RNA virus ecogenomics along a subarctic permafrost thaw gradient

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ABSTRACT

Climate change thaws permafrost, which releases greenhouse gases partly from dormant microorganisms awakening and metabolizing organic matter. Though DNA viruses that infect these soil microbes have been studied, little is known on soil RNA viruses, which typically infect microeukaryotes. Here we identify and characterize 2,651 RNA viruses from a 4-year time series of bulk soil metatranscriptomes derived from the climatically fragile Stordalen Mire ecosystem —a long studied permafrost peatland. RNA virus diversity was structured by habitat (palsa, bog, and fen), and these patterns correlated with pH and carbon dioxide and methane emissions. Further, host prediction, virus-encoded metabolite-transforming and information-processing functions suggested roles in ecosystem-scale carbon fluxes and contribute to greenhouse gases emissions. Together, these RNA virus ecogenomic data in permafrost provide essential baseline information for integration into predictive models to support hypothesis testing.

Climate change accelerates permafrost thaw through reduced ice cover lessening albedo (*1*). To study this, a model ecosystem – Stordalen Mire in northern Sweden – was established decades ago and revealed that permafrost frozen for thousands of years (*2*) now thaws near-annually (*3*). Concomitant with such thaw, dormant Stordalen Mire microorganisms are also annually awakened and in turn metabolize organic matter that accelerate greenhouse gas (GHG) emissions (*4*, *5*). Globally , such processes could unlock permafrost soil carbon reserves three times greater than the carbon currently in the atmosphere (*6*, *7*), though how specific soils and their microbiomes respond are among the greatest consequential unknowns in climate models (*8*). Thus, mechanistic understanding of the interplay between thawing permafrost, microbial communities (bacteria and archaea) and their viruses, and carbon dynamics represent a key grand challenge for climate science.

Previous studies in Stordalen Mire have characterized three distinct habitats representing a thaw gradient: palsas (partially frozen), bogs (intermediate thaw), and fully-thawed fens – using metagenomic sequencing, functional genomics, and ecosystem measurements (*5*, *8–10*). These studies identified dominant microbial phyla (e.g., Acidobacteria and Actinobacteria) and demonstrated that microbial communities varied across the permafrost thaw gradient. Additionally, key microbial metabolic pathways, including polysaccharide degradation and methane metabolism, revealed their roles in altering ecosystem greenhouse gas (GHG) outputs across the thawing gradients (*5*). Among the notable findings, the syntrophy between *Candidatus* Methanoflorens and *Candidatus* Acidiflorens has been shown to be ubiquitous for regulating carbon dynamics in such soils (*5*, *9*, *11*). Further, double-stranded (ds)DNA viruses – important in marine microbial biogeochemistry (via lysis, gene transfer, and metabolic reprogramming) (*12–14*) and frequently overlooked in microbiome studies and climate change modeling (*15*, *16*) – also appear important to GHG and ecosystem processes at Stordalen Mire. Specifically, ecogenomic analyses predict that dsDNA viruses are diverse and modulate microbial ecosystem outputs via infecting key microbial hosts and encoding auxiliary metabolic genes (AMGs) (*17–19*).

Despite these advances, the roles of other biological entities, such as mobile genetic elements and RNA viruses, remain largely unexplored in permafrost ecosystems. Recent reviews suggest soil RNA viruses influence food web structure, energy flow, and nutrient cycling, potentially shaping soil health and fertility (*20*). Given the unique environmental conditions of permafrost ecosystems, RNA viruses may play an even more pronounced role in shaping microbial communities and regulating carbon cycling and GHG. Here, we address this gap by investigating RNA viruses (the focus here) and mobile genetic elements (see Companion Guo et al., mobilome) in Stordalen Mire. By leveraging four years of bulk soil metatranscriptomes, we provide the first comprehensive analysis of RNA viruses across the permafrost thaw gradient, revealing their diversity, distribution, and functional potential in regulating ecosystem processes. Specifically, we screened the data to identify 25,337 viral contigs that were dereplicated into 2,651 virus operational taxonomic units (vOTUs), which we classified and ecologically contextualized. Similar to their dsDNA counterparts, Mire RNA viruses were largely novel and likely play diverse and significant roles in ecosystem processes. Our analyses revealed that RNA virus distribution is influenced by environmental factors such as pH and CH_4 concentrations. Moreover, detailed examination of host-virus interactions and functional potential suggested these viruses play a crucial role in regulating carbon cycling and greenhouse gas emissions at the ecosystem scale. Studying RNA viruses also offers a unique lens into the underexplored eukaryotic communities in these environments, further underscoring their importance in permafrost regions.

Stordalen Mire hosts thousands of novel RNA viruses

To identify RNA viruses in Stordalen Mire, here we focused on RNA viruses of the kingdom *Orthornavirae*, which replicate their genomes via RNA-directed RNA polymerase (RdRp). Hence, we screened the RdRp gene across ≈630 Gbp of bulk soil RNA metatranscriptomes sequencing data (*n*=55) collected from Stordalen Mire. These samples were obtained from three canonical permafrost thaw habitats (palsa, bog, and fen) and spanned across the soil active layers over the four years (2010, 2011, 2012 (*5*, *17*) and 2016 (*10*, *19*)). To enhance the detection of divergent RNA viruses, we iteratively searched and refined RdRp hidden Markov models (HMMs), a strategy previously successful (*21*, *22*) (**fig. S1**). Together these efforts identified 25,337 RdRp-containing contigs (RdRps length mean ± s.d.: 185.47 ± 112.30) and removed 3,225 false positives (see Methods). Of the resultant 22,112 RdRps that could be used for ecological analyses, 1,702 were complete or near-complete such that they could be used in deeper taxonomic analyses (**fig. S2**).

To determine the megataxa (phyla and classes) of RNA viruses present in this ecosystem, we utilized a previously benchmarked approach based on Markov clustering algorithm (MCL) analysis of RdRps (*22*). First, because global phylogenies of the >3-billion-year-old RdRp-encoding gene are challenging (*23*), we applied machine learning to pre-sort RdRp sequences into five orthornaviraen phyla that are recognized by the International Committee on Taxonomy of Viruses, or ICTV (*Duplornaviricota, Kitrinoviricota, Lenarviricota*, *Negarnaviricota,* and *Pisuviricota*) (*23*, *24*). Note that our study did not consider the recently established sixth RdRp-encoding phylum, *Ambiviricota* (*23*, *25*), as analyses were closed before ambiviricots were described. For the machine learning pre-sorting step, we specifically applied a network-based, iterative clustering approach to the 1,702 complete/near-complete RdRp sequences and another 704,003 complete RdRp references sequences derived as follows: 6,732 from the global *Tara* Oceans project (*22*), 3,325 from coastal seawater near the Yangtze River estuary (*26*), and 695,648 from GenBank (version 245). This merged network analysis of near-complete RdRp sequences revealed a 96% adjusted rand index (98.7% agreement, the number of correct assignments per total number of assignments to ICTV taxonomy) and 18 distinct "megaclusters" (**fig. S3; Table S5**). Of the ten megaclusters that contained Stordalen Mire sequences, seven matched ICTV- taxa within the five established phyla of *Duplornaviricota, Kitrinoviricota, Lenarviricota*, *Negarnaviricota,* and *Pisuviricota* (**Fig. 1A**). In addition to the established phyla, lineages of *incertae sedis* viruses, i.e., Permutotertaviridae ('permutotetra-like') and 'kitrino-like' viruses, were included as well. These 5 phyla include a total of 20 classes and Stordalen Mire sequences are assigned to 16 of them (**Table S5**), and at vOTUs/"species"-level, RNA viruses are all different (**fig. S2F**).

Beyond ICTV-recognized taxa, from our analysis, we also identified the recently reported megataxa '*Taraviricota*' (*25*) of Stordalen Mire RNA viruses. This new phyla is not currently ICTV-recognized due to a lack of complete genomes, but other studies support the taxonomic assertions (*27*, *28*) (**fig. S3**; see **Table S5**). Moreover, we identified four novel virus operational units (vOTUs) encoding unclassified RdRps that clustered within *Lenarviricota* as a novel class tentatively named novel class "*Stormiviricetes*" (**fig. 1A, Fig. S3**). Their genomes ranged from 2.5 to 5.8 kb in length, and the longest one encoded two partially-overlapping open reading frames (**fig S4**). Given they are lenarviricots, we presume these are positive-sense, single-stranded RNA viruses.

To assess the similarity of Stordalen Mire RNA viruses with known reference sequences, we performed phylogenetic distance analysis using UniFrac. Phylogenetic analyses revealed permafrost virus-enriched clusters within the phyla *Lenarviricota, Kitrinoviricota, Negarnaviricota, Duplornaviricota (chrymotiviricetes),* and *Pisuviricota*, often forming phylogenetically distinct blocks (UniFrac distance >0.9) with limited reference sequences outside Stordalen (**Fig. 1B; fig. S5-S12**). Aligned to the previous literature (*29*, *30*), which found

that permafrost hosts harbor phylogenetically distinct RNA viruses—including reoviruses (Duplornaviricota), hypoviruses (Pisuviricota), rhabdoviruses (Negarnaviricota), and leviviricetes—our findings reinforce the uniqueness of RNA viruses in thawing permafrost (*29*). Comparing these patterns to other soil ecosystems, we find distinct differences in RNA virus composition. For example, in agricultural soils, leviviricetes (*Lenaviricota*) are most represented (*31*), whereas soils with added root litter show increased levels of barnavirids (*Pisuviricota*), megabirnavirids, and quadrivirids (*Duplornaviricota*), along with mitovirids (*Lenarviricota*) and leviviricetes (*32*).

