

Bacteria Subsisting on Antibiotics

Gautam Dantas *et al.*
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blockade of let-7 processing. It is possible that Lin28 and Lin28B may require both the cold-shock domain and CCHC zinc fingers for blocking activity. Lin28 and Lin28B are the only animal proteins known to contain both of these domains (15).

Our results show that Lin28 is necessary and sufficient for blockade of pri-miRNA processing of let-7 family members both in vitro and in vivo. There are several possible reasons why ES and EC cells possess a mechanism for post-transcriptional regulation of let-7 miRNA expression. First, posttranscriptional activation of miRNA processing would allow for rapid induction of several let-7 miRNAs by down-regulation of a single factor. Second, disruption of DGCR8, a dsRNA binding protein and essential component of the Microprocessor complex, interferes with ES cell differentiation, which suggests that activation of miRNAs may be important for silencing the self-renewal machinery (16). It has been suggested that posttranscriptional control could prevent even small amounts of let-7 from being produced in ES and EC cells, tightly maintaining the undifferentiated state (7). Third, posttranscriptional control of miRNA expression could serve as a means for dissociating expression patterns of intronic miRNAs from expression patterns of their host transcripts.

The precise mechanism by which Lin28 blocks miRNA processing as well as the range and determinants of its substrate selectivity are unknown. Our data suggest that Lin28 has a preference for selectively blocking the processing of let-7 family pri-miRNAs at the Microprocessor step. However, we cannot rule out the possibility that Lin28, alone or in concert with other factors, may block other pri-miRNAs in different physiological contexts. Others have reported that miRNA processing can also be regulated at the Dicer step, when pre-miRNAs are cleaved to their mature

form (5, 8). Additional factors may yet be discovered that posttranscriptionally regulate miRNA processing. Lin28 is predominantly localized to the cytoplasm, although it can also be found in the nucleus (10, 11); Lin28B is translocated into the nucleus in a cell cycle-dependent fashion (13). Lin28 may posttranscriptionally regulate miRNA processing in embryonic cells in a cell cycle-specific manner.

Recently, Lin28 was used in conjunction with Nanog, Oct-4, and Sox2 to reprogram human fibroblasts to pluripotency (14). Our data thus suggest that modulating miRNA processing may contribute to the reprogramming of somatic cells to an embryonic state. Additionally, global inhibition of miRNA processing by knockdown of the Drosha component of the Microprocessor was shown to promote cellular transformation and tumorigenesis; this phenotype was found to be, in large part, due to loss of let-7 expression (17). Let-7 has been reported to play a tumor suppressor role in lung and breast cancer by repression of oncogenes such as *Hmga2* (18) and *Ras* (19, 20). We suggest that disruption of let-7 processing by activation of Lin28 could promote the oncogenic phenotype. Notably, several human primary tumors show a general lack of correlation between expression of pri-miRNAs and the corresponding mature species (7, 21). This suggests that a block in miRNA processing may contribute to the low miRNA expression observed in many human cancers (22). Future study of Lin28 promises to reveal how miRNA processing contributes to the dedifferentiation that accompanies both somatic cell reprogramming and oncogenesis.

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Materials and Methods

Figs. S1 to S8

Table S1

References

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Bacteria Subsisting on Antibiotics

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Antibiotics are a crucial line of defense against bacterial infections. Nevertheless, several antibiotics are natural products of microorganisms that have as yet poorly appreciated ecological roles in the wider environment. We isolated hundreds of soil bacteria with the capacity to grow on antibiotics as a sole carbon source. Of 18 antibiotics tested, representing eight major classes of natural and synthetic origin, 13 to 17 supported the growth of clonal bacteria from each of 11 diverse soils. Bacteria subsisting on antibiotics are surprisingly phylogenetically diverse, and many are closely related to human pathogens. Furthermore, each antibiotic-consuming isolate was resistant to multiple antibiotics at clinically relevant concentrations. This phenomenon suggests that this unappreciated reservoir of antibiotic-resistance determinants can contribute to the increasing levels of multiple antibiotic resistance in pathogenic bacteria.

