significantly more protein interaction transcripts than FP-negative turtles. DE transcripts across leech comparisons showed no functional enrichment, whereas DE transcripts across FP comparisons showed some GO terms were enriched in FP-positive and FP negative turtles. Notably, only FP-negative turtles were enriched for GO terms involved in acquired and inflammatory immune gene regulation. Overall, our DE transcripts included several candidate genes that may play important roles in *C. mydas* resistance to or recovery from FP, highlighting that transcriptomics provides a promising venue to understand this impactful disease. Continued investigation of *C. mydas* responses to FP and leech affliction is imperative for species persistence and the conservation of marine ecosystems worldwide due to the essential role of sea turtles in ecosystem function and stability.

1. Introduction

Infectious diseases are currently ranked among the top causes of global species declines and their role in historical extinctions may be underestimated (MacPhee and Greenwood, 2013; McCallum, 2012). Fibropapillomatosis (FP) is an emerging infectious disease (EID) in marine turtles (Jones et al., 2016) that is characterized by benign tumors that can impair vision, diving and feeding behavior (Herbst, 1994). FP prevalence varies among species and regions with some regions exceeding 50% of observed green turtles (Chelonia mydas; (Hargrove et al., 2016). FP impacts *C. mydas* by affecting immunocompetence, submergence intervals, blood chemistry, and cleaning symbiosis

Abbreviations: GO, Gene Ontology; FP, Fibropapillomatosis; DE, Differential expression; ChHV5, Chelonid herpesvirus 5; EID, Emerging infectious disease; RIN, RNA integrity number; mRNA, Messenger RNA; cDNA, Complementary DNA; PCR, Polymerase chain reaction; FDR, False discovery rate; BLAST, Basic local alignment search tool.

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interactions (Aguirre et al., 1995, 2004; Cray et al., 2001; Aguirre and Balazs, 2000; Brill et al., 1995). Although FP is largely a non-fatal disease, FP prevalence is used as an indicator of the overall health of green turtle populations, and possibly the health of the water or ecosystem itself (Gorham et al., 2016). FP was first identified in *C. mydas* in 1938 where an individual presented multiple tumor like legions at the New York Aquarium (Smith and Coats, 1938). This individual was native to Florida USA where immature and breeding assemblages of *C. mydas* are abundant (Foley et al., 2019; Hart and Fujisaki, 2010). Immature *C. mydas* individuals also constitute the majority of documented FP cases (Jones et al., 2016). This disease has been investigated for over eighty years, yet its epidemiological dynamics are still not fully understood (Hargrove et al., 2016). Recovery from FP is possible (Patrício et al., 2016), but the proportion and rate at which marine turtles recover or succumb to FP, either directly or indirectly (e.g., impaired foraging behavior), remains uncertain. The Florida Keys, Indian River Lagoon and Florida Bay report 50–70% of green sea turtles in these regions are affected by FP (Ehrhart et al., 2016) suggesting that the negative fitness consequences of FP on these green turtle assemblages could be substantial. Because FP commonly affects immature turtles, it may cause cascading sublethal consequences over the lifespan of these long-lived organisms (Greenblatt et al., 2005), such as reducing lifetime fecundity.

Prior work associating herpesviruses with immune evasion and suppression (Alibek et al., 2014) and detection of Chelonid herpes virus 5 (ChHV5) in FP tumors (Alfaro-Núñez et al., 2016; Lackovich et al., 1999) provided the basis for association of FP with viral variants of ChHV5. Although this association is widely accepted, the relationship between exposure to ChHV5 and development of FP in green turtles remains unclear. High concentrations of ChHV5 DNA are found within FP tumors, but recent studies identified that asymptomatic turtles are often carriers of ChHV5 (Alfaro-Núñez et al., 2016; Lawrance et al., 2018). Clinically (and visibly) healthy turtles are known carriers, and viral latency, viral load thresholds, or other ecological factors may play transformative roles in tumor expression (Alfaro-Núñez et al., 2016). Sea turtles are highly migratory animals that spend time in distinctly different habitats at different life stages (Bolten, 2003). Marine turtle migratory patterns across life stages, and sampling difficulties associated with their oceanic habitat, present challenges to understanding the relationship between ChHV5 and FP (Chaves et al., 2017). ChHV5 genetic lineages show some geographical separation (Greenblatt et al., 2005), but do not predict the presence or severity of FP tumors (Lawrance et al., 2018). Ene et al. (2005) provides support for marine turtles becoming infected with ChHV5 variants after they return to neritic habitats after an initial oceanic stage. Thus, the precise role ChHV5 plays in FP tumor appearance remains to be fully elucidated, and whether ChHV5 is the primary cause of FP remains a topic of debate.

The role of parasitic infections in biological outcomes (e.g., lifetime fecundity, survival rates, population-level trends) is currently underrepresented in the literature, but their impact remains undeniable (Preston and Johnson, 2010). Marine leeches *Ozobranchus margoi* and *O. branchiatus* are ectoparasites of marine turtles worldwide (Bunkley-Williams et al., 2008). Leeches potentially decrease turtle fitness directly by blood-feeding, and *O. margoi* and *O. branchiatus* are also proposed as vectors for ChHV5 (Jones et al., 2016). *Ozobranchus* spp. harbor sufficient ChHV5 loads to be a mechanical vector (Greenblatt et al., 2004) and leech parasitism is significantly positively associated with FP occurrence in *C. mydas* (Rittenburg et al., 2021). Unlike other leech species, *Ozobranchus* spp. are semipermanent parasites via their reproductive behavior where they mate and deposit cocoons on their host (Burreson, 2020), with prolonged contact possibly increasing the likelihood that a leech harboring ChHV5 will infect its marine turtle host. Recent transcriptomic research identified salivary loci from *O. margoi* that provide further context to studies of leech-induced disease in turtles and will also be useful in broadening understanding of host-parasite relationships (Tessler et al., 2018). Investigation into *C. mydas* immune system responses to these parasites may therefore provide further insight into leech-ChHV5-FP relationships and could offer alternative management strategies for this disease. Additionally, in other taxa tumor burden is associated with decreases in host immune response to pathogens (Broder and Waldmann, 1978). One consequence of this decreased immune response may be increased vulnerability to secondary infections (Aguirre et al., 2004), which may contribute to host death or negatively impact host persistence (Domiciano et al., 2017; Work et al., 2004). Thus, the combined presence of tumors and leech parasitism in *C. mydas* individuals may make them more vulnerable to other infections.