Ecological patterns of Stordalen Mire RNA viruses

We next sought to assess the ecological distributions of RNA viruses. We used a recent community consensus definition (*33*) for OTUs of RNA viruses, preclustered at 90% average nucleotide identity across 80% of the shorter sequence length and 1 kb minimum contig length (materials and methods) (*21*, *22*). Clustering using these parameters collapsed the 22,112 Stordalen Mire RdRp sequences to 2,651 vOTUs (**fig. S13-S15**). Compared to other studies, this number is relatively large and suggests high soil RNA viral diversity considering it derives from a single sampling location and yet is nearly half that (2,651 vs 5,504 vOTUs) found throughout the global oceans (*22*). Consistent with such high diversity, individual samples contributed from ~83 to 166 vOTUs on average, and accumulation curves revealed sampling was not yet saturated (**fig. S13C)**; though most large-scale RNA virus surveys of marine, soil, and engineered environments fail to saturate RNA viral diversity (*27*, *34*, *35*).

To assess this further within soils - a biome where microbiomes are considered particularly diverse (*36*, *37*) - we collated and reanalyzed all available soil metatranscriptomes using our iterative HMM RdRp search strategy. This revealed the following number of RNA virus vOTUs: 83,027 vOTUs across 288 diverse Chinese soil samples (*38*), 3,161 vOTUs across 83 Warwick-England crop agricultural soil samples (*31*), 2,112 vOTUs across 48 California rhizosphere soil samples (*39*), and 1,412 vOTUs across 16 Welsh upland peatland samples (*32*). The comparison, based on the vOTU clustering at 90% nucleotide identity and 80% coverage, across these different soil RNA studies revealed that only 62 vOTUs overlapped to Chinese soils and 28 vOTUs to those Welsh upland peatland (**fig. S16C**). Further, 98% of the Stordalen Mire vOTUs were unique across these soil studies, similar to the high proportion of study-specific unique viruses detected in other soil datasets (**fig. S6C**).

We next explored vOTU-based community structure and patterns as follows. First, we explored beta-diversity of RNA vOTU relative abundances via ordination and hierarchical clustering of Bray-Curtis dissimilarity, which revealed three distinct meta-communities that matched the major soil types (palsa, bog, and fen) (PERMANOVA, $p = 0.001$; **Fig. 2B, fig. S13-S15**). This approach added RNA viruses to habitat-specific clustering of plants, microbiomes, and dsDNA viruses at this site (*17*, *19*, *40*), similar to RNA virus community differentiation across soil types observed in other studies (*31*, *32*, *38*, *39*, *41*). This structuring in Stordalen Mire may be shaped by the distinct plant communities associated with different thawing stages, as Sphagnum mosses, hare's-tail cottongrass (*Eriophorum vaginatum*), and common cottongrass (*Eriophorum angustifolium*) dominate the palsas, bogs, and fens, respectively (*42–47*).

Next, we assessed alpha-diversity (Shannon's *H*) of RNA viruses and compared it to broader Stordalen Mire taxa including dsDNA viruses and microbial communities (eukaryotes and prokaryotes). Mire RNA viral diversity increased along the thaw gradient, with the highest diversity observed in fens followed by bogs and the lowest at palsas, just as seen for gene-marker-inferred alpha-diversity for eukaryotes assayed here (*18S rRNA*; **Fig. 2F**) and prokaryotes assayed elsewhere (16S rRNA; complementary paper). Interestingly, dsDNA virus diversity peaks in bogs but does not differ significantly from that in fens (**Fig. 2D**). However,

192 alpha diversity measured for plant diversity exhibited distinct trends, no significant difference
193 across palsa, bog and fen and peaks in palsas for plants, respectively (**Fig. 2F, 2H**). These
194 differences likely reflect the varying resolution of the markers used, *COX1* and *RPS3* provide
195 higher taxonomic resolution (*39*, *48*), whereas 18S rRNA is more conserved and better suited
196 for broad taxonomic overviews but lacks the resolution to capture finer-scale variation (*49*).
197 Though prior soil RNA virus studies have documented RNA virus diversity (*31*, *32*, *38*, *39*, *41*),
198 few assessed broader communities taxa within their sites (*31*, *39*). Interestingly, while dsDNA
199 virus diversity peaked in bogs, its Shannon's *H* in fens was not statistically significant. Given
200 that fens represent fully thawed, inundated permafrost systems, these results suggest that
201 permafrost thaw is associated with increased RNA viruses, eukaryotes, and microbial diversity.
202 However, the broader ecological consequences of these changes remain uncertain.
203
204 **Ecological drivers of orthornaviraens in thawing permafrost**
205 We next applied ecological driver analyses to understand how biotic and abiotic factors
206 impact Stordalen Mire RNA viral community structure across habitats and environmental
207 factors. Myriad analyses revealed that Stordalen Mire RNA virus community structure was most
208 impacted by pH, followed distantly by $\delta^{13}C$ (CH_4), $\delta^{13}C$ (CO_2), and total relative abundance of
209 organisms (eukaryote) (goodness of fit and Mantel tests in **Fig 3B-C**, relative importance
210 analysis in **fig. S17,** and redundancy analysis in **fig. S18**). Previously, prokaryotic dsDNA
211 viruses have shown pH-influenced community structure at local and global scales (*50*). In
212 thawing permafrost systems, pH can impact ecological parameters such as microbial activity,
213 nutrient availability, and plant species composition by modulating the decomposition of newly
214 thawed organic matter. Thawing also leads to organic acid accumulation in low-pH bogs, while
215 the higher pH in fens promotes bicarbonate formation, making them more alkaline (*51*, *52*),
216 which in turn could have an indirect effect on RNA virus communities via host composition
217 selection (as seen prior for prokaryotes in this ecosystem, Emerson et al (*17*)). Alternatively, pH
218 might directly alter virus community structure through conformational changes critical to virus
219 integrity and infectivity, as known for viral pathogens (*53*). As pH emerges as a large-scale
220 prominent driver of RNA virus community structure, supporting prior findings for prokaryotic
221 dsDNA viruses (*50*) linking RNA virus diversity to pH, nutrients, and organic content in soil
222 (*38*).
223 Having better understood drivers of community structure, we next sought to assess
224 which vOTUs best predicted ecosystem features of interest across Stordalen Mire. To this end,
225 we used weighted gene/genome correlation network analysis (WGCNA) to group vOTUs into
226 modules and assess modular correlations to environmental factors as developed for ocean biota
227 (*54*) including application for identifying key ocean DNA and RNA viruses that best predict
228 carbon flux (*21*, *55*). Of 13 environmental traits assessed, four factors, including carbon (%),
229 nitrogen (%), isotopic signature of carbon dioxide, and carbon in peat, are not the best predictors
230 of RNA virus diversity. Instead, air and soil temperature, pH, CH_4, CO_2, and the relative
231 abundance of microbes across the thawing permafrost were prominent predictors (**Fig. 3C**).
232 Moreover, our analysis also showed 12 of 20 vOTU modules correlated to these environmental
233 factors ($p \leq 0.05$). Among these, the WGCNA gray module strongly correlated with CH_4
234 concentrations (corr. = 0.87, p = 7.3e-57; **Fig. 3D**). While dsDNA viruses have been speculated
235 to indirectly impact methane cycling via methane-related AMGs (*17*, *19*, *56*) or microbial lysis
236 dynamics (*57*, *58*), our result provides a connection between RNA viruses and CH_4
237 concentrations in thawing permafrost systems. However, the mechanism of RNA viruses
238 influencing CH_4 in thawing permafrost remains unclear, possibly through host interactions.
239 Toward the latter consideration, our analysis also found the WGCNA yellow module showed
240 the strongest correlation with organismal abundance (corr. = 0.94, p = 4e-69; **Fig. 3E**). This
241 extends long-standing theories such as "kill the winner," (*59*) which suggests that host-driven

242 shifts in microbial ecosystems could also indirectly influence RNA viruses and their ecological
243 roles, including potential links to methane-related processes. A subsequent correlation analysis
244 of RNA virus richness and diversity (Shannon's *H*) with host abundance supported this
245 hypothesis, showing a significant positive relationship (**Fig. 3F**). Together, these findings raise
246 new questions about the ecological roles of RNA viruses in rapidly changing environments and
247 underscore the need for future mechanistic studies to explore their contributions to methane-
248 related biogeochemical processes.
249
250 **RNA viruses infect diverse eukaryotic hosts that are key components of food webs and**
251 **biogeochemistry in Stordalen Mire**
252 Past studies have begun to uncover RNA viruses that affect multiple trophic levels
253 (bacteria, protists, fungi, and invertebrates), as well as inferred carbon cycling impact through
254 host lysis (*32*, *39*). However, soil RNA virus ecological roles are largely underexplored.
255 To provide context for the organisms inhabiting the ecosystem, we screened for hallmark
256 genes for eukaryotes (*COX1*, *RPS3*, 18S rRNA) and prokaryotes (*RPS3*, 16S rRNA) (**Fig. 4A-**
257 **B**, **fig. S19**) and estimated their abundances across the metagenomes (**fig. S19**). Bacteria and
258 archaea are well known at the site due to past genome-resolved studies (*5*), but known RNA
259 viruses more commonly infect eukaryotes (*21*, *60*), so we expected the same here. Since the
260 eukaryotic communities at Stordalen Mire are not yet characterized, we describe their patterns
261 here. Despite discrepancies found in the organisms inferred from the different gene screenings
262 —likely due to bioinformatic or biological biases) (**fig. S19** and **fig. S20**—, they suggested a
263 diverse taxonomic repertoire of protists, fungi, plants, and invertebrates with habitat-dependent
264 abundance profiles as follows. Palsas were enriched in plants (monocotyledons of Poales, likely
265 hare's-tail cottongrass, *E. vaginatum* (*42*)), providing primary production that supports
266 heterotrophs with diverse ecological roles: saprophytic and mycorrhizal (Agaricales and
267 Helotiales) and pathogenic fungi (Mycosphaerellales), litter-decay insects (Entomobryomorph
268 and Poduromorph springtails), rodents, and parasitic apicomplex protists (Eugregarinorida).
269 Wetter bogs were dominated by fungi (Heliotales), tardigrades (Parachela), bacteriovorous
270 rotifers (Adinetida, Ploima) and ciliates (Haptorida). Inundated fens were dominated by
271 oligochaetean worms (Haplotaxida and Lumbriculidae), likely feeding on the rich bacteria and
272 archaea biomass fueled by peatland carbon, and monocotyledons of Poales (likely, *Carex* sp.
273 and *Eriophorum* sp.). In general, plants, fungi and herbivorous invertebrates were well
274 distributed along the thawing gradient. In bogs and fens, prokaryotic life is grazed by
275 bacterivorous protists and invertebrates (*61*), with protists possibly being outcompeted by
276 invertebrates (**fig. S20**) as previously reported in inundated environments (*62*). With this
277 mapped backdrop of potential host taxa across the Mire, we next assessed whether our newly
278 identified RNA viruses might infect any of these organism taxa as predicted by the following *in*
279 *silico* approaches. Unlike DNA viruses, RNA virus host prediction is not as well developed
280 because orthornaviraens lack programmed endogenization into the host genome. Thus, as done
281 previously (Zayed et al (*22*)), we combined host predictions from multiple approaches based on
282 different types of features derived from RNA virus contigs as follows: (*i*) prior host information
283 of known virus taxa classified using the RdRp sequences (**fig.S21,23**), (*ii*) similarity to closely-
284 related endogenous virus elements that reside in known cellular genomes (**fig. S21**), (*iii*)
285 prediction of alternative genetic codes that suggest possible host organisms and subcellular
286 compartments (**fig. S21**), and (*iv*) prediction of Shine-Dalgarno motifs and CRISPR spacers that
287 suggest bacterial hosts (**fig. S21, 22**). Where more than one line of evidence existed, the final
288 host prediction was assigned using a hierarchical scoring approach that differentially weighted
289 the predictions according to confidence (see materials and methods).
290 Overall, the approach worked well to provide at least some taxon rank host prediction
291 for the vast majority (1,871 or 70.9%) of the 2,561 vOTUs (**Fig. 4C**, individual host inference

