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RESEARCH ARTICLE

Glutamine deprivation initiates reversible assembly of mammalian rods and rings

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Abstract Rods and rings (RR) are protein assemblies composed of cytidine triphosphate synthetase type 1 (CTPS1) and inosine monophosphate dehydrogenase type 2 (IMPDH2), key enzymes in CTP and GTP biosynthesis. Small-molecule inhibitors of CTPS1 or IMPDH2 induce RR assembly in various cancer cell lines within 15 min to hours. Since glutamine is an essential amide nitrogen donor in these nucleotide biosynthetic pathways, glutamine deprivation was examined to determine whether it leads to RR formation. HeLa cells cultured in normal conditions did not show RR, but after culturing in media lacking glutamine, short rods (<2 μm) assembled after 24 h, and longer rods (>5 μm) formed after 48 h. Upon supplementation with glutamine or guanosine, these RR underwent almost complete disassembly within 15 min. Inhibition of glutamine synthetase with methionine sulfoximine also increased RR assembly in cells deprived of glutamine. Taken together, our data support the hypothesis that CTP/GTP biosynthetic enzymes polymerize to form RR in response to a decreased intracellular level of glutamine. We speculate that rod and ring formation is an adaptive metabolic response linked to disruption of glutamine homeostasis.

Keywords Cytidine triphosphate synthetase · Glutamine · Inosine monophosphate dehydrogenase · Rods and rings

Abbreviations

RR	Rods and rings
CTPS	Cytidine triphosphate synthetase
IMPDH	Inosine monophosphate dehydrogenase
DON	6-Diazo-5-oxo-L-norleucine
CTP	Cytidine triphosphate
GMP	Guanine monophosphate
MPA	Mycophenolic acid
FBS	Fetal bovine serum
GTP	Guanosine triphosphate
UTP	Uridine triphosphate
mRNA	Messenger RNA

Introduction

Cytoplasmic structures referred to as rods and rings (RR) were identified by indirect immunofluorescence with human autoantibodies in cancer cell lines and mouse embryonic stem cells [1, 2]. Co-staining experiments demonstrated that RR are not part of, nor do they colocalize with, the Golgi complex or other known organelles. Although RR are filament-like structures, they are not enriched in actin, tubulin, or vimentin. Two proteins, cytidine triphosphate synthetase type 1 (CTPS1) and inosine monophosphate dehydrogenase type 2 (IMPDH2), have been identified as components of RR [1, 3–7]. Moreover, two CTPS1 inhibitors [6-diazo-5-oxo-L-norleucine (DON), and acivicin] and two IMPDH2 inhibitors (ribavirin and

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mycophenolic acid) are highly effective in inducing RR formation in mammalian cells, a polymerization process that is dependent on concentrations of and treatment times with these inhibitors [1, 8]. To date, the four metabolic inhibitors that induce RR assembly share a common feature, namely retarding key enzymatic reactions in de novo purine and pyrimidine nucleotide pathways.

The rate-limiting enzyme in pyrimidine biosynthesis, CTPS1, catalyzes the glutamine-dependent conversion of uridine triphosphate to cytidine triphosphate (CTP), an essential precursor for DNA, RNA, and CMP-linked phospholipids. CTP synthetase exists as two isozymes, CTPS1 and CTPS2, with 74 % identity in humans. CTPS1 is a 591-residue protein (MW = 67 kDa), composed of an N-terminal CTP-synthesizing domain and a C-terminal glutamine-hydrolyzing domain. Neutral ammonia liberated at the glutaminase active site is transferred by means of an inter-subunit tunnel to the synthetase active site, where nascent ammonia attacks 4-phosphoryl uridine triphosphate (UTP) to form CTP. CTP synthetase activity is affected by all four ribonucleoside triphosphates in vitro [9]. CTP acts as a potent product inhibitor that binds to the synthetase domain [10, 11], whereas GTP is an allosteric activator [12]. As a pacemaker enzyme in the purine biosynthesis pathway, IMPDH2 catalyzes the conversion of inosine monophosphate (IMP) into xanthosine monophosphate (XMP). The latter is then converted to guanosine monophosphate (GMP) [13]. IMPDH is also a widely exploited chemotherapeutic target for controlling proliferation and differentiation of normal and neoplastic cells [14, 15]. Humans produce two isoforms, IMPDH1 and IMPDH2, with 84 % sequence identity and a molecular weight of 55 kDa. IMPDH activity can be affected by way of IMP site-directed inhibitors, NAD^+ site-directed inhibitors, as well as allosteric inhibitors [16].

As the most abundant free amino acid in the body, glutamine plays a central role in nitrogen metabolism, protein synthesis, and cell proliferation [17]. Glutamine is also involved in changes in protein activity, gene and protein expression, and the formation of other intracellular metabolite concentrations [18]. Extracellular administration of glutamine can stimulate purine and pyrimidine synthesis. Notably, the first committed steps in purine and pyrimidine pathways are glutamine-dependent. In the former, glutamine-derived ammonia is incorporated into 5′-phosphoribosylamine, and in the latter, glutamine-derived ammonia is incorporated into carbamoyl-phosphate [19]. So essential is exogenously supplied glutamine for the growth and vitality of cultured cells that standard growth media are supplemented with 1.0–4.5 mM [20].

