Extracellular vesicle-mediated export of fungal RNA

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Extracellular vesicles (EVs) play an important role in the biology of various organisms, including fungi, in which they are required for the trafficking of molecules across the cell wall. Fungal EVs contain a complex combination of macromolecules, including proteins, lipids and glycans. In this work, we aimed to describe and characterize RNA in EV preparations from the human pathogens Cryptococcus neoformans, Paracoccidioides brasiliensis and Candida albicans, and from the model yeast Saccharomyces cerevisiae. The EV RNA content consisted mostly of molecules less than 250 nt long and the reads obtained aligned with intergenic and intronic regions or specific positions within the mRNA. We identified 114 ncRNAs, among them, six small nucleolar (snoRNA), two small nuclear (snRNA), two ribosomal (rRNA) and one transfer (tRNA) common to all the species considered, together with 20 sequences with features consistent with miRNAs. We also observed some copurified mRNAs, as suggested by reads covering entire transcripts, including those involved in vesicle-mediated transport and metabolic pathways. We characterized for the first time RNA molecules present in EVs produced by fungi. Our results suggest that RNA-containing vesicles may be determinant for various biological processes, including cell communication and pathogenesis.

The extracellular release of macromolecules is essential for a number of physiological events in fungal cells, including nutrition, intercellular communication, biofilm formation and, in pathogenic fungi, activation of immune cells.¹,² Fungal cells are encased within a dense cell wall, so mechanisms are required for the transport of molecules across the wall for extracellular release in these organisms. In recent years, a number of studies have shown that the trafficking of molecules across the fungal cell wall requires extracellular vesicles (EVs).³–¹⁸

Fungal EVs contain a complex combination of macromolecules, including many proteins, neutral lipids, glycans and pigments.³,⁴,⁷,⁹,¹⁴,¹⁵,¹⁸ This complex molecular mixture, including many cytoplasmic components, is consistent with the proposed origin of fungal EVs as cytoplasmic subtractions.¹⁹ This would suggest that the complexity of the composition of fungal EVs may be underestimated, because it is not possible to rule out the presence of other macromolecules present in the cytoplasm. Fungal and mammalian EVs have similar morphological features and compositions.²⁰ Mammalian EVs efficiently transport mRNA and other macromolecules to the extracellular space.¹ Interestingly, mRNAs from mammalian EVs can be translated into proteins by target cells.²¹,²² It has recently been shown that EVs also contain small noncoding RNA species.²²,²³, EV-associated RNA produced by pathogens could affect the physiology of host cells, as suggested by experiments with mammalian EV RNA.²⁴,²⁵

The presence of RNA in fungal EVs was suggested by the findings of a single flow-cytometry study focusing on the human pathogen Cryptococcus neoformans.² However, the structural aspects of EV-associated fungal RNA remain to be explored. In this study, we selected four species of EV-producing fungal cells for an analysis of the presence of RNA sequences. Our results for the model yeast Saccharomyces cerevisiae and the human pathogens C. neoformans, Paracoccidioides brasiliensis, and Candida albicans, demonstrated that fungal cells use EVs to export RNA molecules of different natures and with different biological functions.

Results and Discussion

Comparative transcriptomic analysis of fungal extracellular vesicles (EVs). Total RNA was isolated from fungal EVs in three independent biological replicates. The RNA was then fractionated into samples enriched...
in small RNAs (less than 200 bp) and mRNAs. The small RNA-enriched fractions were then analyzed, to determine their molecular size distribution (Fig. 1A). EV small-RNA fractions consisted of molecules of various sizes, mostly fewer than 250 nucleotides long. One preparation obtained from *P. brasiliensis* Pb18 illustrate this profile (Fig. 1A), and similar observations were made for all the species studied. We conclude that fungal EVs carry small RNA molecules with similar size distributions.

We checked that RNA molecules were not loosely aggregated with fungal EV rather than being packaged within these vesicles, by subjecting control samples to RNAse treatment before RNA extraction (Fig. 1B). The small-RNA profile of RNAse-treated EVs was not modified, confirming that the small RNA molecules were within the vesicles, which protected them from hydrolysis. We also assessed RNase activity, by adding exogenous, total fungal RNA EV preparations before RNase treatment. The total RNA was degraded, consistent with functional RNase-mediated hydrolysis (Fig. S1A to D).