approach results are in **fig. S21, S22** and **Table S6**). These efforts revealed that Stordalen Mire RNA viruses most commonly infected fungi (e.g., Microascales, Pezizales), invertebrates (e.g., Hymenoptera, Rhabditida), and protists (e.g., Ciliophora, Trypanosomatida). Similarly to what we report here, RNA viruses that putatively infect fungi are consistently found as abundant in diverse soils (*32*, *38*, *39*, *63*) and those that infect other eukaryotic hosts (protists and invertebrates) are more rare (*32*, *38*), although this fact may derived from methodological differences. Notably, by integrating RdRp assignment to *Leviviricetes* and prediction of Shine–Dalgarno motifs and CRISPR spacers, only 69 vOTUs (2.7%) putatively infect bacteria, and these were not particularly abundant as they proportionally comprised less than 2% of the vOTU abundances (**fig. S19, S20**). By contrast, prior soil surveys have observed more than an order of magnitude higher relative abundances of bacterial RNA viruses, with more than a third of the leviviricete vOTUs (*32*). However, the predominance of RNA viruses likely infecting eukaryotes is consistent with findings in the global oceans (*21*, *22*, *60*).

Beyond these qualitative inferences, we next sought to more quantitatively assess virus-host interaction biology across the Mire by assessing vOTU abundances in light of their predicted hosts (**fig. S3**). In palsas, RNA viruses commonly infected hymenopteran insects (19.4%), unknown fungi (7.5%), mortierellalian fungi (6.6%), and microascalian fungi (4.0%) (**fig. S19**, **S20**), all of which ultimately derived carbon from plants that support herbivorous, parasitic, symbiotic, and saprotrophic life around them in this habitat (*5*, *31*). In bogs, RNA viruses infected unknown fungi (24.7%), plants, lepidopterans (9.1%), hymenopterans (5.8%), and protists (ciliates and trypanosomatids, 5.6%) (**fig. S19, fig. S20**). By contrast, in the fully inundated fens, RNA viruses mostly infected microascalian fungi (33.1%), unknown plants and lepidopterans (19.5%), hymenopterans (5.7%), priapulids (5.3%), and rhabditids (4.9%) (**fig. S19, S20**). In wetter habitats like bogs and fens, peatlands provide a rich source of biogenic carbon, supporting abundant bacteria and archaea (which increase CO_2 and CH_4 emissions), which in turns fuels active grazing. This shift in the carbon sources could explain the higher frequency of protists in bogs and non-arthropod invertebrates in fens compared to bogs.

Extrapolating from what is known about these predicted hosts, we propose the following ways RNA virus infection might modulate key ecological roles that impact soil carbon cycling. First, impacts on primary production of the Mire ecosystem through infection of plants and photosynthetic protists. Second, infection of fungi could impact their multiple roles in rhizosphere building, saprotrophic degradation of litter and peatland and parasitism. Third, among other functions, bacterial and archaeal predation could be influenced by the infection of protists and non-arthropod invertebrates (e.g., priapulids, nematodes). Critically, because RNA viruses that infect eukaryotes are known to utilize a range of infection strategies from cryptic or persistent to acute infections, associated with different levels of impact on the hosts, this viral infection continuum (*64*, *65*) leaves open many questions about the ecological footprint of RNA viruses. Even after assuming acute infection for all RNA viruses, hence suppressing the ecological roles of their hosts, the effects on the carbon cycling often remain complex to assess. Plant RNA viruses could likely reduce photosynthetic primary production and increase dead plant material, thus decreasing CO_2 absorption and increasing respiration by saprotrophs. However, given the diverse ecological roles of fungi, the impact of their RNA viruses across the three habitats could range from decreased CO_2 levels by suppressing phytopathogenic and saprotrophic fungi in litter and peatlands to reduced CO_2 assimilation due to hampered mycorrhizal fungi. Furthermore, the higher prevalence of RNA viruses infecting potential grazers in bogs and fens could reduce pressure on bacteria and archaea, thereby enhancing the mobilization of carbon from peatlands into CO_2 and CH_4 in these wetter habitats (*66*).

Auxiliary metabolic genes of Stordalen Mire RNA viruses likely suppress ecological activities performed by their hosts via molecular shutoff of gene expression

342 Another axis for understanding the ecological impact of RNA viruses would be to assess how
343 they might metabolically reprogram their hosts during infection. While impossible to assess in
344 complex natural communities at any relevant scale, one window into the metabolisms viruses
345 could impact during infection include auxiliary metabolic genes, or AMGs (*67*). While well-
346 established for dsDNA viruses (*17–19*), previously identified AMGs in RNA viruses included
347 diverse protein domains and motifs linked to both metabolite-transforming and information-
348 processing pathways, requiring functional annotation with multiple databases for accurate
349 detection. To this end, we annotated amino acid sequences encoded by RNA virus contigs using
350 multiple databases (NCBI CDD, NCBI NR, KEGG, UniRef90, PFAM, dbCAN, MEROPS; see
351 Methods) and revealed 52 AMGs across 51 RNA virus contigs longer than 500 nts, and 33
352 AMGs across ~1.2% of the vOTUs (*n*=32) (**S8 Table**). As previously reported, AMGs encoded
353 cellular-like protein domains and motifs (instead of complete ORFs), likely shaped by purifying
354 selection on RNA virus genomes (facing strong streamlining constraints) after acquiring AMGs
355 through illegitimate recombination with host mRNA (*21*, *34*). As a control, we searched the
356 vOTU sequences against co-sampled cellular-fraction metagenomics, short-reads and long-
357 reads, found no hits, which we interpret as consistent with the genes being RNA virus derived
358 rather than from host genomes or endogenous virus elements (EVEs).
359 What kinds of AMGs were observed? Functional annotation revealed AMG-encoded
360 proteins were associated with transcription, translation, posttranslational modifications,
361 membrane proteins, and miscellaneous metabolic pathways (**Fig. 5**), with many functions
362 similar to those for RNA viruses from oceanic plankton and land invertebrates (*22*, *24*, *68*).
363 Closer examination of the AMG sequences revealed that AMGs encoded protein domains (e.g.,
364 NADAR) and motifs (e.g., motifs for SUMOylation), which could indicate the selection of
365 specific catalytic activities with cellular proteins (**S8 Table**). To explore deeper into the effects
366 of these AMGs on the host cells, we identified a conservative subset of 16 AMG-encoded
367 protein sequences (indicated with double asterisks in **Fig. 5**; **S8 Table**) that were not broken by
368 contig ends and had alignments with cdd profiles of cellular domains.
369 Focusing just on these conservative 16 AMGs, activities predicted from the specific
370 motifs found suggested translational and posttranslational interferencemechanisms as follows.
371 *First*, five RNA viruses that encode proteins with two RNA-binding zinc knuckles (motifs of
372 the domains AIR1 and UMSBP), that would possibly block host mRNA translation to redirect
373 it toward virus RNA translation. Given that the only putative host of these viruses is a
374 trypanosomid, suppression of gene expression by blocking translation in infected cells would
375 hamper their parasitic activity on invertebrate hosts, which could increase grazing on bacteria
376 and archaea (and hence, decrease microbial respiration-derived CO_2 and methanogenesis) in
377 fens. *Second*, five viruses encoded specific motifs within the domains DnaJ (HSP70-interacting
378 motif (*69*, *70*)) and Siz/PIAS RING (CHC2- and C3H2C3-type motifs (*71*)), which putatively
379 increase protein turnover via ubiquitination and SUMOylation, likely providing amino acids for
380 building virus proteins. Although hosts (hymenopterans) could be assigned only to one virus,
381 unbalanced increase of protein degradation over synthesis would lead the host cell to a catabolic
382 state, hence diminishing the associated ecological roles. *Finally*, seven viruses encoded NUDIX
383 hydrolases, and two viruses encoded NADAR. Both domains have been proposed to counter the
384 antiviral defense based on NAD^+-dependent ADP ribosylation of virus macromolecules (*72*):
385 NUDIX cleaves the required NAD^+ for this system and NADAR deconjugates ADP-ribosyl
386 moieties from virus proteins and RNAs. Only one virus-encoding NUDIX was assigned to a
387 specific host taxon (dinoflagellates of the order Suessiales). Depletion of NAD^+, a cofactor
388 required in many essential cellular processes, would disrupt the functions of dinoflagellates
389 (photosynthesis and grazing), with concomitant impact on biogeochemical cycling by
390 decreasing CO_2 absorption or increasing CH_4 methanogenesis. Together, these interference

mechanisms boost virus fitness while decreasing host fitness with subsequent suppression of their ecological roles in the Stordalen mire with diverse impacts on carbon cycling.