The seemingly unchecked spread of multiple antibiotic resistance in clinically relevant pathogenic microbes is alarming. Furthermore, an important environmental reservoir of antibiotic-resistance determinants, termed the antibiotic resistome, has been discovered (1, 2). The primary microbial antibiotic-resistance mecha-

nisms include efflux pumps, target gene-product modifications, and enzymatic inactivation of the antibiotic compound (3, 4). Many of the mechanisms are common to several species of pathogens and spread by lateral gene transfer (5). Although enzymatic inactivation is often sufficient to annul the antimicrobial activity of these chemicals, the

biochemical processing of these compounds is unlikely to end there, and we hypothesize that the soil microbiome must include a substantial reservoir of bacteria capable of subsisting on antibiotics. Although many bacteria growing in extreme environments (6) and capable of degrading toxic substrates (7) have been previously reported, only a few organisms have been shown to subsist on a limited number of antibiotic substrates (8–10).

We cultured clonal bacterial isolates from 11 diverse soils (table S2) that were capable of using one of 18 different antibiotics as their sole carbon source. These antibiotics included natural, semisynthetic, and synthetic compounds of different ages and from all major bacterial target classes. Every antibiotic tested was able to support bacterial

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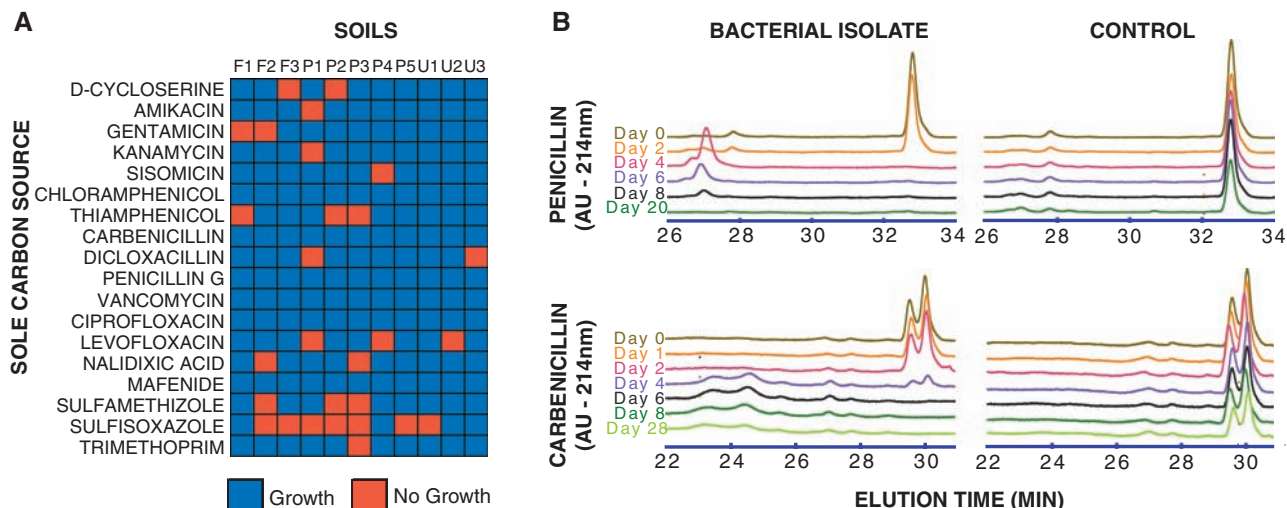


Fig. 1. Clonal bacterial isolates subsisting on antibiotics. **(A)** Heat map illustrating growth results from all combinations of 11 soils with 18 antibiotics, where blue squares represent the successful isolation of bacteria from a given soil that were able to use that antibiotic as their sole carbon source at an antibiotic concentration of 1 g/liter. Soil samples labeled F1 to F3 are farm soils

and those labeled U1 to U3 are urban soils. P1 to P5 are pristine soils, collected from non-urban areas that had minimal human exposure over the past 100 years (table S2). **(B)** HPLC traces at 214 nm of representative penicillin- and carbenicillin-catabolizing clonal isolates and corresponding un-inoculated media controls for different time points over 20 or 28 days of growth, respectively.