Genome RNA EV mapping statistics are listed in Table 1. For *C. neoformans* samples, about 90% of the RNA EV reads mapped to intronic regions, the remaining 10% mapping to exons, in mature mRNA. A similar profile was clearly observed in *P. brasiliensis* samples, in which 17% of the reads mapped to intronic regions and 82% mapped to exons. However, 21% of the reads in *P. brasiliensis* mapped to exon-intron regions, versus less than 1% of the mapped reads in *C. neoformans*. In *S. cerevisiae* and *C. albicans*, about 90% of the reads mapped to exons. This observation is consistent with the introns lower number in these species [26,27], by contrast to *P. brasiliensis* and *C. neoformans*.

Genome RNA EV mapping information is illustrated in Figure 2. Reads mapping to an entire mRNA (Fig. 2A), intergenic (Fig. 2B) and intronic regions (Fig. 2C), or aligning with specific positions in the
mRNA (Fig. 2D) were observed for all species studied. This suggests that, in addition to the small RNA molecules previously observed, mRNAs are also present in fungal EVs. Most of the reads aligning with intergenic and intronic regions were found to be in the reverse orientation, complementary to the transcript (Fig. 2D). This pattern is characteristic of sequences processed by the small-RNA interference (RNAi) machinery.

**Small RNA characterization: miRNA-like sequences**. For the identification of conserved miRNA sequences among the small RNAs identified in EVs from *P. brasiliensis*, *C. neoformans*, *C. albicans*, and *S. cerevisiae*, we used a database of mature miRNA sequences from all organisms characterized to date (http://www.mirbase.org/). We identified a total of 1,246 conserved miRNA-like sequences, only 20 of which were common to all four fungal species studied (Table 2). The total numbers of miRNAs identified were 145 (1,953 mean reads) for *P. brasiliensis*, 344 (4,796 mean reads) for *C. neoformans*, 423 (2,477 mean reads) for *C. albicans*, and 532 (4,349 mean reads) for *S. cerevisiae* (Table S1). We then used Baggerly’s test to compare the levels of miRNA-like molecules in EVs among the four species. We found that 47 of these molecules were differentially distributed (Fig. 3). *C. neoformans* had the largest number of overrepresented sequences (16), followed by *C. albicans* (8), *P. brasiliensis* (12), and *S. cerevisiae* (1). We also identified five miRNA-like sequences exclusive to *P. brasiliensis* and one exclusive to *S. cerevisiae*. The distribution of miRNA-like molecules was more similar in *C. neoformans* and *C. albicans* EV, although the values for *C. neoformans* were considerably higher for most sequences (Fig. 3).

Five miRNA-like sequences were exclusive to *P. brasiliensis* EVs (Fig. 3). One of them, dre-miR-125a-2, belongs to the miR-125 family, which is highly conserved in eukaryotes and is involved in many different cellular processes, including cell proliferation, differentiation and apoptosis, through the targeting of different transcription factors, matrix-metalloproteases, growth factors, and nonsense-mediated mRNA decay pathway. In addition, miR-125 controls the differentiation of immune cells, thereby affecting responses to bacterial and viral infection.

Many fungal species have RNAi machinery, which may be non-canonical in *Schizosaccharomyces pombe*, for e.g., or absent from others (e.g. *S. cerevisiae*), and functional in most filamentous fungi. *C. neoformans* bears a fully functional RNAi machinery.

### Table 1 | Sequence mapping statistics

|          | *C. neoformans* | *C. albicans* | *P. brasiliensis* | *S. cerevisiae* |
|----------|-----------------|---------------|-------------------|-----------------|
| Exon-exon| 5029.5          | 10664         | 113654.5          | 1003971         |
| Exon-intron| 10664          | 43            | 0                 | 90.3            |
| Total exon| 113654.5        | 13853         | 281083            | 1117625         |

### Figure 2 | Genome RNA EVs mapping (A), high-coverage exon; (B) intron; (C), intergenic regions; (D) low-coverage exon. Gray bar, genomic region, with length in bp, shown above; black lines above the reads, consensus; vertical columns, number of sequences; yellow bars, exons (access codes shown); blue bars, introns. For the sequence colors: forward reads, red; reverse reads, green and non-specific match, yellow.
identifying roles in at least two species. In mammalian cells, the transcription machinery accounts for 8% of the sequences and rRNAs accounting for 13%. The reads were fairly evenly distributed between snoRNAs and tRNAs. Only eight long ncRNA species were identified in our samples which accounted for 22 to 75% of all reads (Fig. 4).