In summary, Stordalen More RNA viruses likely impact ecosystem processes through infection of biogeochemically important microbial eukaryotic hosts and AMGs that specifically target processes underpinning the cellular physiology that dictates biogeochemical cycling. However, quantifying these impacts awaits perturbation experiments and/or modeling designed leveraging these initial empirical observations.

CONCLUSIONS

Our study provides a baseline for understanding ecological patterns and the footprint of soil RNA viruses in thawing permafrost, emphasizing their potential implications amid Arctic environmental and climate change. By exploring diversity patterns, we posit a near-future scenario in which permafrost thawing and proliferation of palsas will conceivably activate and expand the existing RNA virus communities, leading to increased diversity and complexity compared to current levels. We evaluated the potential roles of RNA viruses to modulate the mineralization rates of peatland carbon into CO_2 and methane through infection. We suggest that pathogenesis and killing along with AMG-driven suppression of gene expression by RNA viruses have the potential to impact greenhouse gas emissions. Fungal hosts assume extremely diverse ecological roles, from being saprotrophs feeding on plant litter or peatland to biotic interactions (e.g., being parasites of plants or invertebrates and mutualists in lichens). Infection of saprotrophic, parasitic, and mutualistic fungi and heterotrophic protists has the potential to increase CO_2 emissions from peatland. It could also increase methanogenesis by infecting (*i*) grazers (e.g., priapulids, nematodes, arthropods, ciliates), (*ii*) invertebrates stimulating microbiota, and (*iii*) anaerobic protists and ciliates (in particular, those associated with methanogenic archaeal endosymbionts). The manner, intensity, and direction of such effects in diverse hosts need to be addressed to allow the enable footprint of RNA viruses to be integrated into predictive ecological models. This knowledge will empower us to improve the accuracy of ecosystem models and improve the prediction of the ecological implications of climate change.

ACKNOWLEDGMENTS

This material is based upon work supported by the U.S. Department of Energy, Office of Science, Office of Biological and Environmental Research, under Award Number DE-SC0023307 to M.B.S. Funding was provided by DOE grants to MBS. This research is a contribution of the EMERGE Biology Integration Institute, funded by the National Science Foundation, Biology Integration Institutes Program, Award #2022070.. The authors thank the Swedish Polar Research Secretariat and SITES (Swedish Research Council grant 4.3-2021-00164) for the support of the work done at the Abisko Scientific Research Station. This work was supported in part through a Laulima Government Solutions, LLC, prime contract with the U.S. National Institute of Allergy and Infectious Diseases (NIAID) under Contract No. HHSN272201800013C. J.H.K. performed this work as an employee of Tunnell Government Services (TGS), a subcontractor of Laulima Government Solutions, LLC, under Contract No. HHSN272201800013C. K.C.W. funding was provided by EMERGE (#2022070), NSF Cyverse [#2149505 ,Collaborative Research: Updating iVirus -- the CyVerse-powered analytical toolkit for viruses of microbes], and was partially funded by the Genomic Science Program of the United States Department of Energy Office of Biological and Environmental Research, grants #DE-SC0023456.

We thank Anya Crane (National Institutes of Health National Institute of Allergy and Infectious Diseases [NIAID] Division of Clinical Research [DCR] Integrated Research Facility at Fort Detrick [IRF-Frederick], Frederick, MD, USA) for editing the manuscript. The views and conclusions contained in this document are those of the authors and should not be interpreted as

necessarily representing the official policies, either expressed or implied, of the U.S. Department of Health and Human Services or the institutions and companies affiliated with the authors, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government. A.A.P is currently funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) under Germany's Excellence Strategy – EXC 2051 – Project-ID 390713860. G.D.H is currently funded by the Spanish Ministry for Ecological Transition and the Demographic Challenge.

Data availability

The sequencing data described in this article, including metagenomic and metatranscriptomic data also described in Woodcroft et al., Emerson, et al, Sun et al. and McGivern et al. (*5*, *10*, *17*, *19*), are submitted under NCBI BioProject accession number PRJNA386568. The metagenomics assembled genomes (MAGs) used in this study were acquired from a previous study (Woodcroft et al.,(*5*)) and deposited at DDBJ/ENA/GenBank under the accession numbers provided in **Table S6**.

456 **SUPPLEMENTARY**
457
458 **Methods**
459 **Sampling, purification of nucleic acids, library preparation, and short-read sequencing**
460 A total of 55 metatranscriptomics were used in this study (**S1 Table**). Twenty-eight
461 metatranscriptomics were collected during 2010, 2011, and 2012 (*5*, *11*); and 27
462 metatranscriptomics samples were collected in 2016 (*10*) (≈0.63 terabases [Tb] of data). A
463 detailed description of active permafrost sampling strategies and protocols was previously
464 described (*5*, *11*). Briefly, triplicate samples were taken from the active layer across the thaw
465 permafrost gradient comprising palsa (intact permafrost), bog (partially thawed), and fen (fully
466 thawed). The soil cores were taken at different depths of 2.5–48.5 cm (**S2 Table**). Soil cores
467 were kept at saturation with three volumes of LifeGuard solution (MoBio Laboratories) and
468 stored at -80°C until processing.
469 The protocols for extraction and purification of RNA for 2010–2012 samples were described
470 previously (*5*). The total RNA of samples was subjected to removal of residual DNA using
471 DNAse I (Roche) following the manufacturer's instructions. Next, ScriptSeq Complete
472 (Bacterial)-low input kits (Epicentre) were used for library preparation. To check the quality of
473 RNA and libraries during processing, Agilent 2100 Bioanalyzer and Agilent 2200 Tapestation
474 (Agilent Technologies) with Qubit (ThermoFisher Scientific) were used. Before the deeper
475 NextSeq (Illumina) sequencing, the resulting metatranscriptomic libraries were assessed on
476 either a HiSeq (Illumina) or MiSeq (Illumina) to evaluate library quality. For 2016 samples, the
477 TruSeq Stranded mRNA Library Prep (Illumina cat. No. 20020594, 20020595) was used for
478 library preparation. Using this, polyA-containing mRNA molecules were purified in two rounds
479 with oligo-dT magnetic beads. During the second elution process of the polyA RNA, the RNA
480 was fragmented and primed for cDNA synthesis.
481
482 **Metatranscriptome assembly, virus identification, and evaluation of completeness of**
483 **putative RdRps**
484 The description of the metatranscriptome read assembly, virus identification, and taxonomic
485 classification methods have been detailed previously (*21*, *22*). Briefly, the raw-read RNA
486 sequences downloaded from the National Institutes of Health (NIH) National Center for
487 Biotechnology Information (NCBI) Sequence Read Archive (SRA) database
488 (https://www.ncbi.nlm.nih.gov/sra) were first processed to eliminate adapter and low-quality
489 sequences by using Trimmomatic v0.36 (*73*) with default parameters. PhiX contamination was
490 also removed by BBDuk (bbduk.sh; ktrim=*r*, minlen=40, minlenfraction=0.6, mink=11, tbo tpe
491 k=23, hdist=1, hdist2=1, ftm=5). Next, *de novo* assembly was performed using MEGAHIT
492 v1.1.3 (*74*) with default settings. To avoid problems associated with alternative genetic code
493 and non-canonical translation events, EMBOSS Transeq was used (instead of protein prediction
494 with prodigal) to yield RdRp footprints.
495 Approaches to identify the RNA virus genomes or transcripts from assembled
496 metatranscriptomes and the authentication of virus RdRp were described previously (*22*). To
497 improve the identification of the divergent RdRp, the Hidden Markov Model (HMM) was
498 applied over 10 iterations to search for virus RdRp domains (**fig. S1**). To evaluate the
499 authenticity, thus true positive hits of virus RdRp sequences, competitive HMM searches were
500 conducted of 70 RdRps derived from the lineages defined previously (*24*), eight recently
501 discovered lineages (*22*), and 16,268 PfamA (v33.0) non-RdRp HMMs. Further, the putative
502 RdRp hits with the best match to an RdRp HMM and a bit-score ≥30 was kept as true positives
503 for proteins containing the virus RdRp domain. In addition, to confirm that the RNA virus
504 contigs were not from endogenous viruses, the identified sequences were searched against the
505 available metagenomes.

