Title: Transient compartmentalization of RNA replicators prevents extinction due to parasites

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One sentence summary: Trait group selection in natural compartments would allow survival of a genomic set of unlinked replicators before the evolution of reproducing protocells

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Abstract: The appearance of molecular replicators (molecules that can be copied) was probably a critical step in the origin of life. However, parasitic replicators take over, and would have prevented life from taking off, unless the replicators were compartmentalized in reproducing protocells. Paradoxically, control of protocell reproduction would seem to require evolved replicators. We show here that a simpler population structure, based on cycles of *transient* compartmentalization (TC) and mixing of RNA replicators, is sufficient to prevent takeover by parasitic mutants. TC tends to select for ensembles of replicators that replicate at a similar rate, including a diversity of parasites that could serve as a source of opportunistic functionality. Thus TC in natural, non-biological compartments could have allowed life to take hold.

Main text: The earliest molecular replicators (*1, 2*) must have been plagued by freeloading parasitic replicators (*3-6*). For example, when the RNA genome of the Qβ virus was replicated *in vitro* using the viral replicase, 83% of the genome was deleted due to selection for RNAs with the fastest replication rate (*7*). Eventually, reproducing compartments (protocells) must have arisen, taming parasites by spatially limiting their propagation and allowing group selection at the compartment level, preventing functional collapse (*5, 8-10*). Indeed, serial fusion-division cycles of water-in-oil emulsion

droplets, that function as artificial cell-like compartments (*11*), allows propagation of a compartmentalized replicative cycle catalyzed by Qβ replicase (*12*, *13*).

However, a more rudimentary type of population structure (*14*), in which replicators underwent repeated cycles of mixing and interaction in local groups, similar to Wilson's trait group model (*15*), may have existed before the evolution of reproducing protocells (*9*, *16*, *17*).

Here we demonstrate that repeated cycles of mixing and TC in non-biologial compartments, for example atmospheric aerosol droplets (*18*), microcompartments in hydrothermal vents (*19*), ice eutectic phases (*20*), clusters on mineral surfaces (*21*, *22*), or lipid vesicles (*23*), is sufficient to maintain functional replicators and provides a simple solution to allow the evolution of molecular complexity prior to the appearance of the first protocells.

We used a droplet-based microfluidic system (*24*) to investigate TC (Fig. 1, fig. S1). The functional replicator (MDV-VS RNA) comprises the *trans* Varkud satellite (*trans* VS) ribozyme (*25*), inserted into the midivariant (MDV-1) of Qβ genomic RNA (*26*), which is replicated by Qβ replicase (fig. S2). Replication was studied under three conditions, in duplicate: (1) uncompartmentalized (Bulk), (2) with TC in ~1 million 12 pL (28 μm diameter) droplets (Unselected Compartmentalized), and (3) with TC in ~1 million 12 pL droplets and selection of the droplets based on ribozyme activity (Selected Compartmentalized). In each case, 50 μL of aqueous phase, containing $1.5 \cdot 10^6$ molecules (290 fg) of MDV-VS RNA (corresponding to a Poisson distribution with a mean, λ, of ~0.4 RNA molecules per droplet), was used to start the experiment. Replication was followed using a green fluorescent RNA intercalating dye and hydrolysis of the substrate RNA by the ribozyme was measured simultaneously by the increase in orange fluorescence (fig. S2C and E). After 3h replication, for Bulk and Unselected Compartmentalized experiments, RNA was simply pooled. However, for the Selected Compartmentalized experiments, all droplets with orange fluorescence >150 RFU (containing functional ribozymes) were sorted and RNA from the sorted droplets was pooled. To initiate the next round, 290 fg of purified RNA was inoculated into 50 μL of fresh reagents. This process was repeated for a total of 4 to 9 rounds.

In bulk, MDV-VS RNA disappeared by round 4 (Fig. 2A), as did the corresponding catalytic activity, due to the appearance of parasitic RNAs that are shorter than MDV-VS RNA (figs. S3 to S5), replicate faster (fig. S2C and G) and limited in diversity (fig. S6A).

Without selection for ribozyme activity, compartmentalization slowed down, but did not prevent, the extinction of MDV-VS RNA, which had essentially disappeared by round 7 (Fig. 2B), outcompeted by parasitic RNAs (figs. S3 to S5). The fraction of droplets containing replicated RNA (high green fluorescence, in the "active" and "inactive" gates) that contained active ribozymes (high orange fluorescence, in the "active" gate) fell from >87% in round 1, to <0.05% by round 9 (Fig. 2E, fig. S7A and B), indicating that by round 9 almost all occupied droplets contained parasitic RNAs with no catalytic activity.