Recent transcriptomic studies are beginning to uncover the functional genetic underpinnings of FP. For example, Duffy et al. (2018) compared transcriptome-wide gene expression in FP tumors to healthy tissue from the same individual and found that FP tumors express transcripts previously identified as cancer promoting genes in human cancers. Investigation of FP tumor similarity to human cancer revealed FP tumors appear to share vulnerabilities with human basal cell carcinoma; only 18% of turtles treated with the human anti-cancer drug fluorouracil showed tumor regrowth (Duffy et al., 2018). Signs of immunosuppression and chronic inflammation are also associated with flow cytometry profiles of FP-afflicted turtles (Rossi et al., 2016). The flow cytometry profiles compared FP-infected individuals to clinically healthy turtles, while the differential expression study characterized FP tumors without comparing to unafflicted individuals. Characterizing gene expression differences among FP infected and uninfected turtles is an important next step in understanding the host factors potentially driving FP susceptibility or the potential for the evolution of resistance.

Applying human precision medicine approaches to wildlife tumor diseases may enhance biodiversity conservation (Whilde et al., 2016). For example, identifying gene expression profiles at each stage of an oncogenic phenomena can assist in the development of immunotherapies and ultimately disease management strategies. In human oncogenic progression, immune responses are extensively studied and known to play roles in not only tumor suppression but also tumor progression (Schreiber et al., 2011). Similarly, *C. mydas* show altered immune responses when afflicted with FP tumors (Cray et al., 2001), and FP tumors alter host gene expression (Duffy et al., 2018). Differential gene expression approaches have been used to understand immune gene responses to parasites in diverse taxa ranging from grass carp to honeybees to hard clams (Chang et al., 2005; Jiang et al., 2016; Perrigault et al., 2009), but this approach has not yet been implemented in natural assemblages of marine turtles. Discovering hallmarks of gene expression that identify a host that is able to avoid and/or survive FP, viral infection, and other parasites may provide insight into key immune responses associated with sea turtle health.

In this study, we used RNA-seq to generate a *de novo* reference transcriptome from healthy skin biopsies sampled from immature *C. mydas* individuals in the Indian River Lagoon, FL, USA. We quantified global gene expression profiles to determine whether gene expression in the skin of immature *C. mydas* individuals differs significantly among (1) turtles with and without FP tumors, and (2) turtles with and without leech parasites. This study provides the first transcriptomic analysis of disease and parasitism in coastal immature sea turtles and offers insight into how *C. mydas* individuals functionally respond to these potentially fitness-reducing conditions.

2. Materials and methods

2.1. Turtle sampling

The University of Central Florida's Marine Turtle Research Group sampled the six wild-captured immature *C. mydas* individuals that were used in this study from Sebastian Inlet, Indian River Lagoon, Florida, USA (27° 49′ N, 80° 26′ W). All individuals were sampled during bimonthly collection trips in March through May of 2018. Large mesh tangle nets (455 m long) were used to capture turtles over a 3-hour soak

sampling period; captured turtles were brought onboard a work-up boat where they were weighed, sampled and tagged (per Ehrhart et al., 2007; Long et al., 2021). Skin biopsies were collected using 4 mm biopsy punches from a (tumor-free) trailing edge of the rear flipper in all animals. Thus, we compared equivalent skin biopsies from all turtles (Table 1). Three of the turtles sampled had visible signs of FP (tumors) with partial regression, and the remaining were visibly (i.e., externally) tumor-free. Four of the sampled turtles had approximately 1–4 leeches attached to their bodies (including all three turtles with FP and one turtle without FP) and two samples did not have any visible signs of leech parasitism at the time of sampling (both turtles without FP; Table 1). Examples of *C. mydas* individuals that are clinically healthy, leech-positive, and FP-positive are depicted in Fig. 1. Skin biopsies were not collected in locations near leeches or FP tumors. Skin biopsies were placed in individual 1.5 mL tubes containing ~ 300 μL of RNAlater (Invitrogen, CA, USA) for preservation and stored in an −80 °C freezer until RNA isolation. We did not record the sex of the immature *C. mydas* individuals; visual and external sex determination in immature sea turtles is not possible without invasive laparoscopic techniques (Wyneken et al., 2019). All research was conducted full compliance with protected species laws and guidelines of the United States and State of Florida under Florida Marine Turtle Permits (MTP)-231 and MTP-19–225 and National Marine Fisheries Service Endangered Species permit #19508.

2.2. Transcriptome library generation

We isolated and extracted total RNA using Trizol (Invitrogen, CA, USA) and confirmed RNA quality (RIN) using the TapeStation 2200 (Agilent, CA, USA). We isolated messenger (m)RNA from total RNA using the Dynabeads mRNA kit (Invitrogen, CA, USA). We then synthesized cDNA using the Invitrogen first strand cDNA synthesis module (Invitrogen, CA, USA) with random hexamer primers and the NEBNext Second Strand Synthesis Module (New England Biolabs, MA, USA) using 0.25 of the recommended amount of enzyme per sample. We sheared all cDNA libraries to a mean fragment size of 400 bp using a Covaris DNA Sonicator.

To prepare cDNA libraries for Illumina sequencing we blunted the ends of the sheared mRNA using DNA Polymerase I, Large (Klenow) Fragment (New England Biolabs, MA, USA). We completed dC-tailing using Klenow (3′-5″ exo) kit (New England Biolabs, MA, USA). We performed quick ligation of stubby adapters using a quick ligation kit (New England Biolabs, MA, USA). Finally, we used Nextera-style Illumina adapters with unique combinations of 8-mer barcodes and 18 cycles of PCR with HotStart Kapa Taq (Roche, CA, USA) to create transcriptome libraries that could be multiplexed for sequencing. After each procedure listed above, we removed excess enzymes and fragments <200 bp using Sera-Mag Magnetic SpeedBeads (MilliporeSigma, MO, USA). We quantified each library using three dilutions and duplicate reactions with the Kapa Library Quant kit (Roche, CA, USA). We created equimolar pools of all samples, performed a final bead clean-up to remove any remaining small fragments, and sequenced all libraries on one lane of the Illumina HiSeq 3000 platform using 2×150 bp chemistry.

2.3. Transcriptome assembly

We used Prinseq v. 0.20.4 (Schmieder and Edwards, 2011) to trim low-quality sequences from raw reads using a minimum mean Phred score of 20 and end-trimming with a sliding window of ten for high quality results. We checked results using FastQC v. 0.11.5 (Andrews, 2010) and assembled a *de novo* transcriptome using all retained reads from all samples with Trinity v. 2.5.1 (Grabherr et al., 2011). We used default Trinity assembly parameters, including in silico normalization, a minimum contig length of 200 base pairs, and a kmer size of 25.