The ncRNA groups most frequently represented in all fungal EV preparations, many of which were specifically associated with fungal EV preparations. We identified a total of 114 different ncRNA sequences and the RNAi machinery includes a noncanonical Dicer, and the RNA-dependent RNA polymerase (Dicer and the RNA-dependent RNA polymerase). In this organism, RNAi-dependent mechanisms are involved in the sex-induced silencing of transgenes during the sexual stage of the fungus. In addition, C. neoformans miRNA and pre-miRNA may be similar to their mammalian counterparts. In C. albicans, the RNAi machinery includes a noncanonical Dicer, and small-RNA molecule preparations from this species are enriched in 22-mer sequences. The occurrence of RNAi machinery in different fungal pathogens suggests a possible role for miRNA-like molecules during with the interaction of fungi with their host cells, possibly involving the mimicking of endogenous miRNA. This putative mechanism might regulate gene expression in host cells and modify sensitivity to infection. Further investigations, determining the roles of small RNAs in fungal EVs, are required to test this hypothesis.

Identification of non-coding RNAs (ncRNAs). Our analysis of RNA sequences in fungal EVs included classes of non-coding RNA other than miRNAs. These classes were identified with the use of ncRNA data from the Saccharomyces Genome Database as a template. We identified a total of 114 different ncRNA sequences in EV preparations, many of which were specifically associated with C. neoformans (46). P. brasiliensis (38), C. albicans (68), or S. cerevisiae (106). Eleven ncRNAs were common to all four fungal species (Table 3 and Table S2): one tRNA, two snRNAs, two rRNAs and six snoRNAs. None of the roles of these ncRNAs had previously been characterized during host-parasite interactions.

The ncRNA groups most frequently represented in all fungal EV preparations were small nuclear RNAs (snRNAs) and tRNAs, which accounted for 22 to 75% of all reads (Fig. 4). S. cerevisiae had a high proportion of snRNA (75%), whereas tRNAs accounted for 60% of all reads in C. albicans. In C. neoformans, for the most part, reads were fairly evenly distributed between snRNAs and tRNAs. P. brasiliensis had a slightly different profile, with long ncRNAs accounting for 8% of the sequences and rRNAs accounting for 13%.

Only eight long ncRNA species were identified in our samples (Table S2). None was common to all fungal EVs, but most were identified in at least two species. In mammalian cells, the transcription of several long ncRNAs is regulated by factors critical for mammalian development. This suggests possible key roles in development for these ncRNAs, through the regulation of protein synthesis by mechanisms independent of the Dicer RNAi pathway. Table S2 highlights the involvement of ICR1, a cis-interfering long intergenic ncRNA that regulates transcription of the FLO11 locus in S. cerevisiae. ICR1 and PWR1 form a circuit with a bidirectional toggle, including the upstream signaling pathway transcription factors Str1 and Flo8, and the chromatin remodeler Rpd3L, which is essential for phenotypic transitions in yeast. The ncRNA RPR1 was identified in S. cerevisiae EV samples. It interacts with nine other proteins to form the ribonuclease P (RNase P) complex, a ubiquitous endonuclease that cleaves precursor tRNAs to generate mature 5’ termini. RNase P is also involved in the turnover of normally unstable nuclear RNA. The RNA component of mitochondrial RNase P, RPM1, was found in EVs from three fungal species. Another component of a mitochondrial RNase – NME1 – was found in EV samples from C. albicans and S. cerevisiae. NME1 is a subunit of the essential ribonucleoprotein endoribonuclease, RNase MRP, which is required for the processing of pre-rRNA. In vitro, it promotes the cleavage of pre-5.8S RNA and the degradation of specific mRNA sequences involved in cell cycle regulation.

snRNAs constitute a small group of highly abundant, non-polyadenylated ncRNA transcripts present in the nucleoplasm. They form the core of the spliceosome and catalyze the removal of introns from pre-mRNA. Four different snRNAs were found in fungal EVs (Table S2). LSR1 and snR19 were present in all species, snR6 was absent from P. brasiliensis, and snR14 was observed only in C. albicans and S. cerevisiae. As mentioned above, 72 different snoRNA species were found in fungal EVs (Table S2). These RNAs can be classified into two groups on the basis of sequence motifs and secondary structures: C/D and H/ACA boxes, which are involved in the methylation and pseudouridylation of mammalian rRNA nucleotides, respectively. They may also play a role in RNA modification, participating in the control of expression. The snoRNAs also guide rRNA nucleotide modifications and participate in pre-rRNA cleavage. A few snoRNAs, such as snR10, have both functions. This molecule is required for normal cell growth, in addition to pre-rRNA processing in S. cerevisiae. It was found in EVs from all species except C. neoformans. Other snoRNAs identified included U3 (SmnR7a/b), U14 (SmnR128), and snR30, which are essential for eukaryotic growth. The collection of snoRNAs found in fungal EVs included snR191RNA, which has been reported to support cell growth in S. cerevisiae, whereas the snR70, snR71, SnR65, and SNR68 mutants display growth defects.