506 To estimate RdRp domain completeness, based on previous efforts (*22*), the extraction of
507 footprint amino acid sequences of the RdRp domain was performed considering the challenging
508 cases of the usage of alternative genetic codes and non-canonical translation events (e.g.,
509 ribosomal frameshifting). Extraction and reconstruction of RdRp domain sequences were
510 performed by running another iteration of the search for the translated amino acid against the
511 70 RdRp profile HMMs, and the hits with a bit score ≥30 and with aligning regions of ≥90% of
512 the average length of the best-matching HMMs (to be considered as "complete" or "near-
513 complete" virus RdRp domain sequences). The 3,560 extracted RdRps that were shorter than
514 100 amino acids were further inspected for RdRp annotations, having detected only 18 of them
515 for which no virus signal was apparent, hence further supporting that 99.5% of hits are RdRp-
516 encoding sequences derived from RNA viruses. Lastly, to estimate genome completeness for
517 the recovered viral contigs, CheckV (*75*) was used with its default parameters (**fig. S2**), which
518 compared amino acid identity and HMMs of complete viral genomes derived from NIH NCBI
519 GenBank and environmental samples.
520
521 **Virus operational taxonomic units (vOTU) and calculations of vOTU relative abundances**
522 **and diversity metrics**
523 To analyze the diversity and ecological pattern of soil RNA viruses, they were first designated
524 as vOTUs or an approximate "species-rank" ecological unit; the detailed analysis of the RNA
525 virus sequence space to establish a "universal" cutoff has been reported previously (*21*, *22*).
526 Using the previously recommended cutoff, 90% average nucleotide identity (ANI) across 80%
527 of the shorter sequence length (*21*, *22*), resulted in 9,567 total vOTUs, of which 2,561 were ≥1
528 kb (**S3 Table**). Further, to assess the robustness of this cutoff in the soil system, an investigation
529 of the ecological patterns was performed across an ANI range of 60–100% (and 80% allele
530 frequency [AF]), including consensus cutoff recommended by (*33*), 95% ANI and 85% AF (**Fig.**
531 **S3**).
532 To generate the relative abundance table of the designated vOTUs, the previously described
533 approach was followed (*21*, *22*). Before the mapping, virus contigs were trimmed off their
534 polyA/polyT sequences; this was done to avoid inflated abundance and better estimate
535 horizontal coverage for polyA-tailed viruses, respectively. Further, a quality-controlled
536 sequence read of metatranscriptomic samples to the vOTUs was recruited, and trimmed mean
537 abundances were calculated with CoverM (https://github.com/wwood/CoverM). The trimmed
538 mean abundance table was then normalized by dividing abundances by the number of quality-
539 controlled reads per sample per 1 Tb to get the final relative abundance table (**Table S4**).
540 The ecological analysis was done using samples collected in 2012 and 2016 to minimize
541 bias introduced by samples variably from 2010 and 2011. Different diversity metrics were
542 calculated, including α-diversity (Shannon's H, Simpson, Inverse–Simpson, Richness, and
543 Perilous J Evenness) and β-diversity (Bray–Curtis dissimilarity method), using R vegan package
544 (*76*). vOTUs that were not present in any samples and samples with no vOTUs present were
545 excluded. The abundance table of vOTUs was log-transformed (function 'decostand', method
546 = 'log') before the calculation of the Bray–Curtis dissimilarity analysis (function 'vegdist',
547 method 'bray'). This was then used to generate principal coordinate analysis (pCoA, function
548 'capscale' of the vegan package). The emerging metacommunities in pCoA were statistically
549 verified using permanova tests (function 'adonis') and the confidence intervals were plotted
550 using the standard deviation method (function 'ordiellipse') at 95 and 97.5% confidence limits.
551 The same Bray–Curtis dissimilarity matrices were used for hierarchical clustering (function
552 'pvclust', method.dist ='co'' and method.hclust = 'average') using 1,000 bootstrap iterations.
553 Only the approximately unbiased (AU) bootstrap values were reported on the dendrograms. The
554 heatmaps of the hierarchical clustering analysis were generated using the heatmap3 package
555 (*77*) (**fig. S3–S5**).

556
557 **Comparing Stordalen Mire RNA virus to other soil RNA virus studies**
558 Metatranscriptome sequences were downloaded from recently published soil RNA virus studies,
559 which included examination of California grassland (*39*), grassland and coastal soils (*32*), crop
560 field soil (*31*), Kansas grassland (*41*), and various soil types across China (*38*). Similar pipelines,
561 including metatranscriptome assembly, virus identification, and evaluation of completeness of
562 putative RdRps, were run as described above. To compare the virus sequences across different
563 soil studies, the identified RNA virus contigs were clustered at 90% ANI and 80% AF to get
564 vOTUs. For RdRp-level comparison, only those "complete"/"near-complete" (≥90% of the
565 average length of the best-matching HMMs) were used. The RdRps were clustered using
566 USEARCH (-cluster_fast) (*78*) at 75% Identity of Amino Acid (IAA), representing taxonomic
567 rank between family and genera (*24*). Subsequently, those clustered at 75%, were considered
568 shared among studies. These results were plotted in UpSet using the UpSetR package in R (*79*)
569 (**fig. S6**). To compare the identified orthornavirans from this study to other soil studies,
570 taxonomy assignment was done using RdRp-scan (*80*).
571
572 **RdRp-based taxonomic annotation of RNA viruses**
573 The taxonomic classification analysis was conducted using both the network-based, iterative
574 clustering approach and the phylogenetic described previously (full details in Zayed et al., 2022)
575 (*22*) (**fig. S7**). Briefly, a total of 705,705 complete and near-complete RdRp domains—including
576 1,702 from the current Stordalen Mire soil study; 6,732 from a *Tara* Ocean study (*21*, *22*); 3,325
577 from coastal ocean RNA viromes (*24*); and 695,648 from GenBank [version 245]—were used
578 in the analysis. First, these sequences were pre-clustered at 50% amino acid identity using
579 Uclust v10.0.240 (usearch -cluster_fast -id 0.50 -sort length) (*78*). Then, these centroid
580 sequences (n = 6,553) were used in pairwise comparisons using blastp v2.10.0+ (-gapopen 9 -
581 gapextend 1 -word_size 3 -threshold 10) (*81*). Further, the e-values for each pair were extracted
582 and negative-long10-transformed in MCL v14-137 (-stream-mirror -stream-neg-log10 -stream-
583 tf 'ceil(200)') (*82*). The transformed e-values were used in an MCL network for iterative
584 clustering, changing the granularity parameter at each iteration (range 0.1–8). From this point,
585 'adj.rand.index' value was used to evaluate all the cluster sets from MCL and concurrently
586 compared to the previously established ICTV taxonomy (**Table S5**). The clusters with the
587 highest rand index value at the phylum and class ranks were picked and the taxonomic
588 delineations were then assigned for sequences within each cluster.
589 Moreover, phylogenetic trees were generated for each assigned taxonomic network-derived
590 cluster (at the phylum and class ranks) as established previously (*22*). Briefly, from each
591 resultant network-derived major cluster, sequences from each cluster were aligned separately
592 using the E-INS-I strategy over 1,000 iterations in MAFFT v7.017 (*83*). Aligned sequences were
593 subsequently trimmed using Trimal (*84*). Sites with ≥20% gaps were removed, and the
594 alignments were manually inspected. Before phylogenetic analysis, sequences were screened
595 for possible recombination events using 3Seq (*85*), with a recombinant event determined by a
596 Bonferroni-corrected p-value cutoff of 0.05. Phylogenetic relationships of sequences within a
597 cluster were first assigned the appropriate evolutionary model using ModelFinder (*86*). Then, a
598 subsequent maximum-likelihood phylogenetic tree was generated using bootstrap support
599 generated for 1,000 iterations in IQ-TREE (*87*). Given the high number of reference sequences
600 within the phylum-level phylogenetic trees, a subset of these trees was created to aid in
601 visualization. Redundant reference sequences were reduced while maintaining the diversity
602 captured in the original tree using Treemer, applying stop option -X (50–200) depending on the
603 phylum (*88*). For **Fig. 1**, sequences used to build the trees were preclustered at 50% identity,
604 and clades supported by 100% bootstrap values were collapsed.

605 To taxonomically assign sequence to a family rank, a two-pronged approach was used. First,
606 the class-rank phylogenetic trees were used to establish the placement of sequences on the tree,
607 requiring a sequence to fall within a clade or sister clade to be assigned to the same taxon. Then,
608 sequences were assigned tentative names identical to the reference sequences of the established
609 clades with a '-like' suffix added. Further, the prior analysis was complemented by the
610 taxonomic assignment from the conservative MCL clusters. Subsequently, these two approaches
611 were combined and the more specific taxonomic assignment of the two methods (e.g., without
612 the '-like' suffix or higher-resolution taxonomic assignment) was picked as the final putative
613 classification for the novel sequences.
614
615 **Host inference of hosts and cellular organisms' analysis**
616 To infer the virus-host associations, a few independent approaches—including RdRp-based
617 taxonomy, endogenous virus elements (EVEs), RdRp-based against the cellular non-redundant
618 (nr) database, alternative genetic code, Shine–Dalgarno motifs, and CRISPR-Cas spacers
619 analysis—were used. The following is the detailed approach: (*i*) The RdRp-based taxonomy
620 approach was used to infer the potential virus–host associations. For this approach, the known
621 RNA virus taxa (phyla, classes, and families) that were assigned to the vOTUs after clustering
622 the RdRp domain protein sequence similarity network were used to retrieve the information on
623 putative hosts. (*ii*) EVEs looked for closely-related RdRp-encoded cellular genomes, and the
624 RdRp hits (from virus contigs) suggested that a virus contig can have a putative host
625 taxonomically related to one of the cellular genomes (*68*). All available assembly genomes from
626 cellular organisms in GenBank version 205 (last accessed May 2021) were downloaded; this
627 included archaea (4.89 Gbp), bacteria (234 Gbp), fungi (157 Gbp), invertebrates (618 Gbp),
628 plants (683 Gbp), protozoa (41.6 Gbp), and vertebrates (2.44 Tbp). A more sensitive diamond
629 (version 0.9.36) blastx algorithm against the aforementioned databases was used (--evalue 1e-
630 20–-sensitive–-out–-outfmt 6). The threshold for the search was set to 100 amino acids for
631 length and 1 × 10−20 for e-value. The results were also checked manually to accurately assign
632 the putative host taxonomy. (*iii*) Host information was inferred from cellular nr databases. The
633 query of virus RdRp footprints was then used against this database. The threshold for the search
634 was set to 100 amino acids for length and 1×10^{-5} for e-value. Only the top match hits were
635 used, and the results were manually checked. (*iv*) Using genetic code, the translated sequences
636 were searched once more against the 84 RdRp profile HMMs, and those with a bit score ≥30
637 and aligning regions of ≥90% of the average length of the best-matching HMMs were
638 considered proteins containing "complete" virus RdRp domain sequences. Sequences
639 containing more than one hit in the same or different frames (presumably non-canonical
640 translation cases) were resolved manually by joining the aligning regions into the same protein
641 sequence. (*v*) Shine–Dalgarno motifs, following the same approach described by Menardo et
642 al., 2018 (*88*). (*vi*) To predict potential prokaryotic hosts using the CRISPR-Cas spacer
643 approach, blastn was used against ≈12 million spacer sequences (*89*), and only blastn hits with
644 0 or 1 mismatch over the whole spacer length were considered. Furthermore, spacers were
645 extracted from 1,529 previously described metagenomics-assembled genomes (MAGs) (*5*)
646 using MinCed (https://github.com/ctSkennerton/minced). A minimum of two repeats was
647 required for a CRISPR to be included in the analysis. Further, as in the preceding analysis, only
648 blastn hits with 0 or 1 mismatch over the whole spacer length were considered. The best hit
649 sequence alignment was then visualized using mView
650 (https://www.ebi.ac.uk/Tools/msa/mview/). Moreover, the taxonomic assignment of the
651 identified orthornavirans, which was performed using RdRp-scan (*80*), was also utilized to infer
652 the hosts. Additionally, to identify the RNA virus infecting bacteria, bacteriolytics analysis was
653 conducted using HMM searches, utilizing the HMM profiles previously provided (*34*) and only
654 considering hits with a bit score ≥30 for further analysis (**Fig. S9–S12**). Leviviricetes require