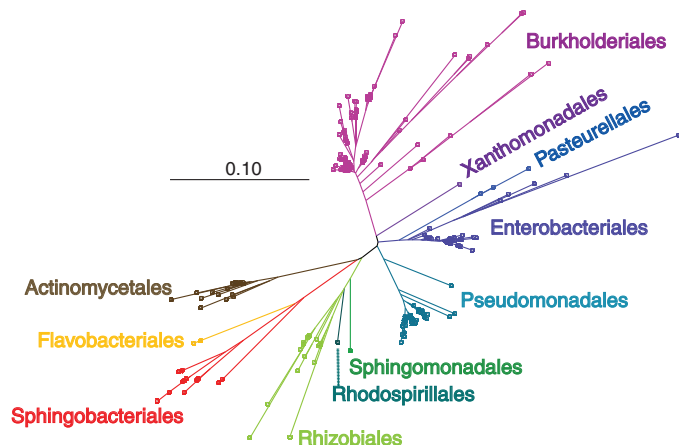
growth (Fig. 1A and fig. S1). Six out of 18 antibiotics supported growth in all 11 soils, covering five of the eight classes of antibiotics tested. Appropriate controls were performed to ensure that carbon source contamination of the source media or carbon fixation from the air were insignificant (11).

We obtained clonal isolates capable of subsisting on penicillin and carbenicillin from all the soils tested, and isolates from 9 out of 11 soils that could subsist on dicloxacillin. Representative isolates capable of growth on penicillin and carbenicillin were selected for subsequent analysis by high-performance liquid chromatography (HPLC) (11). Removal of the antibiotics from the media was observed within 4 and 6 days, respectively (Fig. 1B). Mass spectrometry analysis of penicillin cultures has indicated the existence of a penicillin catabolic pathway (9) that is initiated by hydrolytic cleavage of the beta-lactam ring. Beta-lactam ring cleavage, followed by a decarboxylation step, is the dominant mode of clinical resistance to penicillin and related beta-lactam antibiotics (fig. S3) (11).

We isolated bacteria from all the soils tested that grew on ciprofloxacin (Fig. 1A), a synthetic fluoroquinolone and one of the most widely prescribed antibiotics. Clonal isolates capable of catabolizing the other two synthetic quinolones tested, levofloxacin and nalidixic acid, were also isolated from a majority of the soils (Fig. 1A). Previous studies have highlighted the strong parallels between antibiotic-resistance determinants harbored by soil-dwelling microbes and human pathogens (5, 12, 13). The lateral transfer of genes encoding the enzymatic machinery responsible for subsistence on quinolone antibiotics from soil-dwelling microbes to human pathogens could introduce a novel resistance mechanism so far not observed in the clinic.

Phylogenetic profiling of the clonal isolates (11) revealed a diverse set of species in the Proteobac-

Fig. 2. Phylogenetic distribution of bacterial isolates subsisting on antibiotics. 16S ribosomal DNA (rDNA) was sequenced from antibiotic-catabolizing clonal isolates with the use of universal bacterial rDNA primers. High-quality nonchimeric sequences were classified using the Greengenes database (16), with consensus annotations from Ribosomal Database Project II (17) and National Center for Biotechnology Information taxonomies (18). Phylogenetic trees were constructed using the neighbor-joining algorithm in the ARB program package (19) using the Greengenes-aligned 16S rDNA database. Placement in the tree was confirmed by comparing automated Greengenes taxonomy to the annotated taxonomies of the nearest neighbors of each sequence in the aligned database. Branches of the tree are color-coded by bacterial orders, and clonal isolates are represented as squares.



teria (87%), Actinobacteria (7%), and Bacteroidetes (6%) (Fig. 2 and fig. S2). These phyla all include many clinically relevant pathogens. Of the 11 orders represented, *Burkholderiales* constitute 41% of the species isolated. The other major orders (>5%) are *Pseudomonadales* (24%), *Enterobacteriales* (13%), *Actinomycetales* (7%), *Rhizobiales* (7%), and *Spingobacteriales* (6%).

One explanation for the widespread catabolism of both natural and synthetic antibiotics may relate to organic substructures found in nature. Microbial metabolic mechanisms exist for processing those substructures and may allow for the utilization of the parent synthetic antibiotic molecule. It is interesting that more than half of the bacterial isolates identified in this study belong to the orders *Burkholderiales* and *Pseudomonadales*. Organisms in these orders typically have large genomes of

approximately 6 to 10 megabases, which has been suggested to be positively correlated to their metabolic diversity and multiple antibiotic resistance (14). These organisms can be thought of as scavengers, capable of using a large variety of single carbon sources as food (15).