**Table 2 | miRNA-like common sequences from P. brasiliensis, C. neoformans, C. albicans, and S. cerevisiae EVs.** Means of normalized values are shown.

| Feature ID | C. albicans Mean | C. neoformans Mean | P. brasiliensis Mean | S. cerevisiae Mean |
|------------|------------------|---------------------|---------------------|-------------------|
| Aau-MIR172 | 118.37           | 732.16              | 217.01              | 17.58             |
| aly-MIR159c| 353.98           | 1852.68             | 434.03              | 116.95            |
| aly-MIR408 | 707.96           | 905.99              | 840.84              | 8.79              |
| ats-MIR172 | 3643.60          | 2667.29             | 1369.77             | 512.57            |
| cin-mir-4000c| 3539.19          | 961.54              | 217.01              | 108.15            |
| cre-MIR909 | 101.07           | 133.12              | 623.83              | 116.95            |
| cre-MIR916 | 8351.86          | 4633.87             | 311.92              | 604.06            |
| gma-MIR408b| 1181.44          | 2107.91             | 434.03              | 76.93             |
| mmu-mir-5102| 15297.93         | 8184.08             | 217.01              | 6716.77           |
| mmu-mir-5110| 1230.16          | 1923.08             | 311.92              | 116.95            |
| osa-MIR156f| 1805.63          | 3494.66             | 623.83              | 935.94            |
| osa-MIR408 | 202.14           | 1172.23             | 311.92              | 125.74            |
| pea-MIR2916| 2899.44          | 3110.15             | 1871.49             | 12065.74          |
| pta-MIR1310| 293384.35        | 116870.19           | 153791.50           | 147045.22         |
| pvu-MIR166a| 118.37           | 2536.97             | 311.92              | 610.65            |
| sbi-MIR396c| 1314.85          | 13800.48            | 651.04              | 316.95            |
| sha-mir-710a| 91615.15         | 126265.66           | 2699.07             | 47842.09          |
| sha-mir-716b| 168637.59        | 326375.49           | 642856.87           | 665774.04         |
| sme-mir-2152| 51962.07         | 110498.84           | 528.93              | 4609.00           |
| smo-MIR1082a| 1267.48          | 20639.54            | 31127.89            | 356.52            |
It is tempting to speculate that ncRNAs present in the *P. brasiliensis*, *C. neoformans*, *C. albicans*, and *S. cerevisiae* EV preparations may be involved in intercellular regulation, mostly through the regulation of protein synthesis.

The tRNAs constitute an interesting group of ncRNAs. The EVs of the fungal species studied containing 22 to 60% tRNAs, depending on the species analyzed (Fig. 5 and Table S2). In the protozoan parasite *Trypanosoma cruzi*, which has no RNAi machinery molecules, a PIWI-like protein has been characterized. However, the cells of this parasite contain a homogeneous population of small RNAs derived from mature tRNAs, known as tsRNAs. tsRNAs are produced by an alternative small RNA pathway. Recent studies have shown that these molecules are packaged into vesicles that are shed by the parasite and can be transferred to other parasites and/or host cells. The cargo of these vesicles can change the expression profile of the host cells, leading to changes in the cytoskeleton and extracellular matrix, increasing susceptibility to infection. We detected fragments of mature tRNAs in the EVs of all the fungal species studied (Table 3 and Fig. 5). Fungal EVs were enriched in nuclear and mitochondrial tRNAs, which are not the most abundant population in the cell (Table 3). Specific enrichment in the 3' region of the tsRNA was also observed (Fig. 5). This scenario is illustrated by the situation for arginine tRNA (CCU), which is present at a relatively low concentration in cellular compartments. This tRNA, which was found in fungal EVs, is required for growth on non-fermentable carbon sources at high temperatures, for the synthesis of the heat shock protein Ssc1p, and for Ty1 retrotransposition, through the regulation of translational frameshift. Our observations indicate that fungal EVs contain tsRNAs that might modify and affect gene expression in host cells.