However, selecting the compartments based on ribozyme activity prevented extinction of the MDV-VS RNA. By round 9, full-length MDV-VS RNA was still readily observed (Fig. 2C), and >86% of high green fluorescent droplets also showed high orange fluorescence and therefore contained functional replicating ribozymes (Fig. 2F, fig. S7C and D), but a high diversity (fig. S6A) of shorter parasitic RNAs were also present (figs. S3 to S5). The fraction of MDV-VS RNA was dependent on the mean initial number of RNA molecules per droplet, λ: when round 9 (Experiment 1) was performed at λ of 6.9 the fraction of MDV-VS RNA was 0.28, but increased to 0.83 at λ of 0.60 (Fig. 2C), slightly higher than in the previous round (where the fraction of MDV-VS RNA was 0.78 at λ of 4.4).

The ability to purge parasites was confirmed by performing an experiment at $\lambda \approx 0.6$ starting with a 1:4 mixture of MDV-VS RNA and a parasite, cloned from the bulk selection, that replicates 1.46-times faster than MDV-VS RNA (fig. S2C and G). The fraction of MDV-VS RNA rose quickly from 0.19 to reach a plateau of 0.92 by round 3 (Fig. 2D). The

fraction of droplets containing replicated RNA (high green fluorescence) that contained active ribozymes (high orange fluorescence) also increased from ≤10.3% in round 1 to ≥90.6% by round 3 (Fig. 2G, fig. S8A and B). Thus, MDV-VS RNA and parasites converge to equilibrium from above or below the equilibrium position, provided the droplets are selected for catalytic activity.

We can surmise that if catalytically active replicators provide a selective advantage at the compartment level, TC can prevent takeover by parasites and functional collapse. However, despite selection of the compartments, parasitic replicators were not fully purged, and the fraction of parasite is a function of λ.

To better understand the system dynamics and the nature of the parasites we developed a model that takes into account the stochastic first appearance (SFA) of parasites by mutation (see Supplementary Materials). There is a close fit between the experimental and SFA modeling results using experimental λ values (Fig. 2A to D, fig. S6). The model shows that, in bulk (Fig. 3A) and with unselected compartments (Fig. 3B) the parasite with the highest replication rate (1.60 times faster than MDV-VS RNA) takes over, driving MDV-VS RNA to extinction. However, when the compartments are selected, MDV-VS RNA reaches an equilibrium with parasites. The fraction of MDV-VS RNA at equilibrium is a function of λ and it is a parasite with a replication rate only slightly faster than MDV-VS RNA (1.18 times faster) that is most abundant (Fig. 3C, see also fig. S9). The low replication rate of these "soft" parasites allows them to co-exist with co-compartmentalized MDV-VS RNA, which catalyzes enough product formation to allow droplet sorting. Indeed, the enzymatic activity of MDV-VS RNA becomes vital for survival of the "soft" parasites, which function as *bona fide* parasites, not simply RNAs that replicate quickly. However, "hard" parasites, with higher replication rates, are not sorted, because the final fraction of co-compartmentalized MDV-VS RNA (and thus product) is too low. The higher frequency of "soft" parasites in the Selected Compartmentalized experiment than in Bulk was confirmed experimentally (Supplementary Materials and fig. S8C). Similar results were obtained using a deterministic minimum model (Supplementary Materials and fig. S10), indicating that stochastic compartmentalization effects (*27*) are not required to alleviate parasite spread.

The results indicate that, before reproducing protocells, trait group selection based on repeated cycles of TC and mixing could have allowed life on earth to take off. Here, compartments that did not reach the selection threshold were eliminated, but trait group selection would operate whenever transiently arising groups containing a larger number of functional replicators contribute more replicators to the pool seeding the next "generation" of compartments than those containing a larger number of parasites (*9, 16, 17*). This would be the case, for example, if an RNA replicase ribozyme was replicating both itself and parasites. "Soft" parasites that do not "kill" the functionality of the compartments are like well-adapted contemporary parasites that do not kill their host. The persistence of a diversity of "soft" parasites (fig. S6), which have lost their original activity, could be an important source of genetic diversity as long-term selection could turn some parasites into replicators with novel (even surprising) functionality for the group (*28*) in the same way that novel function evolves from gene duplication (*29*). Our results are relevant whether one adheres strongly or weakly to the RNA world (*2*) or not at all, provided there were macromolecular replicators with the possibility of heterocatalytic function.