2.4. Differential expression and GO enrichment analysis

We used RSEM to map the quality-trimmed read libraries against the transcriptome and quantify the number of each expressed transcript (Li et al., 2014). We used Trinity scripts to create a matrix of rows of transcript counts with columns as samples across FP status and leech presence. We used Trinity scripts that incorporate edgeR in R (Robinson et al., 2009) to convert the transcript counts to log-counts per million and model the variance among samples in pair-wise comparisons (Robinson et al., 2009). We classified any transcripts with a false discovery rate (FDR) *P*-value of <0.05 as significantly differentially expressed (DE) and created expression heat maps with the ComplexHeatmap package in R (Gu et al., 2016).

We used Annocript to annotate all assembled transcripts, which incorporates BLAST+ to query all sequences against the Uniref90, SwissProt and Conserved Domain databases (E-value $<1e^{-5}$) (Camacho et al., 2009; Musacchia et al., 2015). We used the resulting best-hit annotations and assigned gene ontology (GO) terms for our differential expression and enrichment analysis. We performed GO term enrichment analysis using GOSeq (Young et al., 2010). GOSeq uses Fisher's exact tests to identify GO terms that are under or overrepresented in the significantly differentially expressed transcripts and associate them with different tiers of biological processes and molecular functions. We first performed this analysis for all of the significant DE transcripts relative to the reference transcriptome. Next, we performed this analysis for (1) FP-positive versus FP-negative turtles and (2) leech-negative versus leech-positive turtles. In both cases, we used a threshold of FDR-corrected *P*-value < 0.05 to infer significantly over and underrepresented transcripts. We compared the total number of significant DE transcripts to the number with tumor- and immune-related GO terms for turtles with and without leeches and FP using chi-square tests. Because leeches were found on all FP-positive turtles and our sample size did not allow for investigation of whether a combination effect of leech affliction and FP tumors exists, we investigated whether the differential expression analyses were unique by comparing the overlap of differentially expressed

Table 1
Turtle samples with characterization on categories for differential expression analysis.

Sample ID	Collection date	FP tumors present	Leech parasitism	Papilloma grade	Condition	Straight carapace length (cm)
HH4847	May 2018	No	None seen.	0	Flipper damage, no shell damage	48.6
HH1669	May 2018	No	None seen.	0	Flipper damage, some shell damage	45.7
HH1667	May 2018	No	Leeches on axillary and inguinal	0	No flipper damage, no shell damage	48.2
HH4852	March 2018	Yes	Leech eggs on plastron and flippers	2, partial regression	No flipper damage, no shell damage	43.2
HH4857	March 2018	Yes	Leeches on inguinal and axillary Leech eggs on inguinal and axillary	1, partial regression	No flipper damage, no shell damage	45.8
HH4872	March 2018	Yes	Leeches on both rear and both front flippers. Leech eggs on flippers, under marginals, and plastron	3, partial regression	No flipper damage, no shell damage	50.3

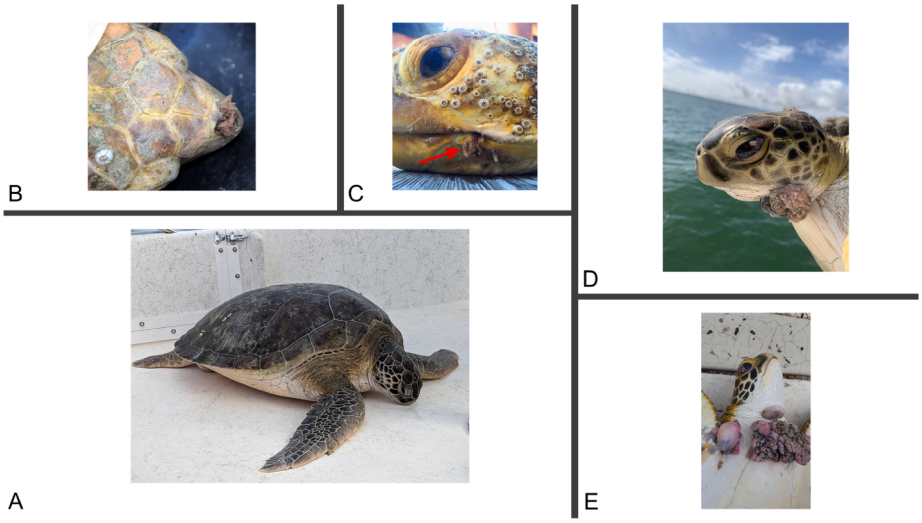


Fig. 1. *Chelonia mydas* individuals. A) Clinically healthy individual, B & C) Leech-positive individuals, D & E) FP-positive individuals. Photo credit: Gustavo Stahelin, UCF Marine Turtle Research Group; Permits: MTP-231 and NMFS 19508.

transcripts across the FP and leech comparisons. We used Inkscape (Harrington et al., 2004) to make a Venn diagram showing the overlap of differentially expressed transcript lists across all comparisons. We used chi-square tests to evaluate significant differences in DE transcripts with specific GO terms among the four turtle comparison groups.