**mRNA identification.** The sequencing of EV-associated small RNA-enriched fractions led to the identification of 253 different mRNAs in *C. albicans*, 84 in *S. cerevisiae*, 353 in *C. neoformans*, and 73 in *P. brasiliensis* (Table S3). The ten most abundant mRNAs from each species found are listed in Table 4. These sequences were obtained from high-coverage sRNAs (Fig. 1A), suggesting that molecules of more than 200 nt were copurified despite the use of a strategy favoring the purification of small RNAs. We validated the mRNA sequencing data obtained for fungal EVs, by performing RT-qPCR on two representative sequences corresponding to exons with and without high coverage rates (Fig. S2). The RNA-seq data demonstrated that the EV mRNA molecules corresponded to 1–2% of those found in intracellular compartments. The most abundant EV mRNAs differed from the most abundant cellular transcripts, consistent with regulated compartmentalization for extracellular export. This observation is exemplified by the ASH1 mRNA from *S. cerevisiae*, which had an RPKM value of more than 200 in vesicles. This mRNA is not particularly frequent in total cellular mRNA, and is produced preferentially during cell budding. Other examples include mRNAs required for the synthesis of the fatty acid desaturase Ole1, the heat-shock proteins Hsp104, Hsp82 and Hsp70, the regulator of endochitinase (CTS1) expression Nce2m, glyoxalate pathway regulators, a putative estradiol-binding protein, and a putative sterol transporter from *P. brasiliensis*. All these mRNAs are upregulated in yeast cells, but all were absent from EV preparations. The transcripts encoding urease and the cell division control protein CDC42, which may be involved in the pathogenicity of *P. brasiliensis*, were identified in the vesicles. In *C. albicans*, the most abundant vesicular mRNAs were the CYB5 transcript (RPKM > 12,000), which encodes cytochrome b, and the *RTT109* transcript.
encoding histone acetyltransferase. These transcripts are present at a relatively low abundance in *C. albicans* cells.

Gene ontology analysis revealed enrichment in RNA molecules relating to specific cellular processes in fungal EVs. Some of these processes, such as vesicle-mediated transport and metabolic pathways, were common to all four species analyzed in this study (Fig. 6). Other processes were limited to two species. For example, mycelium development and filamentous growth were restricted to *C. albicans* and *C. neoformans* (Fig. 6). Similarly, EV samples from *C. neoformans* and *P. brasiliensis* were enriched in mRNAs relating to cellular responses to stress, whereas those from *C. neoformans* and *S. cerevisiae* were enriched in mRNAs involved in vesicle-mediated transport (Fig. 6). These observations indicate that fungal EVs carry mRNAs common to different species, but they may also carry molecules restricted to one or two species. The biological significance of this finding is unknown.

We compared vesicular mRNA sequences from the four species studied, to identify sequences that were species-specific and characteristics common to EV preparations from *S. cerevisiae*, *C. neoformans*, *P. brasiliensis*, and *C. albicans*. In *C. neoformans*, the most abundant mRNAs with sequences corresponding to known functions (RPKM > 3,500) were those encoding citrate synthase, ubiquitin-activating enzyme, succinate dehydrogenase, VpsA (vacuolar protein sorting A), L7 ribosomal protein, ATP-binding cassette transporter, cellulase and the AGC/AKT protein kinase associated with the TOR complex. In *P. brasiliensis*, the most abundant transcripts with annotated functions corresponded to cell division control proteins, translation initiation factor RLI 1 and nucleotide-binding proteins. Most of the mRNAs identified in *C. albicans* encoded hypothetical proteins, but those encoding cytochrome b5, histone acetyltransferase, cell division control protein (CDC43) and arginine biosynthetic process 1 were also identified. Only three of the transcripts in *S. cerevisiae* samples had a RPKM value greater than 1,000; the most abundant mRNA were those encoding Myb-related transcription factor, the Dam-1 complex associated with cell division, a cyclin involved in cell cycle progression, a pheromone-regulated protein associated with mating, a component of autophagosomes, pre-mRNA splicing and inhibitor of HO transcription.