We determined the magnitude of antibiotic resistance for a representative subset of 75 clonal isolates (table S3). Each clonal isolate was tested for resistance to all 18 antibiotics used in the subsistence experiments at antibiotic concentrations of 20 mg/liter and 1 g/liter in rich media (11). The clonal isolates tested were on average resistant to 17 out of 18 antibiotics at 20 mg/liter and 14 out of 18 antibiotics at 1 g/liter (Fig. 3). Furthermore, for 74 of the 75 isolates, we found that if a bacterial isolate was able to subsist on an antibiotic, it was also resistant to all antibiotics in that class at 20 mg/liter.

Previous work showing that strains from the genus *Streptomyces* are on average resistant to seven to eight antibiotics at 20 mg/liter has highlighted the importance of producer organisms as a reservoir of antibiotic resistance (2). Here we describe bacteria subsisting on antibiotics as a substantial addition to the antibiotic resistome in terms of both phylogenetic diversity and prevalence of resistance. The bacteria isolated in this study were super-resistant, because they tolerated concentrations of antibiotics >1 g/liter, which is 50 times higher than the

antibiotic concentrations used to define the antibiotic resistome (2).

The Greengenes database (16) identified isolates among the bacteria subsisting on antibiotics that are closely related to known pathogens, such as members of the *Burkholderia cepacia* complex and *Serratia marcescens*. In principle, relatedness allows for easier transfer of genetic material, because codon usage, promoter binding sites, and other transcriptional and translational motifs are likely to be similar. It is therefore possible that pathogenic microbes can more readily use resist-

ance genes originating from bacteria subsisting on antibiotics than the resistance genes from more distantly related antibiotic producer organisms.

To date, there have been no reports describing antibiotic catabolism in pathogenic strains. However, because most sites of serious infection in the human body are not carbon-source-limited, it is unlikely that antibiotic catabolism confers a strong selective advantage on pathogenic microbes as compared to just resisting them. Therefore, it is likely that only the resistance-conferring part of the catabolic machinery would be selected for in pathogenic strains.