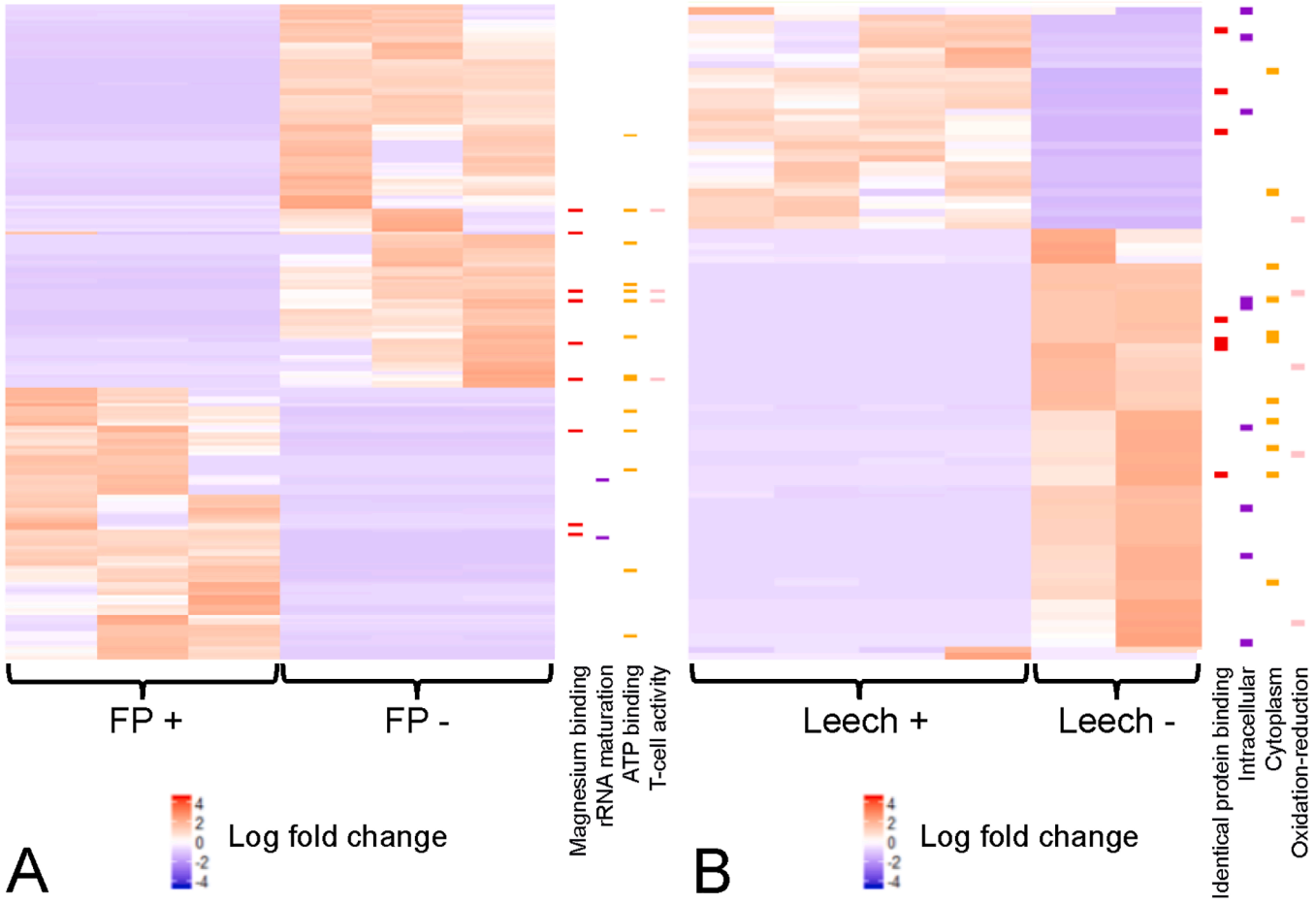
3. Results

Sequencing generated an average of 10.4 million (range: 5.9–12.3) paired end reads per library after quality filtering (SRA accessions SRR14483578-SRR14483583). The final *de novo* assembly contained 455,901 transcripts with a mean length of 622 bp (TSA accession GJFS00000000). Annotation yielded 121,957 transcripts with at least one database hit. Specifically, 37,685 transcripts had top BLAST hits to annotated proteins from the green sea turtle (*C. mydas*), followed by 16,651 top hits to the Chinese softshell turtle (*Pelodiscus sinsensis)* and 14,347 top hits to the painted turtle (*Chrysemys picta belli*). The remaining 53,274 transcripts matched database sequences from Durocryptodira (9260), Cryptodira (5175), Archelosauria (3388), Amneiota (2799), Neognathae (1789), Sauria (1304), and the rest of the hits were split amongst crocodilians and other chelonians. The most abundant coding domains in the assembly included zinc-finger domains and reverse transcriptases (Tables S1–S4). Regulation of transcription, oxidation–reduction processes, proteolysis, signal transduction binding and ATP binding were the most common biological process and molecular function GO terms associated with sequences in the assembly.

Comparing turtles with and without FP (Fig. 2A) and with and without leeches (Fig. 2B), we identified numerous transcripts present under all four conditions (Table 2). Turtles with leeches had an overall pattern of decreased transcript expression relative to turtles without leeches, while transcript up- and down-regulation was more evenly split between turtles with and without FP. Of the four most common GO terms associated with significant DE transcripts in the FP comparison, two (magnesium binding and ATP binding) were spread across FP-positive and FP-negative turtles, whereas rRNA maturation transcripts were only upregulated in FP-positive turtles and T-cell activation transcripts were only upregulated in FP-negative turtles (Fig. 2A). Likewise, the four most common GO terms associated with significant DE transcripts in the leech comparison were spread across leech-positive and leech-negative turtles, except for oxidation–reduction which was only significantly upregulated in turtles without leeches (Fig. 2B). Significant DE transcripts were mostly unique, with only 10 DE transcripts shared by the leech and FP comparisons (Fig. 2C; Table 3). This was especially important to confirm the uniqueness of grouped responses to FP or leeches since three of the leech-positive individuals also had FP tumors. Nine of these transcripts were significantly upregulated in the leech-negative and FP-negative turtles, whereas one (period circadian protein homolog 1) was significantly upregulated in both leech-positive and FP-positive turtles.

We identified 175 significant DE transcripts between the four leech-positive individuals compared to the two leech-negative individuals (Fig. 2B), of which 52 were significantly upregulated in the leech-positive group (Table S3) and 123 were significantly upregulated in the leech-negative group (Table S4). Of the 94 significant DE transcripts that matched at least one GO term (Supplementary Tables 3 and 4), 32 were significantly upregulated in the leech-positive and 62 in the leech-negative group. Turtles without leech parasites significantly upregulated three transcripts with immune function and/or viral interaction GO terms (Table 2; Fig. 3A). Turtles with leech parasites also significantly upregulated three transcripts with immune function and/or viral interaction GO terms, as well as one oncosuppression transcript (Table 2; Fig. 3B). The group of all significant DE transcripts between leech-positive and leech-negative individuals was significantly enriched for queuosine biosynthetic processes, translation reinitiation, IRES-dependent translational initiation, and translation initiation complex (Table S5). Leech-positive turtles had no significantly enriched GO terms compared to leech-negative turtles (Tables S9 and S10).

Comparing the three individuals with and without FP, we identified 200 significantly DE transcripts (Fig. 2A), including 116 that were significantly upregulated in FP-positive turtles and 84 that were significantly upregulated in FP-negative turtles (Tables S1 and S2). Of the 97 significant DE transcripts that matched at least one GO term (Supplement Tables 1 and 2), 57 were significantly upregulated in FP-positive and 40 in FP-negative turtles. Turtles with and without FP had similar numbers of significantly upregulated transcripts with immune function GO terms (12 and 9, respectively; Table 2; Fig. 3C and D). Interestingly, FP-positive individuals displayed significant upregulation of 15 tumor inhibition transcripts while FP-negative individuals had 5 significantly upregulated tumor inhibition transcripts (Fig. 3C and D; Table 2). Six tumor progression transcripts were also significantly upregulated in the FP-positive individuals, while no tumor progression transcripts were significantly upregulated in the FP-negative individuals (Fig. 3C and D; Table 2). Finally, FP-positive individuals significantly upregulated 28 protein interaction transcripts while FP-negative individuals significantly upregulated 5, a significant difference relative to the total number of significant DE transcripts in turtles with and without FP (Chi-square