Many of the mRNA sequences found in EV preparations had a higher abundance than expected from genomic data (Table 5). For instance, in *S. cerevisiae*, vesicular mRNAs from the cellular protein modification process (2.18-fold) and small molecule metabolic process (1.82-fold) categories were more frequent than would be expected given the relative distribution of the corresponding genes in the genome (Table 1). Similar findings were obtained for the categories chromatin repression by histone regulators (5-fold) and mitosis regulator (2-fold) in *P. brasiliensis*. In *C. albicans*, vesicle samples were enriched in sequences required for vesicle-mediated transport (2.43-fold), catabolic processes (2.1-fold), and transport

### Table 3 | ncRNA sequences identified in EV preparations from *C. neoformans* (Cn), *P. brasiliensis* (Pb), *S. cerevisiae* (Sc), and *C. albicans* (Ca)

| RNA   | Ca | Cn | Pb | Sc | Type                | Function                                                                 |
|-------|----|----|----|----|---------------------|--------------------------------------------------------------------------|
| 15S_rRNA | X  | X  | X  | X  | rRNA mitochondrial  | MSU1 allele suppresses ochre stop mutations in mitochondrial protein-coding genes |
| 21S_rRNA | X  | X  | X  | X  | intron encodes the lScel DNA endonuclease          |
| snR128 | X  | X  | X  | X  | snoRNA              | C/D box small nucleolar RNA                                               |
| snR17a | X  | X  | X  | X  | snoRNA              | small nucleolar RNA U3, part of Small ribosomal SubUnit (SSU) processosome |
| snR36  | X  | X  | X  | X  | snoRNA              | H/ACA box small nucleolar RNA                                             |
| snR61  | X  | X  | X  | X  | snoRNA              | C/D box small nucleolar RNA                                               |
| snR69  | X  | X  | X  | X  | snoRNA              | C/D box small nucleolar RNA                                               |
| snR76  | X  | X  | X  | X  | snoRNA              | C/D box small nucleolar RNA                                               |
| LSR1   | X  | X  | X  | X  | snoRNA              | U2 spliceosomal RNA                                                       |
| snR19  | X  | X  | X  | X  | snoRNA              | U1 spliceosomal RNA                                                       |
| tR(CCUJ) | X | X | X | X | tRNA | Arginine tRNA; low abundance tRNA required for growth on nonfermentable carbon sources |

Figure 4 | Distribution of noncoding RNA species (other than miRNA-like species) in fungal EVs. The percentages of each RNA species indicated on the x-axis are expressed relative to total RKPM. Pb, *P. brasiliensis*; Cn, *C. neoformans*; Ca, *C. albicans*; Sc, *S. cerevisiae.*
Figure 5 | Schematic graphs of reads aligning specifically with the 3' end of the arginine tRNA(CCU)J identified in EVs from *P. brasiliensis*, *C. neoformans*, *C. albicans*, and *S. cerevisiae*. Coverage is indicated on the y-axis and alignment position is shown on the x-axis.
RNA. The implications of these findings for fungal physiology and pathogenesis remain unclear, but the presence of RNA in fungal EVs is consistent with the hypothesis that the vesicle-mediated export of nucleic acids interferes with gene expression in host and fungal cells. The presence of different RNA classes in fungal EVs opens up new possibilities for investigating the ways in which fungal cells communicate and respond to external stimuli. Our results suggest that the RNA exported by fungi may interfere with the physiology of host tissues.

**Methods**

Fungal strains and growth conditions. The *P. brasiliensis* isolate used in our study was Pb18, which was recovered from the lungs of BALB/c mice after experimental infection. Eight-week-old male BALB/c were obtained from the Center for Development of Experimental Models (CEDEME), at Universidade Federal de São Paulo (UNIFESP). Animals were kept in ventilated isolators at 22 ± 2°C and 55 ± 10% humidity. All animal manipulations were made in compliance with the protocols approved by Federal University of São Paulo Ethics Committee for Animal Experimentation (project approval number 366/07). Pb18 cells were maintained in their standard strain H99 (serotype A), which was maintained at 30°C.