Previous reports identified CTPS1 and IMPDH2 as components of RR, showing that four inhibitors could induce the formation of RR in various cell lines [1, 8]. Separate groups have also shown anti-RR antibodies to have a strict association with hepatitis C patients treated with interferon-α and ribavirin [2, 6, 21–25]. The present report assesses the impact of glutamine deprivation on RR assembly in human cancer cells, providing new insights about how cells adapt to glutamine deprivation.

Materials and methods

Cell culture

HeLa cells (human cervical cancer, ATCC, Manassas, VA, USA) were cultured in DMEM containing 10 % fetal bovine serum (FBS) in a 37 °C incubator with 5 % CO_2. Adherent cell lines were maintained at 50 % confluence. All media contained 100 IU/ml penicillin and 100 μg/ml streptomycin.

Indirect immunofluorescence

HeLa cells were cultured in eight-well chamber slides (BD Falcon) and were used for indirect immunofluorescence as described [26, 27]. The eight-well chamber slides were prepared using ~50,000 cells per well in 500 μl of medium. Fixation method was 3 % paraformaldehyde in PBS at room temperature for 10 min followed by 0.5 % triton-X/PBS for another 5 min. For co-staining studies, rabbit anti-IMPDH2 (Proteintech, Chicago, IL, USA 12948-1-AP) and human anti-RR prototype serum It2006 were used and followed by secondary antibodies Alexa 488 goat anti-human IgG and Alexa 568 goat anti-rabbit IgG.

Induction of RR assembly by glutamine deprivation

HeLa cells were cultured in eight-well chamber slides exactly as described above except in medium without glutamine (DMEM, Cellgro, 15-013-CV; DMEM, Sigma-Aldrich, D6546). Glutamine-deficient medium was replaced each day for 2 days. On the third day, cells were either harvested or supplemented with L-glutamine (Cellgro, 25-005-Cl), cytosine (Sigma-Aldrich, C3506), cytidine (Sigma-Aldrich, C4654), guanosine (Sigma-Aldrich, G6264), thymidine (Sigma-Aldrich, T1895), or uridine (Sigma-Aldrich, U3003) at designated concentrations ranging from 0.1 to 125 mM for various lengths of time ranging from 1 min to 24 h.

Induction of RR formation using CTPS or IMPDH inhibitors

Mycophenolic acid (MPA; Sigma-Aldrich, M5255) was solubilized in ethanol to a 31-mM stock concentration.

DON; Sigma-Aldrich, D2141 was solubilized in DMEM to a stock concentration of 100 mM. Cells were seeded as monolayers and allowed to attach for 24 h. CTPS1 or IMPDH2 inhibitors were then added in various final concentrations ranging from 0.2 µM to 2 mM and cells were kept for an additional 24 h.

Induction of RR formation using glutamine synthetase inhibitors

Cells were maintained in glutamine-deficient medium and treated with DON or methionine sulfoximine (Sigma, M5379). Methionine sulfoximine was solubilized in warm water to a 100 mM stock concentration. Cells were treated for 24 h with either inhibitor.

qRT-PCR

Total RNA samples were harvested using the mirVana Total RNA Isolation Kit (Life Technologies) after 48 h in culture or at a specific time point indicated. Reverse transcription was performed using the High Capacity cDNA Reverse Transcription Kit (Life Technologies). The relative mRNA levels of target genes were measured in duplicates using TaqMan Fast Advanced Master Mix (Life Technologies) with the corresponding TaqMan Gene Expression Assay (Life Technologies). GAPDH was used as an internal control and was run in duplex with the targets.

Western blotting

The expression levels of CTPS and IMPDH were analyzed using rabbit anti-CTPS (Abcam 1:1,000) and rabbit anti-IMPDH2 (Santa Cruz Biotechnology, Paso Robles, CA, USA 1:1,000; Proteintech, Chicago, IL, USA 12948-1-AP, 1:1,000) as described [28]. The level of GAPDH was determined by rabbit anti-GAPDH antibody (Sigma, G9545, 1:2,000) as a normalizer to control for loading. Protein was quantitated using ImageJ and normalized to GAPDH.

Quantification and statistical analysis

Cell counting was performed using Mayachitra Imago (Mayachitra, Santa Barbara, CA, USA) to detect nuclei counterstained with DAPI. Quantification of the size of RR was accomplished using the threshold feature in Mayachitra Imago software. The intensity threshold was set to 50 (detects RR with total intensity pixels higher than 50) for the analysis of images. The size of the structures was defined by their area in pixels. Unpaired, two-tailed Student's t test and Fisher's exact test were used to compare independent groups. Prism for Windows version 5.0 (GraphPad Software, San Diego, CA, USA) was used for all statistical analysis.