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Supplementary Materials

Fig. S1 - S12
Caption for parasite sequence database S1

Figures

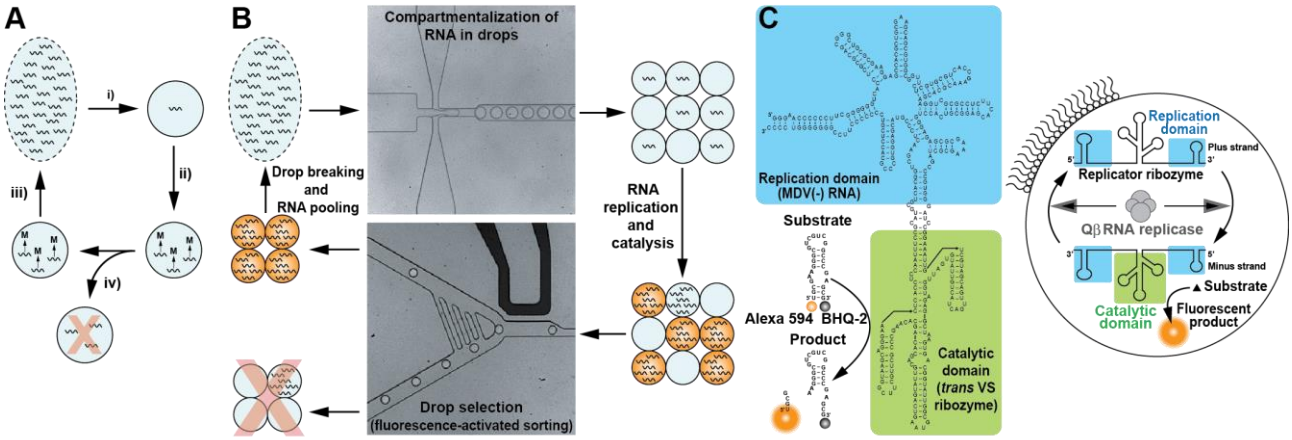

Fig. 1. Primordial TC and its experimental embodiment. (**A**) Primordial RNA molecules are compartmentalized i), replicate in the compartments and ribozyme activity contributes to the "metabolism" (M) of the compartments ii), compartments containing ribozymes that contribute to the "metabolism" pass on more RNAs to the pool iii) than compartments containing parasites iv). (**B**) In the microfluidic system, RNA molecules are compartmentalized in aqueous droplets in an inert carrier oil, replicate in the droplets and catalyze a fluorogenic reaction. Fluorescence-activated droplet sorting (FADS) (*30*) is used to sort droplets containing active ribozymes and the RNA from the sorted droplets is pooled before the next round of selection. (**C**) The *trans* VS ribozyme (*25*), inserted into the minus strand of the midivariant (MDV-1) RNA (*26*), is replicated in droplets using Qβ RNA replicase (*7*). The ribozyme cleaves a non-fluorescent RNA substrate, to generate a fluorescent product.