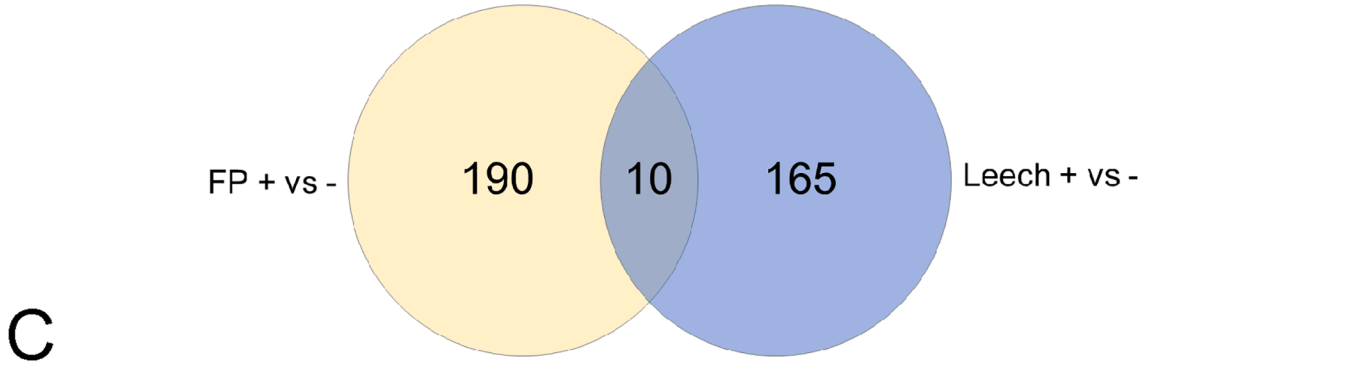


Fig. 2. Significantly differentially expressed transcripts (FDR *P*-value < 0.05) between *C. mydas* individuals (A) with fibropapillomatosis (FP) compared to without FP, and (B) with leech parasites compared to without leeches. Transcripts corresponding to functional GO categories are shown in color-coded bars to the right of each heatmap. (C) Overlap in differential transcript expression associated with FP and leech parasitism among six juvenile *C. mydas* individuals. The number of significantly differentially expressed transcripts unique to FP comparisons are shown in yellow, those unique to leech parasitism comparisons are shown in blue, and overlapping transcripts are shown in center. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

= 8.65, *P*-value = 0.006).

The group of all significant DE transcripts among FP-positive and FP-negative individuals was significantly enriched for GO terms such as positive regulation of T cell cytokine production, positive regulation of activation of Janus kinase activity, DNA/RNA helicase activity, cleavage body, nucleic acid phosphodiester bond hydrolysis, activation of NF-kappaB-inducing kinase, I-kappaB phosphorylation, activation of MAPKK, JNK cascade, IkappaB kinase, positive regulation of interleukin-2 production, nucleolus, Ada2/Gcn5/Ada3 transcription activator, magnesium ion binding, and maturation of 5.8S rRNA (Table S6). FP-positive individuals were significantly enriched (FDR *P*-value = 0.022) for the GO term maturation of 5.8S rRNA compared to FP-negative turtles (Table S7), whereas significant DE transcripts in FP-negative versus FP-positive individuals were enriched for GO terms related to positive regulation of T cell cytokine production, positive regulation of interleukin-2 production, Ada2 /Gcn5/Ada3 transcription

Table 2
Significantly upregulated transcripts in leech-negative compared to leech-positive and FP-negative compared to FP-positive turtles with GO terms related to either tumor inhibition/formation, apoptosis regulation, or immune system.

Gene ID	Biological process GO terms	FDR *P*-value
Upregulated in leech-negative turtles		
serine/threonine-protein phosphatase 2b catalytic subunit gamma isoform isoform x1	immune function/mitotic	0.0131
natural resistance-associated macrophage protein 2	metal ion transport/ immune response	0.0311
midline 2	immune response & regulation/ viral interactions	0.0415
Upregulated in leech-positive turtles		
krueppel-like factor 4 isoform x2	zinc finger c2h2 type/ development and oncosuppression	0.0008
dedicator of cytokinesis protein 9	immune response/viral interactions	0.0020
low quality protein nectin-1	intracellular signaling/viral interactions	0.0037
sorting nexin 16	protein transport/immune response	0.0262
Upregulated in FP-negative turtles		
prostaglandin e synthase 3	viral interactions/immune function/tumor inhibition/ transcription	<0.0001
SPARC	cell proliferation/tumor inhibition/intra-inter cell signaling/embryonic/ development	<0.0001
tyrosine-protein kinase receptor tyro3 isoform x2	immune function/cell proliferation/apoptosis regulation	0.0061
rho-associated protein kinase	apoptosis regulation/structural/ cell proliferation/gene expression	0.0075
tyrosine-protein phosphatase non-receptor type substrate 1	immune function	0.0133
tyrosine-protein kinase	immune function/intra-intercellular signaling	0.0201
microtubule associated serine/ threonine kinase family member 4	cell proliferation/tumor inhibition/mitotic/immune function	0.0216
cop9 signalosome complex subunit 6	dna repair/mitotic/immune function	0.0217
nitrilase family member 2	tumor inhibition/immune function/metabolism	0.0284
serine/threonine-protein phosphatase 2b catalytic subunit gamma isoform isoform x1	immune function/mitotic	0.0295
e3 ubiquitin/isg15 ligase trim25 isoform x1	tumor inhibition/immune function/viral interactions/ protein degradation	0.0451
Upregulated in FP-positive turtles		
prostaglandin e synthase 3	viral interactions/immune function/tumor inhibition/ transcription	<0.0001
RNA binding motif protein 3	cell proliferation/tumor inhibition/tumor progression/ mitotic/protein interactions	<0.0001
e3 ubiquitin-protein ligase rbbp6	cell proliferation/mitotic/viral interactions/immune function/ transcription/translation regulation/protein interactions	0.0005
low quality protein epithelial discoidin domain-containing receptor 1-like	cell proliferation/tumor inhibition/immune function/ embryonic	0.0015
mitogen-activated protein kinase kinase kinase 7	tumor inhibition/immune function/embryonic/ wnt/bmp	0.0028
RNA binding motif protein 3	cell proliferation/tumor inhibition/tumor progression/ mitotic/protein interactions	0.0045
protein wwc3 isoform b	tumor inhibition/cell proliferation	0.0147
semaphorin-3 g	tumor inhibition/immune function	0.0176
nedd4-binding protein 3 like protein	cell proliferation/mitotic/tumor inhibition	0.0216
star-related lipid transfer protein 7 mitochondrial	tumor promoter	0.0216
mitogen-activated protein kinase kinase kinase 7	tumor inhibition/immune function/wnt/bmp	0.0217
adiponectin receptor 2	metabolism/intracellular signaling/immune function	0.0217
eukaryotic peptide chain release factor gtp-binding subunit erf3a	cellular transformation /apoptosis regulation/protein interactions	0.0217
uncharacterized protein	immune function	0.0237
e3 ubiquitin-protein ligase rnf149	tumor inhibition/immune function/regulator of adaptive immunity	0.0284
immediate early response gene 2 protein	protein interactions /transcription/translation regulation/embryonic/stimulates tumor metastasis	0.0284
serine/threonine-protein kinase mrck beta isoform x1	transport/cell proliferation/ apoptosis regulation	0.0352
low quality protein zinc finger protein 710	binding site for transcription factors: development/ oncosuppression	0.0284
sciellin isoform x1	protein interactions/ development/tumor progression	0.0359
semaphorin-3 g	immune function/tumor inhibition	0.0406
mitogen-activated protein kinase kinase kinase 7	tumor inhibition/immune function/ wnt/bmp	0.0406
mitogen-activated protein kinase kinase kinase 7	tumor inhibition/immune function/ wnt/bmp	0.0432
amphiregulin	tumor inhibitor & promoter	0.0470