655 bacterial pili to attach to and infect cells (*90*). Therefore, we performed competitive searches
656 using HMMsearch with nine type-IV pilus (T4P) profiles (PF13681.9, PF11104.11, PF16074.8,
657 PF17456.5, PF11773.11, PF18222.4, PF04350.16, PF04351.16, and PF04964.17) and 13 non-
658 T4P profiles containing the keyword "pilus" (PF07434.14, PF09589.13, PF14855.9,
659 PF09476.13, PF16976.8, PF09460.13, PF13629.9, PF13400.9, PF16968.8, PF09977.12,
660 PF05946.15, PF06340.14, and PF10671.12). The best match to a T4P HMM profile and a bit
661 score ≥50 were considered true positives for proteins containing the bacterial T4P domain. To
662 ensure a true positive match, random hits were manually checked across the datasets with
663 BLASTP using RefSeq and nr databases. The integrated host interference analysis can be seen
664 in **S7 Table**.
665 Next, to get a more comprehensive picture of the eukaryotic community and due to the lack
666 of this information in Stordalen Mire, a further investigation focused on cellular organism
667 community structure using HMM marker genes—cytochrome c oxidase subunit 1 mitochondrial
668 gene (*COX1*); a component of the eukaryotic 40S ribosomal subunit, ribosomal protein S3
669 (*RPS3*) (*39*); and small subunit ribosomal RNA (SSU rRNA). For *COX1* and *RPS3* HMM
670 profiles were used to screen the metatranscriptomic data. Hits with an e-value = 10–5 and a bit
671 score ≥30 were extracted, and the top match for each marker gene was retained. The *COX1* and
672 *RPS3* sequences were dereplicated and clustered at 98 and 99% amino acid identity,
673 respectively, using USEARCH (-cluster_fast) (*78*). The reconstruction analysis was then
674 performed for the second time by running the translated contigs against the marker genes
675 HMMs. The hits with a bit score ≥30 and aligning regions of ≥90% of the average length of the
676 best-matching HMMs (to be considered as "complete" or "near-complete" virus RdRp domain
677 sequences) were retained. Further, a manual check was also done to verify the taxonomy of the
678 identified community. Additionally, SSU rRNA—including 28S and 18S (eukaryotes); 23S,
679 16S, and 23S (bacteria); and 16S (archaea)—were extracted from assembled contigs and
680 subsequently blasted against the Silva database (*91*) to determine their cellular organism
681 taxonomy. The vOTUs of the cellular organisms were then extracted from the aforementioned
682 dereplicated marker genes.
683
684 **Ecological drivers' analysis**
685 To examine the relationships between the relative abundance of orthornaviraens in different
686 habitats and environmental factors, a redundancy analysis (RDA) was performed using the
687 vegan package in R (*76*) and Hellinger. To ensure a suitable ratio of explanatory variables to
688 samples, variance inflation factors (VIF) were used and included soil depth (avg.), air
689 temperature (^{o}C), soil temperature (^{o}C), porewater pH, carbon dioxide (CO_2; mM), methane
690 (CH_4; mM), carbon (C; %), nitrogen (N; %), δ^{13}C of CO_2, δ^{13}C of CH_4, δ^{15}N of peat, relative
691 abundance of cellular organisms (*COX1* and *RPS3*), and relative abundance of cellular organism
692 (SSU rRNA) in the analysis.
693 The environmental variables were fitted to the first two dimensions of the PCoA using a
694 generalized additive model (function envfit; permutations = 9,999 and na.rm = TRUE) and R
695 vegan package (*76*). Then, they were correlated with all the pCoA dimensions using a mantel
696 test (function mantel; permutations = 9,999 and method = "spearman") after scaling (function
697 scale) and calculating their distance matrices (function vegdist; method = "Euclidean" and na.rm
698 = TRUE). Furthermore, Spearman's correlation analysis was performed (function cor; use =
699 "pairwise.complete.obs") and plotted using ggplot2. Finally, Spearman's pairwise correlation
700 was performed with vegan package in R. The "rcorr" function and the Hellinger transformed
701 data were used as input. Correlograms were generated using the corrplot package to visualize
702 the correlation results; only variables with significant correlations highlighted at a significance
703 level of 0.05 were shown.

704 To quantify the relative contributions of environmental variables to the changes in virus
705 community (the first coordinate of a pCoA; pCo1), a relative-importance analysis test was
706 performed. This analysis utilized the "relaimpo" v2.2–348 package (*92*), using commonly used
707 multiple-linear regression models (i.e., LMG, First, Last, Betasq, and Pratt) with 1,000
708 bootstraps. The relaimpo package provided methods for estimating the contributions of
709 explanatory variables within a multi-linear regression model. The Lindeman–Melinda–Gold
710 model (LMG) calculated the marginal contribution of each predictor variable while accounting
711 for the presence of all other predictor variables in the model. The model "last" (also called
712 usefulness) calculated the importance of each variable as the reduction in the residual sum of
713 squares (RSS) when that variable was added last to a model that already contained all the other
714 predictor variables. The model "first" calculated the importance of each variable as the reduction
715 in the RSS when all the other predictor variables were added to a model that already contained
716 that variable. Model "Betasq" calculated the importance of each variable as the square of its
717 standardized regression coefficient. Model "Prat" calculated the importance of each variable as
718 the sum of squared correlations between that variable and all other variables in the model (**fig.
719 S16**).
720
721 **Functional annotation of RNA virus genomes**
722 Functional aspects of the protein sequences encoded by RNA virus nucleotide sequences (partial
723 or complete genomes and transcripts) at 95% ANI and 80% AF were studied by integrating
724 annotations from several tools. Nucleotide sequences of contigs coding RdRp were searched
725 against (*i*) the conserved domain database (CDD) by using CDD search (*93*), HMMER (*94*),
726 and a minimum e-value of 0.01 and (*ii*) against the NCBI nr database using blastx and a
727 minimum bit score of 50. Protein sequences predicted from the RdRp-coding contigs by using
728 Prodigal (apply corresponding genetic codes previously inferred) were (*i*) searched against
729 KEGG, UniRef, CAZY, MEROPS, Pfam, and VOG databases by using DRAM (*95*). Virus
730 coding sequences whose functional annotation suggested a "cellular" origin were considered
731 "auxiliary metabolic genes" (AMGs) after manual inspection if none of the following cases was
732 accomplished: (*i*) the putative cellular domains overlapped typical RNA viral domains (e.g.,
733 RdRp, capsid, helicase, protease), (*ii*) the putatively cellular domain was equivalent to known
734 RNA viral domains, (*iii*) blastx hits also included viral proteins (to avoid poor annotations in
735 the NCBI nr database), or (*iv*) blastx hits were not hypothetical proteins of unknown functions.
736 To determine redundancies and avoid different annotation names for the same gene function,
737 AMG sequences not at the contig ends were considered "complete" and compared against each
738 other through blastp (cutoffs: e-value of 0.001, 20% IAA, and query coverage of 20%). To avoid
739 the possibility of these AMGs being derived from sequenced genomic DNA carrying EVEs,
740 RNA virus contigs were searched against all the available assembled and co-sampled DNA
741 sequencing data (as described in the sections on metatranscriptome assembly, virus
742 identification, and evaluation of completeness of putative RdRps). The final list of RNA virus
743 AMGs can be seen in **Table S8**.