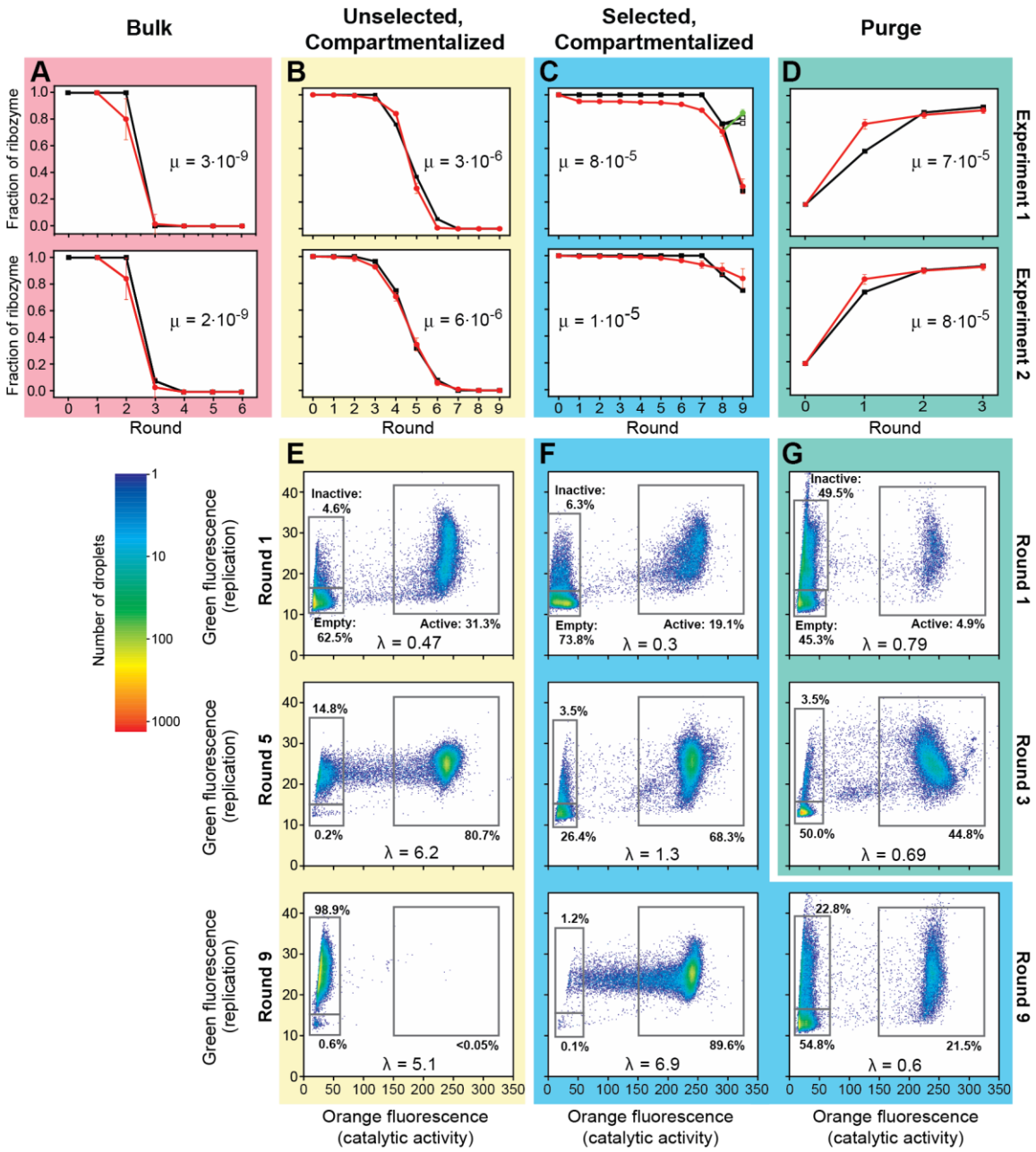

Fig. 2. Survival of functional ribozymes in bulk and with TC in the presence and absence of selection. (**A-D**) Fraction of MDV-VS ribozymes versus round of selection. Experimental results (black squares) are shown for two independent experiments (Experiments 1 and 2) for each condition together with SFA modeling results using experimental λ values (red circles), and mutation rate, μ, used to fit the data. Error bars represent ±1 standard deviation. (**A)** Bulk. (**B**) Unselected Compartmentalized. (**C**) Selected Compartmentalized. Round 9 of experiment 1 was performed at both λ of 6.9 (black squares, experimental results; red circles, SFA modeling) and 0.6 [in duplicate] (white squares, experimental results; green circles, SFA modeling). (**D**) Purge (ability to purge parasites and reach equilibrium). **(E** to **G),** Droplet fluorescence for Experiment 1. Boxes define droplet populations that contain no RNA replicators (empty), parasitic RNA replicators (inactive) and catalytically active RNA replicators (active). The percentage of droplets in each population and λ is indicated. (**E**) Unselected Compartmentalized. (**F**) Selected Compartmentalized. (**G**) Purge.

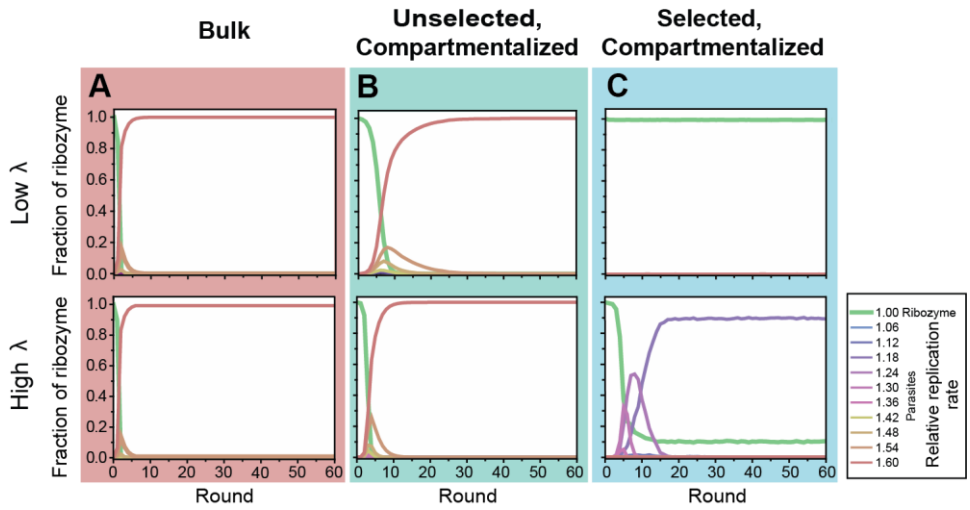

Fig. 3. Modeling of ribozyme and parasites dynamics. Fraction of MDV-VS ribozyme and ten different parasitic RNA replicators, with different relative replication rates compared to the MDV-VS ribozyme, calculated using the SFA model versus round of selection at $\lambda = 0.6$ (Low λ) and $\lambda = 7$ (High λ). This corresponds to 0.6 million and 7 million replicators, respectively, either in bulk or distributed into 1 million compartments (droplets). (**A**) Bulk. (**B**) Unselected Compartmentalized. **(C)** Selected Compartmentalized.