activator complex, and activation of MAPK activity (Table S8).

4. Discussion

Here, we present the first study describing transcriptomic responses of healthy versus tumored and leech parasitized versus non-parasitized *C. mydas* individuals, and we find significant gene expression changes related to immunity and tumor response for both comparisons. The majority of significant DE transcripts in this study were unique to FP and leech comparisons (Fig. 2C). Among the 10 shared DE transcripts across both analyses, the only significantly upregulated transcript in both FP-positive and leech-positive turtles was period circadian protein homolog 1 (PER1) which is involved in regulation of innate immunity and cytokine production (Scheiermann et al., 2018). Because all FP-positive turtles also had leeches, but one leech-positive turtle did not have FP, this suggests increased expression of PER1 could be associated with leech parasitism independent of FP status. Turtles with and without leech parasites each significantly upregulated a handful of transcripts with viral, immunity or tumor response GO terms (Table 2). Turtles with FP significantly upregulated unique transcripts with immune and tumor inhibition functions, and vice versa (Table 2). FP-positive turtles upregulated several tumor progression and suppression GO terms, while turtles without FP did not upregulate any tumor progression transcripts (Fig. 3C and D). FP-positive turtles also upregulated significantly more protein interaction transcripts than FP-negative turtles (Fig. 3C and D). Overall, our gene expression profiles provide novel insight into how juvenile *C. mydas* functionally respond to these pathogen and parasite challenges via immunity and anti-tumor processes, strengthening the

Table 3
Shared significant differentially expressed transcripts among leech and FP comparisons. Significant upregulation in turtles with both leeches and FP are shown in italics.

Transcript ID	Gene ID	Biological Process	Upregulated groups
TRINITY_DN82031_c2_g1_i3	n/a	n/a	FP-negative/Leech-negative
TRINITY_DN83446_c1_g1_i9	n/a	n/a	FP-negative/Leech-negative
TRINITY_DN84693_c0_g2_i2	inositol 1 4 5-triphosphate receptor-interacting protein	embryonic development	FP-negative/Leech-negative
TRINITY_DN84459_c6_g2_i2	stromal interaction molecule 1 isoform x4	calcium response/intra-intercellular signaling	FP-negative/Leech-negative
TRINITY_DN80972_c0_g1_i2	vacuolar protein sorting-associated protein 4a	protein interactions/posttranslational modification	FP-negative/Leech-negative
TRINITY_DN68468_c0_g1_i1	n/a	n/a	FP-negative/Leech-negative
TRINITY_DN84918_c5_g2_i2	serine/threonine-protein phosphatase 2b catalytic subunit gamma isoform x1	immune responses/mitotic	FP-negative/Leech-negative
TRINITY_DN73496_c1_g1_i2	keratin type ii cytoskeletal 1-like	chromosome segregation	FP-negative/Leech-negative
TRINITY_DN67490_c0_g1_i2	uncharacterized protein loc102930756	n/a	FP-negative/Leech-negative
TRINITY_DN83314_c0_g4_i7	period circadian protein homolog 1	signal transduction/regulation of cytokine production involved in inflammatory response	*FP-positive/Leech-positive*

evidence associating leeches, FP and ChHV5 infections.

Among the four significantly upregulated viral interaction transcripts in turtles with leech parasites, two have specific roles in herpesvirus infections: sorting nexin 16 regulates viral replication and overexpression is associated with reduced viral loads (Brankatschk et al., 2011; Le Blanc et al., 2005; Maschkowitz et al., 2018) and nectin-1 is a key molecule used by herpesvirus glycoprotein B to gain entry into host cells (Connolly et al., 2020). Thus, turtles with leeches show responses typical of herpesvirus infections, which putatively cause FP (Alfaro-Núñez et al., 2016) and may be vectored by leeches (Rittenburg et al., 2021). This pattern suggests that leech-positive turtles were infected with herpesvirus, lending further support to the hypothesis that leeches vector ChHV5. The single oncogene that was significantly upregulated in leech-positive individuals is Krueppel-like factor 4 isoform X2, which also plays a key role in wound healing (Liao et al., 2011) and thus may contribute to turtle responses to leech bites. The final significantly upregulated immune transcript in leech-positive individuals, dedicator of cytokinesis protein 9 (DOCK9) is a part of the DOCK protein family which has been associated with regulation and prediction of parasite burden in the wild wood mouse *Apodemus sylvaticus* (Babayan et al., 2018). Additionally, DOCK9 is involved in a wide variety of immune responses (Kunimura et al., 2020), and the association with leech parasitism may be indirectly caused by other pathogen infections transmitted by leech bites such as ChHV5. Alternately, Ectoparasitism is associated with increased metabolic rates in *Drosophila* (Brophy and Luong, 2020) and is a developmental stressor that effects growth-immunity tradeoffs (Eisner Pryor and Casto, 2015). Leech-positive *C. mydas* juveniles may similarly face an energetic tradeoff between immunity and growth/metabolism relative to turtles without leeches, and may be upregulating these immune transcripts in direct response to leech bites. Such an energetic trade-off may have long-term implications for sea turtle maturation rates, fecundity, and population-level trends. Larger samples of simultaneously leech-positive and FP-negative individuals are needed to resolve these patterns.