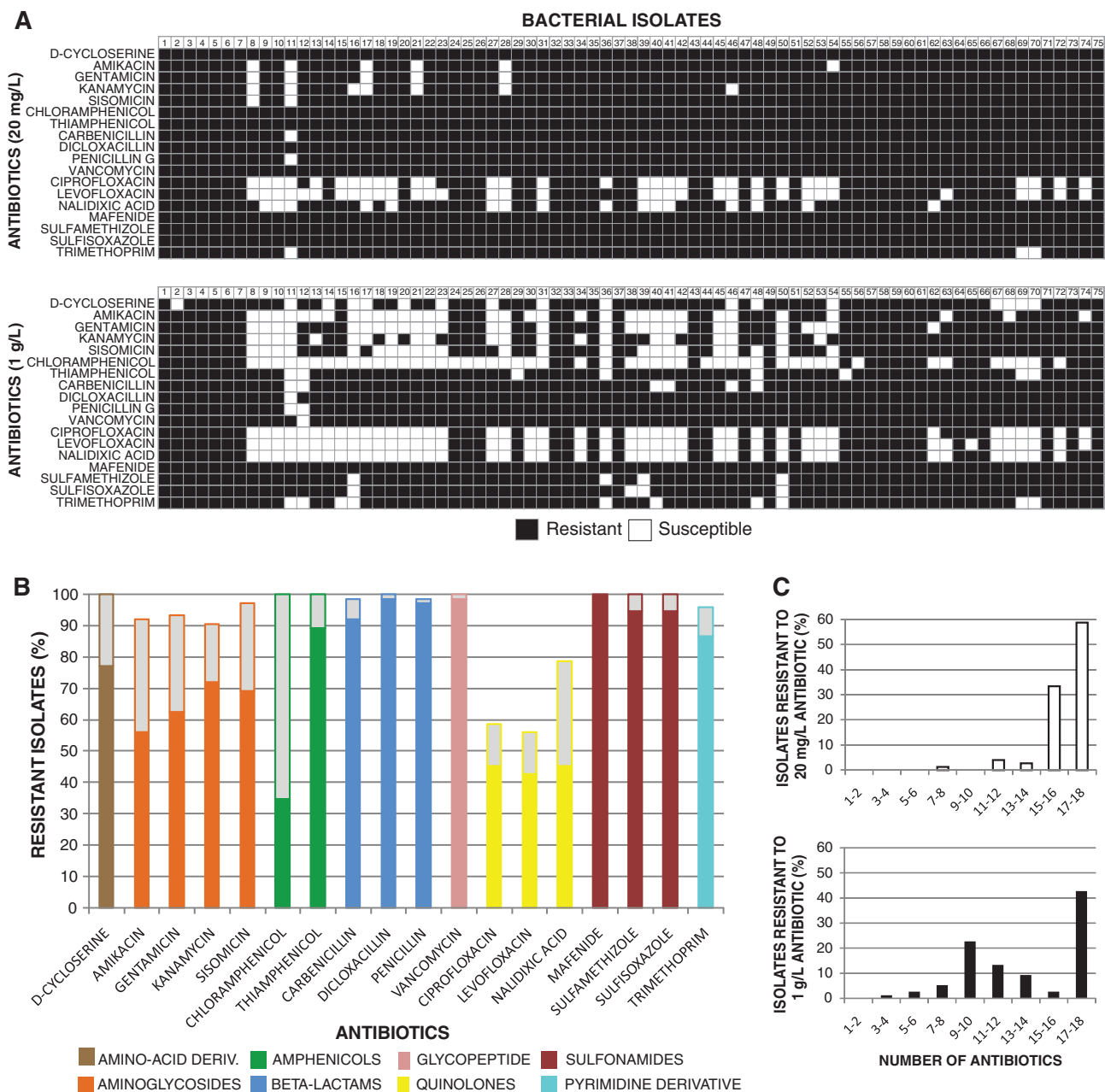


Fig. 3. Antibiotic-resistance profiling of 75 clonal isolates capable of subsisting on antibiotics. **(A)** Heat map illustrating the resistance profiles of a representative subset of 75 clonal isolates capable of using antibiotics as their sole carbon source (table S3). Resistance was determined as growth after 4 days at 22°C in Luria broth media containing antibiotic at concentrations of 20 mg/liter (top) and 1 g/liter (bottom). **(B)** Percentage of clonal isolates resistant to each of

the 18 antibiotics. Antibiotics are color-coded by class; the full height of each bar corresponds to the percentage of clonal isolates resistant at a concentration of 20 mg/liter and the solid colored section of each bar corresponds to the percentage of clonal isolates resistant at 1 g/liter. **(C)** Histogram depicting the distribution of the number of antibiotics that the clonal isolates were resistant to at 20 mg/liter (top) and 1 g/liter (bottom).

In addition to the finding that bacteria subsisting on natural and synthetic antibiotics are widely distributed in the environment, these results highlight an unrecognized reservoir of multiple antibiotic-resistance machinery. Bacteria subsisting on antibiotics are phylogenetically diverse and include many organisms closely related to clinically relevant pathogens. It is thus possible that pathogens could obtain antibiotic-resistance genes from environmentally distributed super-resistant microbes subsisting on antibiotics.

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Supporting Online Material

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Materials and Methods

Figs. S1 to S3
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Reversible Compartmentalization of de Novo Purine Biosynthetic Complexes in Living Cells

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Purines are synthesized de novo in 10 chemical steps that are catalyzed by six enzymes in eukaryotes. Studies in vitro have provided little evidence of anticipated protein-protein interactions that would enable substrate channeling and regulation of the metabolic flux. We applied fluorescence microscopy to HeLa cells and discovered that all six enzymes colocalize to form clusters in the cellular cytoplasm. The association and dissociation of these enzyme clusters can be regulated dynamically, by either changing the purine levels of or adding exogenous agents to the culture media. Collectively, the data provide strong evidence for the formation of a multi-enzyme complex, the "purinosome," to carry out de novo purine biosynthesis in cells.