**Table 4 | mRNA sequences identified in *P. brasiliensis*, *C. neoformans*, *C. albicans*, and *S. cerevisiae* EVs**

| Species          | Feature ID   | Product                                      | Means normalized RPKM |
|------------------|--------------|----------------------------------------------|------------------------|
| C. albicans      | orf19.6834.10| Ortholog of *S. cerevisiae* Tar1p             | 415068.82              |
|                  | orf19.1742   | Hydroxymethylbilane synthase                 | 12255.92               |
|                  | orf19.6140   | Protein with similarity to ferric reductases  | 9716.90                |
|                  | orf19.6336   | Putative GPI-anchored adhesin-like protein    | 1279.89                |
|                  | orf19.5628   | Mitochondrial dicarboxylate transporter       | 1784.62                |
|                  | orf19.3540   | Putative nucleolar DEAD-box RNA helicase      | 1058.57                |
|                  | orf19.101    | Protein required for alkaline pH response     | 1389.34                |
|                  | orf19.5284   | CAWG_01866                                    | 983.61                 |
|                  | orf19.7073   | YCL002C                                       | 947.91                 |
|                  | orf19.1661   | RNA helicase activity, translational termination | 15.58                 |
| C. neoformans    | CNAG_06164   | Hypothetical protein                          | 12715.06               |
|                  | CNAG_03379   | N-acetyltransferase 5                         | 9142.90                |
|                  | CNAG_03382   | Hypothetical protein                          | 6224.73                |
|                  | CNAG_06220   | Allergen                                      | 4180.13                |
|                  | CNAG_02724   | Hypothetical protein                          | 3374.93                |
|                  | CNAG_06101   | Eukaryotic ADP/ATP carrier                    | 2070.80                |
|                  | CNAG_03667   | Hypothetical protein                          | 3112.63                |
|                  | CNAG_01052   | Hypothetical protein                          | 1875.03                |
|                  | CNAG_01743   | Hypothetical protein                          | 1880.59                |
|                  | CNAG_06646   | ypt interacting protein                       | 1574.72                |
| P. brasiliensis  | PADG_06364   | Hypothetical protein                          | 160376.61              |
|                  | PADG_08536   | Conserved hypothetical protein                | 141933.83              |
|                  | PADG_03535   | Nucleotide-binding protein                    | 37239.82               |
|                  | PADG_07966   | ser/Thr protein phosphatase family protein    | 15709.62               |
|                  | PADG_02959   | Predicted protein                             | 4210.27                |
|                  | PADG_08534   | Hypothetical protein                          | 5423.77                |
|                  | PADG_01121   | Conserved hypothetical protein                | 4743.93                |
|                  | PADG_06655   | Coatomer subunit delta                        | 3029.63                |
|                  | PADG_01377   | Hypothetical protein                          | 2335.88                |
|                  | PADG_08533   | Conserved hypothetical protein                | 1293.54                |
| S. cerevisiae    | BAS1         | Myb-related transcription factor              | 2577.53                |
|                  | CLB3         | B-type cyclin involved in cell cycle progression | 1074.90               |
|                  | PRM4         | Phromerone-regulated protein involved in mating | 932.26                |
|                  | DAD2         | Essential subunit of the Dam1 complex         | 886.83                 |
|                  | ATG8         | Component of autophagosomes and Cvt vesicles  | 346.86                 |
|                  | TAO3         | Component of the RAM signaling network        | 340.95                 |
|                  | YIU2         | Essential protein required for pre-mRNA splicing | 278.14                |
|                  | ASH1         | Zinc-finger inhibitor of HO transcription     | 272.97                 |
|                  | PAN1         | Part of actin cytoskeleton-regulatory complex | 219.88                 |
|                  | YKR023W      | Putative protein of unknown function          | 199.01                 |

(2.09-fold). The EVs of *C. neoformans* were enriched in mRNAs corresponding to the transport (3.61-fold), cytoskeleton organization (3.06-fold), signal transduction (2.37-fold), and vesicle-mediated transport (1.94-fold) categories (Table 5).

We then compared the RNA content of extracellular vesicles with that of other vesicles from the species studied. The analysis was performed with OrthoMCL software, which uses the Markov Clustering (MCL) algorithm designed to group orthologous sequences on a genome-wide scale. It identifies molecules orthologous in different species, and functionally redundant or "recent", paralogs within species. Most of the RNAs identified were species-specific: 70% were exclusive to *S. cerevisiae*, 89% to *P. brasiliensis*, 91% to *C. neoformans*, and 92% to *C. albicans* (Fig. 7). None of the mRNA sequences were common to all fungi, but we identified orthologs in two or three species. The largest numbers of orthologs were found in *C. neoformans* and *S. cerevisiae* (16) and in *C. neoformans* and *C. albicans* (11 RNAs). The small numbers of orthologs common to several species may reflect the characteristics of each species and of the RNA cargo, which plays a specific role in each fungus. This result differs from the observations made during proteomic comparisons of the vesicles, for which a core set of proteins was found to be common to different fungal species.