Across all significant DE transcripts, FP comparisons were enriched for a number of GO terms, and only FP-negative turtles were significantly enriched for GO terms involved in acquired and inflammatory immune gene regulation (FDR P-value < 0.05). Turtles with FP had more transcript expression changes involved with tumor response and overall protein interactions, but only the turtles without FP significantly upregulated transcripts that were enriched for immune system regulation. These findings suggest a scenario where turtles with active FP tumors are expressing transcripts aimed towards controlling tumor growth, whereas turtles without FP are increasing expression of transcripts that help them regulate immune responses, perhaps making them less susceptible to contracting FP or constituting a tradeoff between responses. The most significantly enriched GO term in the FP-negative individuals was T cell activation. T cells play a central role in immune memory and response, and chronic diseases including cancer are known to compromise the functionality of T cells (Baitsch et al., 2011; Schietinger et al., 2016). The second most significantly enriched GO term in the FP-negative individuals was positive regulation of interleukin-2 production, which is also directly correlated with T cell activation through its role as a T cell growth promoting factor. The lack of T cell activation in the FP-positive relative to FP-negative individuals may also indicate prolonged exposure to the tumor's microenvironment, which has shown to have immunosuppressant properties in previous cancer research (Motz and Coukos, 2013). The lack of lnterleukin-2 enrichment in FP-positive individuals also provides a possible immune therapy target to enhance host response to FP tumors.

Although FP-negative and FP-positive individuals had similar numbers of significantly upregulated immune-associated transcripts, FP-positive individuals had three times the number of tumor inhibition transcripts upregulated compared to FP-negative individuals (Fig. 3C and D). Of note, the FP tumors were partially regressed in all three of our FP-positive turtles (Table 1), consistent with the significant increase in tumor inhibition gene expression, and implying these animals were successfully controlling tumor growth. Turtles with active tumor growth (either at earlier stages of FP or in completely susceptible individuals) might have significantly different transcriptional profiles, reinforcing the importance of conducting follow-up RNAseq studies using animals with a range of FP tumor severity and stage. The most significantly upregulated tumor inhibition transcripts in FP-positive individuals included RNA recognition motif in cold inducible RNA binding protein (CIRBP), RNA binding motif protein 3 (RBM3), Mitogen-activated protein kinase, and E3 ubiquitin-protein ligase and scaffolding protein WWC3. CIRBP has been implicated in inflammatory response and as a tumor suppressor (Lujan et al., 2018). Ubiquitin-mediated degradation is essential for maintenance of a healthy cell line, and its downregulation has been linked to tumorigenesis and cancer progression (Liu et al., 2015). WWC proteins have been shown to regulate the Wnt and Hippo signaling pathways and increased expression contributes to the suppression of human lung cancer (Han et al., 2017). Additionally, low expression levels of WWC3 have been linked to metastasis in human

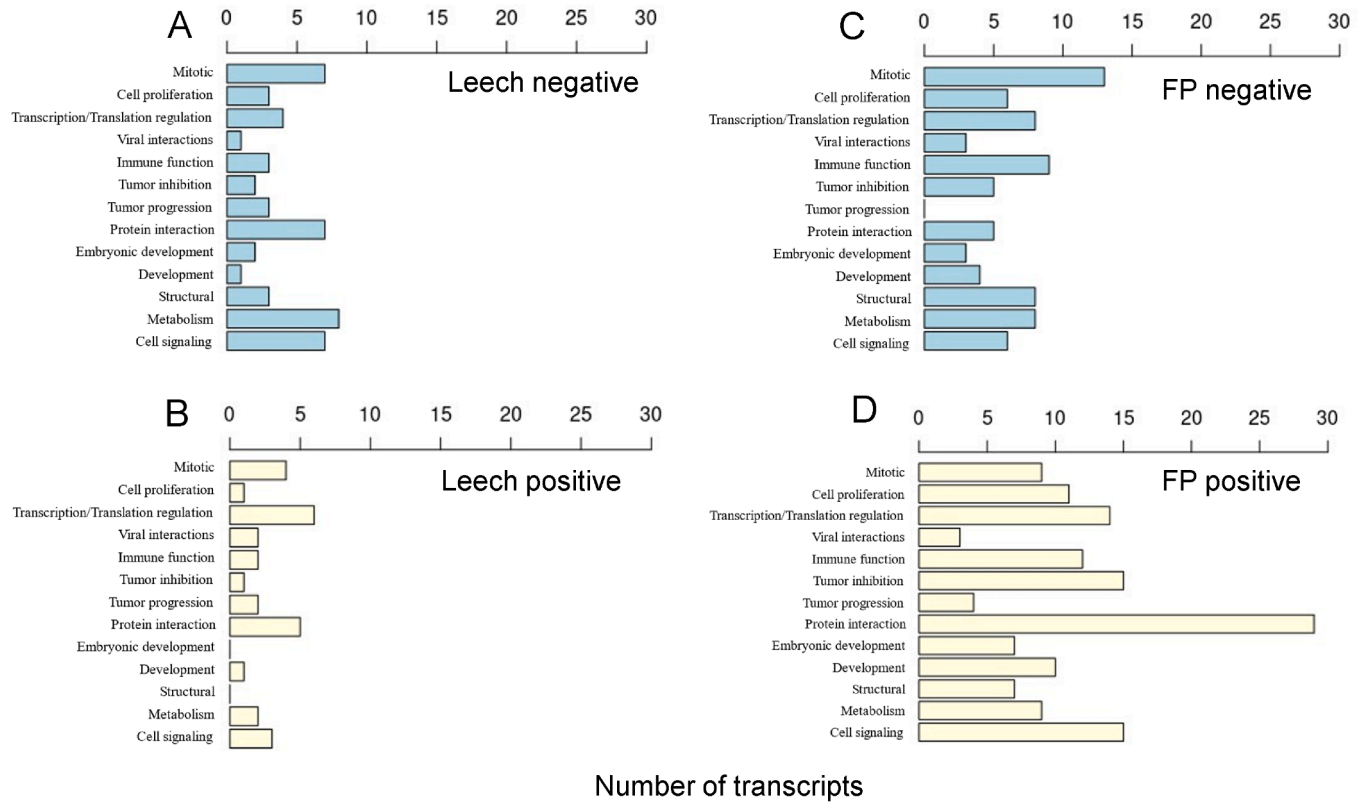


Fig. 3. Significantly upregulated (FDR *P*-value < 0.05) transcripts with gene annotations across highest-level Gene Ontology biological process categories in (A) leech-negative turtles, (B) leech-positive turtles, (C) FP-negative turtles, and (D) FP-positive turtles.

cancers (Han et al., 2017). FP-positive individuals had six total tumor progression/promoter transcripts that were significantly upregulated, whereas FP-negative individuals had none (Fig. 3C and D). Notably, three of these genes (amphiregulin, CIRBP and RBM3) have been implicated in tumor progression or unfavorable prognosis or in inhibition and favorable prognosis in a number of human cancer studies (Busser et al., 2011; Sureban et al., 2008; Taira et al., 2014; Zhu et al., 2016). Taken together, these findings suggest that marine turtles who are controlling their FP tumors mount a physiological tumor inhibition response to FP not only within tumored areas but throughout their dermis, as all of our data derive from biopsies of healthy skin that were not adjacent to tumored areas.