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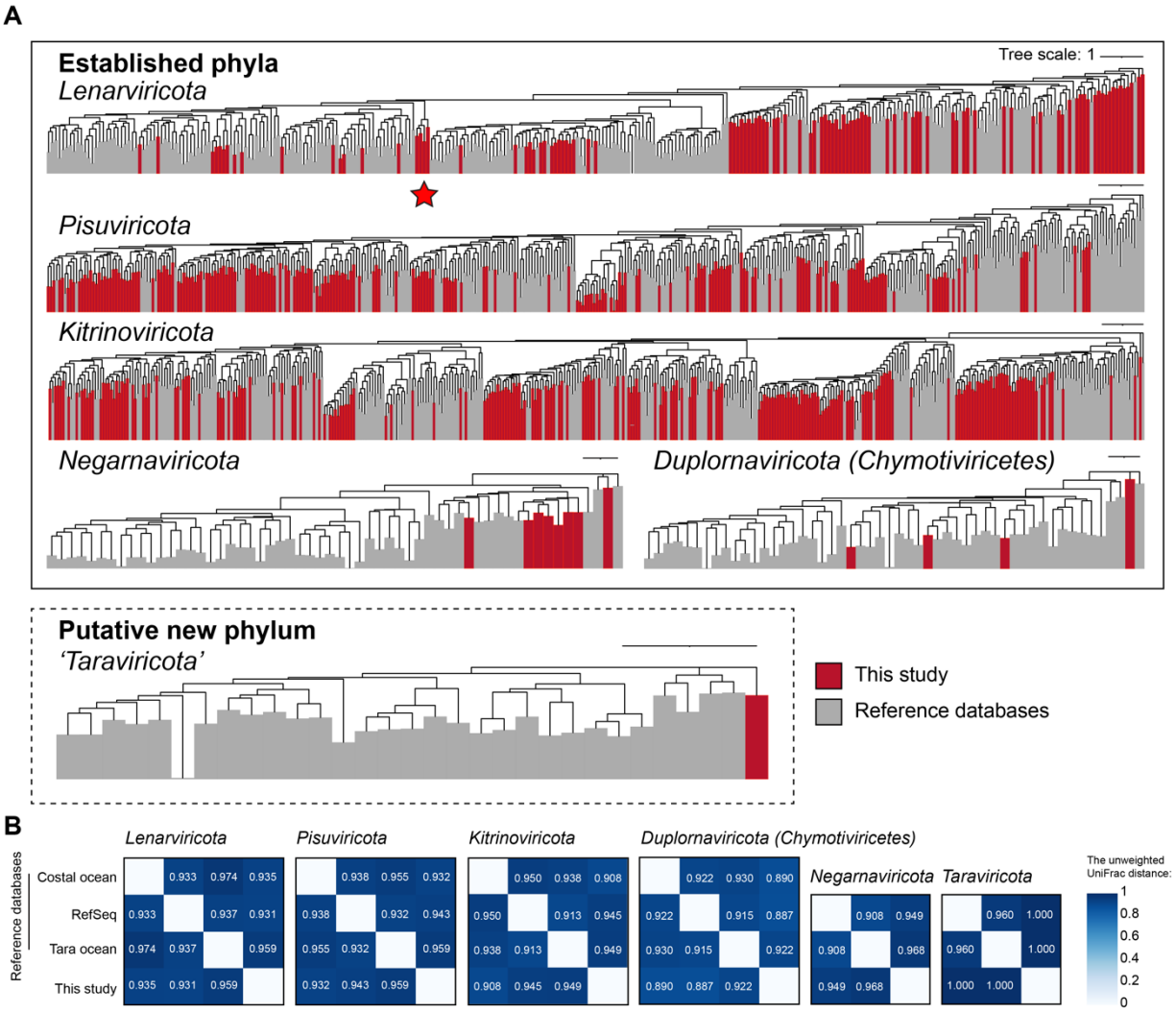

Figure 1. Phylum-ranked, RdRp-based orthornaviran phylogenies.
(A) Six maximum-likelihood phylogenetic trees were built from an RdRp-guided taxonomy analysis of near-complete RdRp sequences. Five trees depict ICTV-established phyla, and one corresponds to a previously suggested phylum, '*Taraviricota*' (Zayed et al.,(*22*)). The red color indicates Stordalen Mire orthornaviran sequences discovered in this study, whereas grey indicates previously known reference sequences (including open-ocean and coastal-ocean RNA viromes). The scale bar indicatesone amino acid residue substitution per site. The ☆ represents a likely new class, Candidatus 'Stormiviricetes'. RdRp, RNA-directed RNA polymerase; ICTV, International Committee on Taxonomy of Viruses. **(B)** Heatmap showing the unweighted UniFrac distances between RNA virus phylum-rank trees across datasets from the coastal ocean (*24*), RefSeq, *Tara* Oceans (*21*, *22*), and Stordalen Mire (this study). Phyla analyzed include *Lenarviricota, Pisuviricota, Kitrinoviricota, Duplornaviricota* (Chymotiviricetes), *Negarnaviricota*, and '*Taraviricota'*. Higher values (darker cells) indicate greater phylogenetic dissimilarity between datasets, whereas lower values (lighter cells) indicate greater similarity.

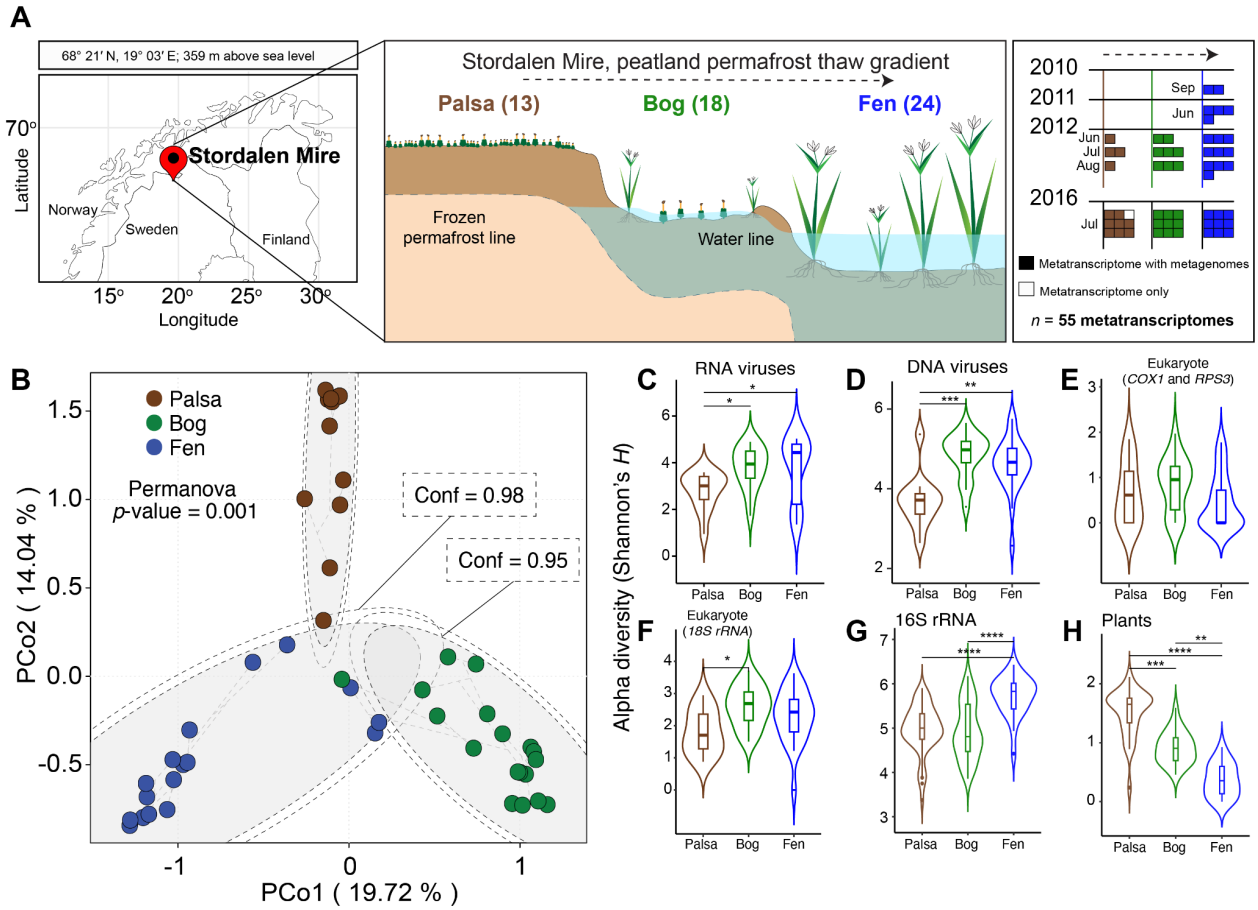

Figure 2. Cross-habitat soil sampling of Stordalen Mire in northern Sweden. **(A)** Soil metatranscriptomes were collected from the active layer across the peatland permafrost thawing gradient with intact palsa (approximately 30 cm), bog (intermediate thawing, approximately 60 cm), and fen (fully thawed) habitats. **(B)** Principal component analysis (PCoA) of a Bray–Curtis dissimilarity matrix calculated from all virus operational units (vOTUs; i.e., species-rank units) uncovered in this study. Dot colors correspond to the colours of the thawing gradient (brown for palsa; green for bog; and blue for fen. Alpha-diversity (Shannon's *H*) of **(C)** RNA viruses, **(D)** dsDNA viruses, **(E)** Eukaryote and prokaryote (based on *COX1* and *RPS3*), **(F)** Prokaryote (based on 18S rRNA), **(G)** 16S rRNA, and **(H)** Plants. Violin plots with boxplots show medians and quartiles. Statistical analysis was performed using the Kruskal–Wallis test with *post hoc* Dunn test and *p*-adjusted Bonferroni. Only the significant values are shown and donated: * *p*-value ≤0.05; ** *p*-value ≤0.01, *** *p*-value ≤0.001, and **** *p*-value ≤0.0001. **Fig. 2** G and H on the complementary papers.