Purines are not only essential building blocks of DNA and RNA, but, as nucleotide derivatives, they also participate in a multitude of pathways in both prokaryotes and eukaryotes (1, 2). Biosynthetically, adenosine and guanosine nucleotides are derived from inosine monophosphate (IMP), which is synthesized from phosphoribosyl pyrophosphate (PRPP) in both the de novo and salvage biosynthetic pathways (Fig. 1). The salvage pathway catalyzes the one-step conversion of hypoxanthine to IMP by hypoxanthine phosphoribosyl transferase (HPRT), whereas the de novo pathway consists of 10 chemical reactions that transform PRPP to IMP. In higher eukaryotes (such as humans), the de novo pathway uses six enzymes, including three multifunctional enzymes: a trifunctional protein, TrifGART, that has glycylamide ribonucleotide (GAR) synthetase (GARS) (step 2), GAR transformylase (GAR Tfase) (step 3), and aminoimidazole ribonucleotide synthetase (AIRS) (step 5) activities; a bifunctional enzyme, PAICS,

that has carboxyaminoimidazole ribonucleotide synthase (CAIRS) (step 6) and succinylaminoimidazolecarboxamide ribonucleotide synthetase (SAICARS) (step 7) activities; and a bifunctional enzyme, ATIC, that has aminoimidazolecarboxamide ribonucleotide transformylase (AICAR Tfase) (step 9) and IMP cyclohydrolase (IMPCH) (step 10) activities. In contrast, prokaryotes, such as *Escherichia coli*, use only monofunctional enzymes throughout this pathway, except for the bifunctional ATIC.

Although studies of the individual enzymes in vitro have revealed much about their respective mechanisms of action, a number of in vitro attempts using kinetic analysis and/or binding measurements to demonstrate protein-protein interactions have not been fruitful, with few exceptions (3–6). In addition, there is scant evidence from in vivo cellular studies for the hypothesis that these enzymes act in concert within a multienzyme complex (7). In this study, we used fluorescence microscopy to investigate whether functional multienzyme complexes involved in de novo purine biosynthesis form in living mammalian cells under purine-rich and purine-depleted conditions, which affect the rate of metabolic flux (8–10).

We selected for study two human (h) enzymes involved with de novo purine biosynthesis as initial candidates for involvement in a multienzyme complex in vivo: the nonsequential hTrifGART protein, which catalyzes steps 2, 3, and 5, and formylglycinamide ribonucleotide synthase (hFGAMS), which catalyzes step 4. These proteins were fused to either a green fluorescent protein (GFP) or an orange fluorescent protein (OFP) and were transiently expressed in HeLa cells that had been cultured in either purine-rich [minimum essential medium and 10% fetal bovine serum (FBS)] or purine-depleted (RPMI 1640 and dialyzed 5% FBS) media (9, 11). When expressed individually in cells grown in purine-rich media, both the hTrifGART (Fig. 2A) and hFGAMS (Fig. 2C) proteins exhibited diffuse cytoplasmic distributions, as previously found in 293T fibroblast cells and with their prokaryotic counterpart, *E. coli* cells (7). However, when these constructs were expressed in purine-depleted cells, we observed cytoplasmic clustering, estimated at ~27% of the hTrifGART-GFP (Fig. 2B) and ~77% of the hFGAMS-GFP (Fig. 2D) proteins. To determine whether the two enzymes colocalized into these clusters in low-purine conditions, we transiently coexpressed hTrifGART-GFP and hFGAMS-OFP in HeLa cells (Fig. 2, G, H, and I); ~60% of the transfected cells exhibited colocalization within the clusters (Fig. 2I).

Z-scan imaging with confocal laser-scanning microscopy confirmed that the hTrifGART-GFP and the hFGAMS-OFP proteins were co-clustered in the cytoplasm (fig. S1). We also observed co-clustering between hFGAMS-GFP and hTrifGART-OFP after we reversed the fusion of the fluorescent probes in HeLa cells that were maintained in purine-depleted media (fig. S2). Additionally, similar cellular localization experiments were carried out with hTrifGART-GFP and hFGAMS-GFP in two additional human cell lines, HTB-125 and HTB-126; the results suggested that the purine-dependent clustering appears to occur in other human cell lines [supporting online material (SOM) Text]. These findings, when combined with the imaging results described

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