Duffy et al. (2018) characterized differentially expressed transcripts between active FP tumors and their non-cancerous host tissues and identified that Wnt/BMP pathways were significantly upregulated in tumors and SHH pathways were significantly downregulated in tumors. The FP-positive individuals in this study displayed evidence of tumor regression and did not display enrichment of Wnt, BMP or SHH signaling but did have four significantly upregulated Wnt and BMP associated transcripts (Table 2, Table S1) while FP-negative individuals had no significantly upregulated transcripts associated with Wnt and BMP or SHH pathways (Table S2). Duffy et al. (2016) provided evidence that the hyperactivation and inhibition of Wnt/ β-catenin signaling can inhibit tumor viability in human derived neuroblastoma, malignant melanoma and colorectal cancer. The Wnt pathway's role in FP progression remains uncertain. Functional assessment of this pathway through gene editing mechanisms as previously done with RAS signaling pathways in relation to cancer progression (Downward, 2003) may provide further insight as none were seen in FP-negative individuals and the interplay between Wnt and Hippo pathways has been implicated in tumor progression and inhibition, respectively (Han et al., 2017).

Studies of animal disease systems help identify expression patterns that impact disease progression (Field et al., 2015; Trone-Launer et al., 2019), determine the underlying mechanics of disease resistance (Eskew et al., 2018; Scott et al., 2020), or allow for application of human precision medicine approaches to EID treatment (Kreiss et al., 2008; Pye et al., 2018). The tumor suppression transcripts upregulated in the FP-positive individuals are candidates for future molecular research and can be used to compare to expression profiles associated with sea turtle recovery. The lack of T cell common GO terms or enriched function in FP-positive individuals may indicate T cell reprogramming as an immunotherapy treatment option for afflicted marine turtles (Katz and Rabinovich, 2020). Immunogenomic guided methods (Mukherjee, 2019) and immune reprogramming of cells (Katz and Rabinovich, 2020) may help identify strategies to restore sufficient antitumor responses in FP afflicted individuals. Tumor promoter transcripts identified in the FP-positive individuals should be further investigated for their role in FP progression and effect on recovery from this disease. FP-positive individuals showed a significant increase in their upregulated transcripts that are associated with protein interactions when compared to FP-negative individuals. Essential biological interactions are performed via biological networks through protein interactions. Characterization of the interactome will help explain the role of these upregulated transcripts in turtles afflicted with FP.

A genomic approach allows for functional assessment of both model and non-model species, expanding the knowledge available for species management. With the ever-increasing rate of wildlife EIDs along with their negative effect on biodiversity and ties to human health (Daszak et al., 2000; Lips et al., 2006; McCallum, 2012; Pimentel et al., 1995; Schmeller et al., 2020; Wilcove et al., 1998; Young et al., 2017), studies that unravel host-pathogen interactions are of critical importance. Understanding how *C. mydas* individuals respond to FP not only during their immature life stage but also in their adult life stage is essential to identify recovery and disease induced mortality patterns. Life stage associated ontogenetic shifts in habitat use, diet, and assessment of anthropogenic threats are also imperative to ultimately managing the

disease outcome and conserving the species. The molecular interactions or biological processes that contribute to the tolerance, resistance, or mortality of FP cases require functional assessments involving temporal and spatial shifts in trophic level habitat selection. In particular, monitoring to determine the rate at which immature *C. mydas* individuals' recover from FP, become reproductive adults, and their long-term reproductive output is a key next step as this can identify tumor inhibition transcripts that are candidates for recovery from FP during early sea turtle ontogeny.

Our study confirms an important role for anti-tumor and acquired immune responses within skin tissues of *C. mydas* turtles that are dealing with infectious tumors and leech parasites. Because our study focused exclusively on larger immature green sea turtles obtained from neritic habitats in Florida, confirmation of our findings in other *C. mydas* developmental habitats, including samples that allow for the investigation of leech and FP tumor combinatorial effects and among other juvenile populations, is needed to better evaluate whether the patterns recovered here can be generalized. Our data along with the recently published *O. margoi* transcriptomes (Tessler et al., 2018) can now be used to further understand host-parasite relationships and possibly the role of anticoagulants in viral transmission. These data also allow for investigation of treatment strategies including reduction of blood-feeding which may reduce the likelihood of ChHV5 transmission and therefore FP affliction. Broader characterization of leech parasitism and associations with ChHV5 infection, FP, and gene expression will help elucidate causal relationships between viral vectoring and tumor development. Although additional ecological, epidemiological, and genomic analyses are still needed before we can fully resolve this knowledge gap, continued research into viral, genomic and anthropogenic drivers of FP is of paramount importance due to the central role of marine turtles in the maintenance of ecosystem function (Duffy et al., 2018; Jones et al., 2016). Florida *C. mydas* nest numbers are currently increasing (Chaloupka et al., 2008), but a substantial increase in the number of stranded *C. mydas* individuals with FP has also been documented (Hargrove et al., 2016; Foley et al., 2005). This is troubling considering the top-down effects of *C. mydas* as dominant herbivorous grazers, and the documented links between turtle declines and collapse of both coral reefs and seagrasses (Christianen et al., 2012; Bjorndal et al., 2003; Jackson, 2001; Heithaus et al., 2014). Thus, understanding the mechanisms that underly marine turtle responses to pathogen and parasite challenges is not only vital to their continued survival, but to the stability of ecosystems worldwide.

CRediT authorship contribution statement

Rachael A. Kane: Conceptualization, Methodology, Formal analysis, Data curation, Writing. **Nicholas Christodoulides:** Data curation, Formal analysis, Editing. **Irelyn M. Jensen:** Investigation, Conceptualization, Formal analysis, Writing - original draft. **Donald J. Becker:** Investigation, Conceptualization, Formal analysis, Writing - original draft. **Katherine L. Mansfield:** Writing - Review & Editing. **Anna E. Savage:** Writing - Review & Editing, Conceptualization, Methodology, Formal analysis, Data curation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data Archiving

The assembled transcriptome has been deposited at NCBI TSA database under Bio- Project PRJNA705796: Chelonia mydas Transcriptome Assembly (TaxID: 8469), TSA database identifier: GJFS00000000.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gene.2021.145800.

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