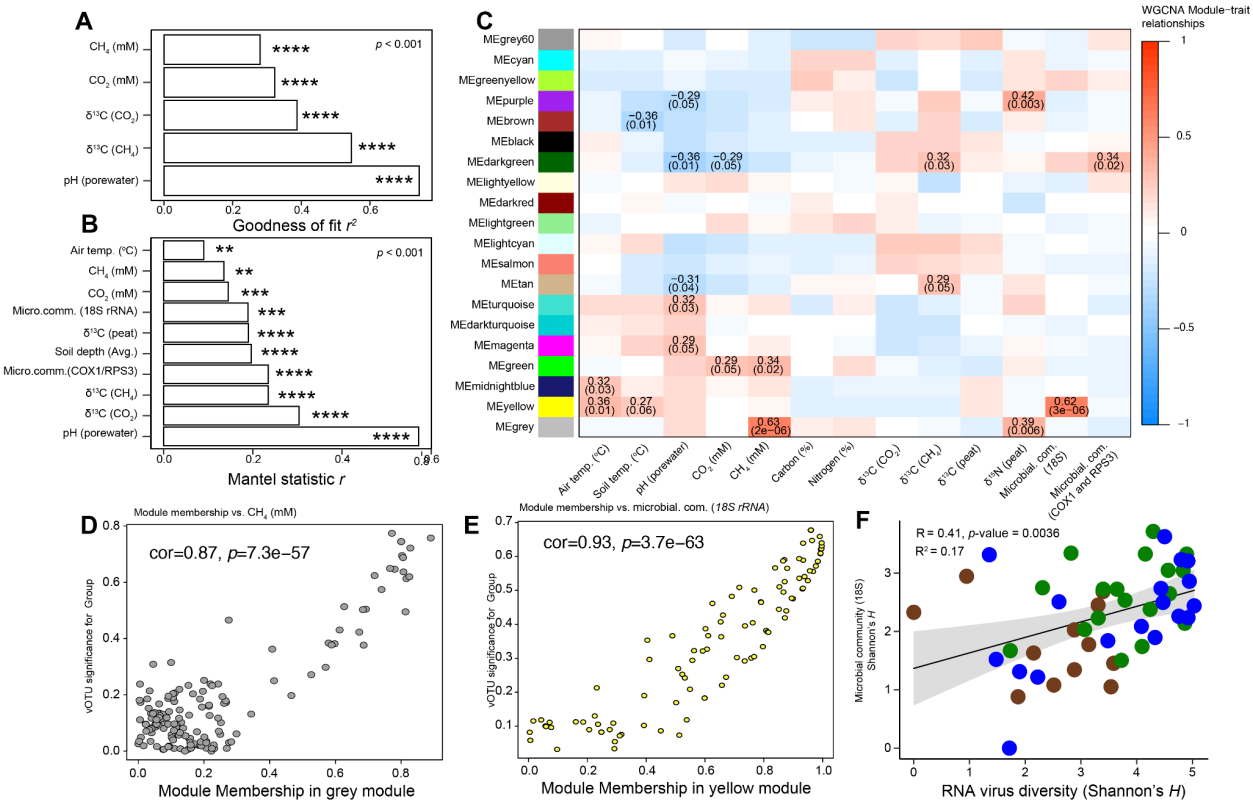

Figure 3. Ecological drivers of Stordalen Mire orthornaviraens.
(**A, B**) The first two dimensions (with a goodness of fit r^2 derived from a generalized additive model) and all dimensions (determined through a Mantel test based on Spearman's correlation) revealed potential ecological drivers and predictors of orthornaviran beta-diversity. pH was consistently identified as the strongest predictor of beta-diversity. Only the significant values are shown and donated as follows: * *p*-value ≤0.05; ** *p* -value ≤0.01, and *** *p* -value ≤0.001. (**C**) Heat map of module-trait relationships derived from Weighted Gene Co-Expression Network Analysis (WGCNA). Each row corresponds to viral operational taxonomic units (vOTUs), grouped into modules (labeled by module colors), and each column represents an environmental trait. The color scale indicates the correlation coefficients. The correlation values and corresponding *p*-values (in parentheses) are shown within each cell; only *p*-values ≤0.05 are shown. (**D, E**) Correlation analysis between module membership in the grey and yellow modules vs. methane concentration (CH_4 mM) and microbial community (*18S rRNA*) relative abundance, respectively. Each point represents a vOTU within the grey and yellow modules. (**F**) Spearman's correlation analysis between orthornaviran and host cellular organism diversity (Shannon's *H*).

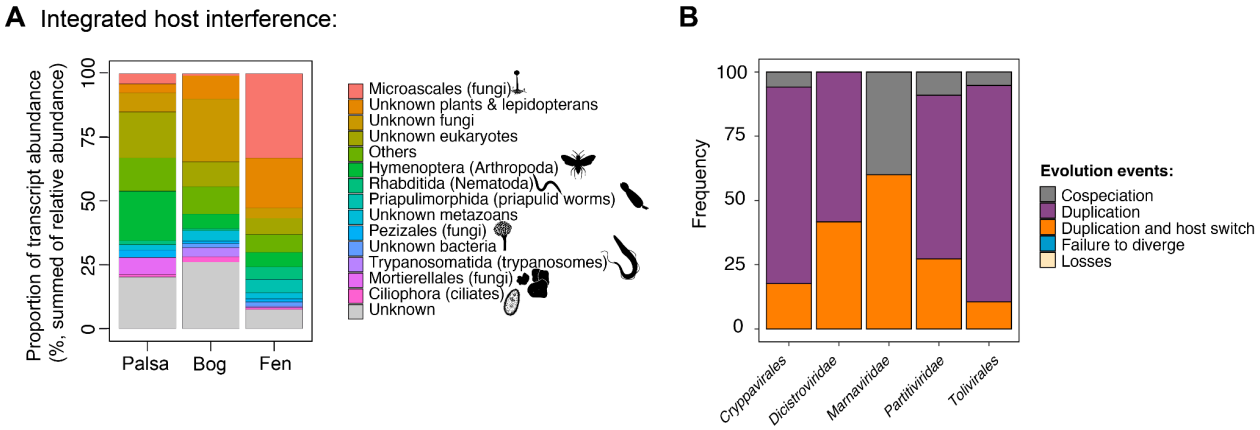

Figure 4. Integrated host inferences, and the co-phylogeny relationship between Stordalen Mire orthornaviraens and their predicted cellular hosts.
(A) Taxonomic assignment of the prokaryotes and eukaryotes in Stordalen Mire identified via *COX1* and *RPS3* gene and 18S rRNA analysis. **(B)** Taxonomy assignment of the integrated host interference. Stacked bar plots illustrate estimated evolutionary events. Cytochrome c oxidase subunit 1 mitochondrial gene (*COX1*); a component of the eukaryotic 40S ribosomal subunit, ribosomal protein S3 (*RPS3*); and small subunit ribosomal RNA (*18S rRNA*).

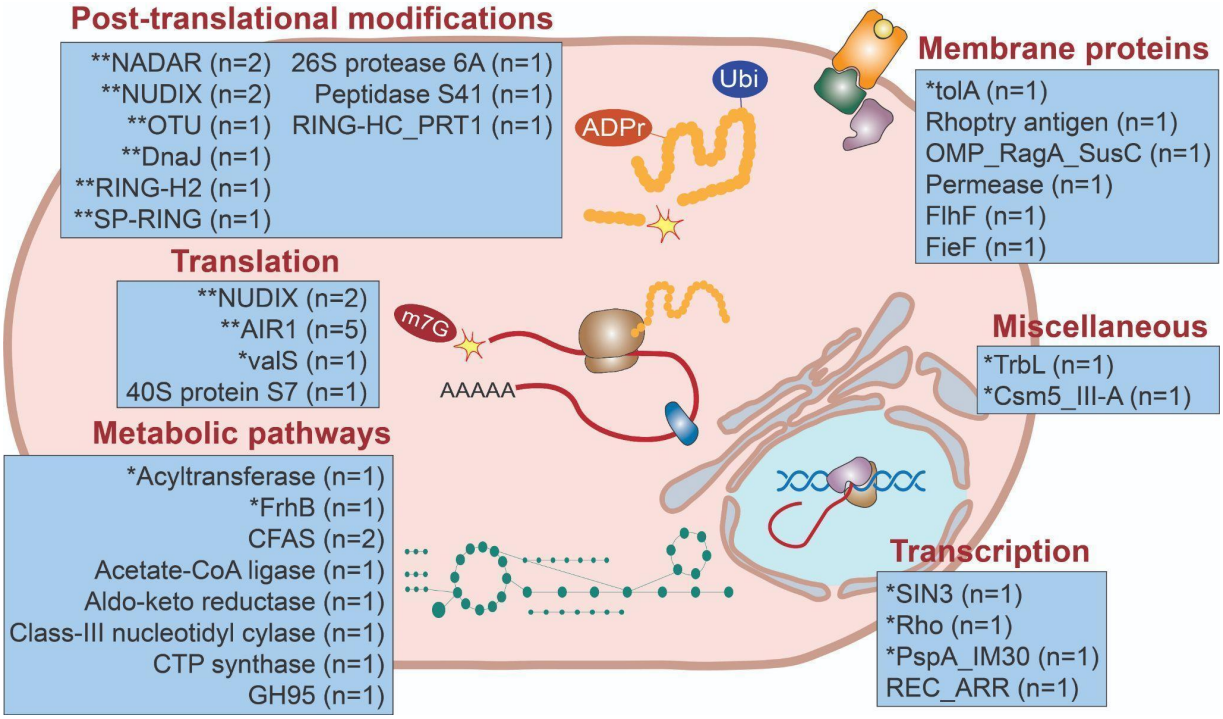

Figure 5. Functional diversity of AMGs carried by Stordalen Mire orthornaviraens. Schematic representation of the hypothesized effects of roles played in the manipulation of host metabolism by orthornaviraen AMGs, which are separated according to functional categories (red text) predicted from using solely functional domains (single asterisk, "permissive" set) and motifs within domains as well (double asterisk, "conservative" set). Blue boxes contain the corresponding protein annotation based on the conserved domain database (CDD), with the corresponding number of vOTUs listed in parentheses. Double asterisks indicate that AMGs that were not located at the contig ends and for which function could be identified studying motifs on the CDD, whereas those with single asterisks indicate that AMGs were not located at contig ends and for which functions could not be identified based on motifs. The absence of an asterisk indicates that AMGs were considered "broken" because given that they are located at contig ends, limiting our ability to predict their functions. AMGs that were found in viral contigs of 500–1,000 nt (**Table S8**) are not reported in this figure. RING-HC_PRT1, RING finger, HC subclass, found in thale cress (*Arabidopsis thaliana* (L.) Heynh.)) proteolysis 1 protein; valS, valine-tRNA ligase; FrhB, coenzyme F420 hydrogenase subunit beta; CFAS, cyclopropane fatty acid synthetase; GH95, glycoside hydrolase family 95; TolA, Tol proteins necessary for the uptake of all the group A colicins; TonB-linked outer membrane protein SusD/RagB family; FlhF, flagellar biosynthesis protein; FieF, divalent metal cation (Fe/Co/Zn/Cd) transporter; TrbL, conjugal transfer protein TrbL; Csm5_III-A, CRISPR system type III-A Csm effector Csm5; SIN3, transcriptional regulatory protein SIN3; Rho, transcription termination factor Rho; PspA_IM3, PspA a protein that suppresses σ^{54}-dependent transcription; REC_ARR, phosphoacceptor receiver domain of type B Arabidopsis response regulators; NADAR, *Escherichia coli* swarming motility protein YbiA and related proteins; NUDIX, hydrolases of Nucleoside Diphosphate linked to another moiety (X); AIR1, arginine methyltransferase-Interacting RING finger protein.