Conclusions. We characterized EVs as biological carriers of fungal RNA. The implications of these findings for fungal physiology and pathogenesis remain unclear, but the presence of RNA in fungal EVs is consistent with the hypothesis that the vesicle-mediated export of nucleic acids interferes with gene expression in host and fungal cells. The presence of different RNA classes in fungal EVs opens up new possibilities for investigating the ways in which fungal cells communicate and respond to external stimuli. Our results suggest that the RNA exported by fungi may interfere with the physiology of host tissues.
used to inoculate minimal medium composed of dextrose (15 mM), MgSO₄ (10 mM), KH₂PO₄ (29.4 mM), glycine (13 mM), and thiamine-HCl (3 mM), and were cultured for three days at 30°C, with shaking. S. cerevisiae (strain SEY6210) cells were maintained at 25°C for 24 h, on Sabouraud dextrose plates. For EV isolation, yeast cells were transferred to Sabouraud dextrose broth and cultured for an additional 24 h at 25°C, with shaking. Strain 11 of C. albicans was isolated from a

Figure 6 | Gene Ontology (GO) function profile of proteins corresponding to the high-coverage RNA sequences identified in the sRNA-enriched fractions isolated from EVs from P. brasiliensis, C. neoformans, C. albicans, and S. cerevisiae. The number of hits identified for each term is indicated on the x-axis.

Table 5 | Gene Ontology (GO) function profile from P. brasiliensis, C. neoformans, C. albicans, and S. cerevisiae EVs. * p-value < 0.05

| Sp       | GO – Cellular Process | EVs/genome ratio* |
|----------|-----------------------|-------------------|
| C. neoformans | Growth                | 3.61              |
|           | Cytoskeleton organization | 3.06              |
|           | Signal transduction    | 2.37              |
|           | Homeostatic process    | 1.97              |
|           | Vesicle-mediated transport | 1.94              |
|           | Cell cycle              | 1.9               |
|           | Protein targeting       | 1.69              |
|           | Reproduction            | 1.57              |
|           | Cellular protein modification | 1.35           |
|           | Vesicle-mediated transport | 2.43              |
|           | Catabolic process       | 2.1               |
|           | Transport                | 2.09              |
|           | Cellular protein modification | 1.7              |
|           | Cellular component assembly | 1.63              |
|           | Cell cycle              | 1.41              |
| C. albicans | Small molecule metabolic process | 1.82 |
|           | Ribosome biogenesis     | 1.73              |
|           | Transport                | 1.61              |
|           | Response to stress      | 1.54              |
|           | Cellular nitrogen compound metabolic process | 1.41 |
male patient4 and kindly provided by Dr. Dornelas from the Institute of Hematology.

**Genomics Workbench**

Data analysis

**In silico RNA sequencing**

We used 100 ng of purified sRNA for RNA-seq from three control genes in EV preparations, a standard curve based on amplified DNA molecules was estimated with a tool available from http://www.sciencelauncher.com/mwcalc.html site. We carried out qPCR with the Fast SYBR® Green Master Mix (Applied Biosystems), according to the manufacturer’s instructions, in a 7500 Fast Real-Time PCR System (Applied Biosystems). Cycling conditions were as follows: 2 minutes at 50 °C, 1 minute at 95 °C, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 minute. An additional cycle of 95 °C for 15 s and 60 °C for 20 s, and 95 °C for 15 s was performed, to determine the dissociation curve at the end of the reaction. The standard curve for absolute quantification from genomic DNA, based on decreasing known numbers of copies (10^1, 10^2, 10^3 and 10^4 theoretical copies), was constructed in parallel, from total cell and EV RNA reactions. Measurements were made in triplicate.

**Data access.** The data have been submitted to the Sequence Read Archive (SRA) database under study accession number (SRA: SRP022849).

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**Author contributions**

R.P.S., D.L.O., L.S.J. and G.V.C.: EV preparation, RNA fractionation, qPCR, RNA data analysis; LRA: RNA-seq and RNA data analysis; R.P.S., R.P., L.N., M.L.R., S.G. and L.R.A.: experimental design, analysis and interpretation of data. R.P.S., R.P. and L.R.A.: preparation of the manuscript. All authors discussed the results, wrote and approved the final manuscript.

**Additional information**

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