Results

The de novo pathways for pyrimidine and purine biosynthesis are shown in Fig. 1, with glutamine-dependent steps indicated. While CTP synthetase requires glutamine as an amide-nitrogen donor, IMP dehydrogenase does not. Even so, the very next reaction in purine nucleotide biosynthesis is glutamine-requiring. We hypothesized that the formation of rod and ring structures (shown in Fig. 1b, c) should be exquisitely sensitive to glutamine availability, which is typically satisfied through the endogenous action of glutamine

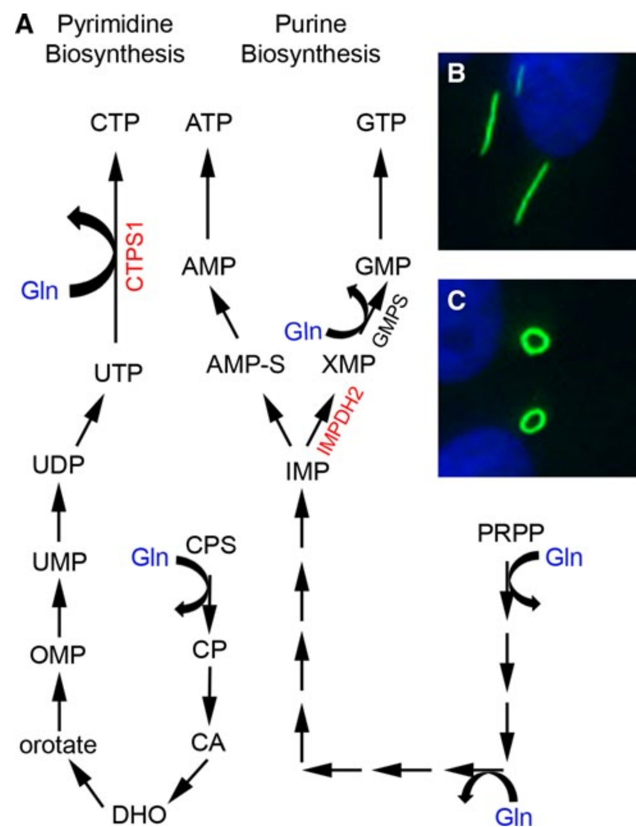


Fig. 1 Purine and pyrimidine biosynthesis pathways and rods and rings. **a** Purine and pyrimidine de novo biosynthesis pathways. Enzymes in these pathways that are known RR components are shown in *red* and steps requiring glutamine in *blue*. In the steps eventually leading to GTP formation, IMPDH2 catalyzes the conversion of IMP to XMP, which is converted to GMP in the glutamine-dependent GMP synthase reaction. CTPS1 is an enzyme involved in the CTP biosynthesis pathway and catalyzes the conversion of UTP to CTP. *Arrows* and *numbers* signify steps in the pathway. The pathway schematic is adapted from Hofer et al. [29]. **b**, **c** Antibodies from prototype anti-RR serum It2006 recognize typical rod (~3–10 µm in length) and ring (~2–5 µm in diameter) structures in the cytoplasm of HeLa cells. Nuclei are counterstained with DAPI

synthetase as well as the commonly practiced exogenous addition of glutamine to cell culture media. The experiments presented below were designed to assess the impact of glutamine on RR formation.

Glutamine deprivation induces rod and ring formation

RR were previously identified as cytoplasmic structures composed of CTPS1 and IMPDH2 with the use of CTPS1 or IMPDH2 inhibitors [1]. CTPS1 and IMPDH2 are involved in the purine and pyrimidine biosynthesis pathways, which both utilize glutamine extensively [29]. In view of glutamine's key role in these pathways, we began by examining whether glutamine deprivation leads to RR formation and accumulation. Cells were cultured with or without glutamine at various concentrations of FBS: 10, 5, 1, 0.1, and 0 %. Cells maintained in medium containing 10 % FBS, but deprived of glutamine for 2 days, formed RR in an average of 48.7 % of cells after 2 days (Fig. 2a). RR formation was confirmed by co-staining using a rabbit anti-IMPDH2 antibody and the prototype human anti-RR serum It2006 as previously described [1]. In contrast, cells cultured in normal medium containing glutamine showed diffused cytoplasmic staining with no RR structures in evidence (Fig. 2c). Cells cultured in 1 % FBS, but lacking glutamine, had RR in 10.7 % of cells (Fig. 2b), with significant cell death noted. In culture medium containing glutamine, a decrease in FBS from 10 % (Fig. 2c) to 1 % induced only few RR (Fig. 2d). At FBS concentrations below 1 %, there was extensive cell death and no RR were detected.

Varying glutamine levels over the 0.5–15 mM range was previously found to affect protein and gene expression [30]. Messenger RNA (mRNA) levels of CTPS1 and IMPDH2 were elevated after cells were deprived of glutamine in the presence of 10 % FBS when compared to cells maintained in medium with 10 % FBS and glutamine (Fig. 2e). In contrast to the ~twofold increase in mRNA, only a small increase in protein levels was observed for CTPS1. A 56 % increase in IMPDH2 protein levels was observed in cells

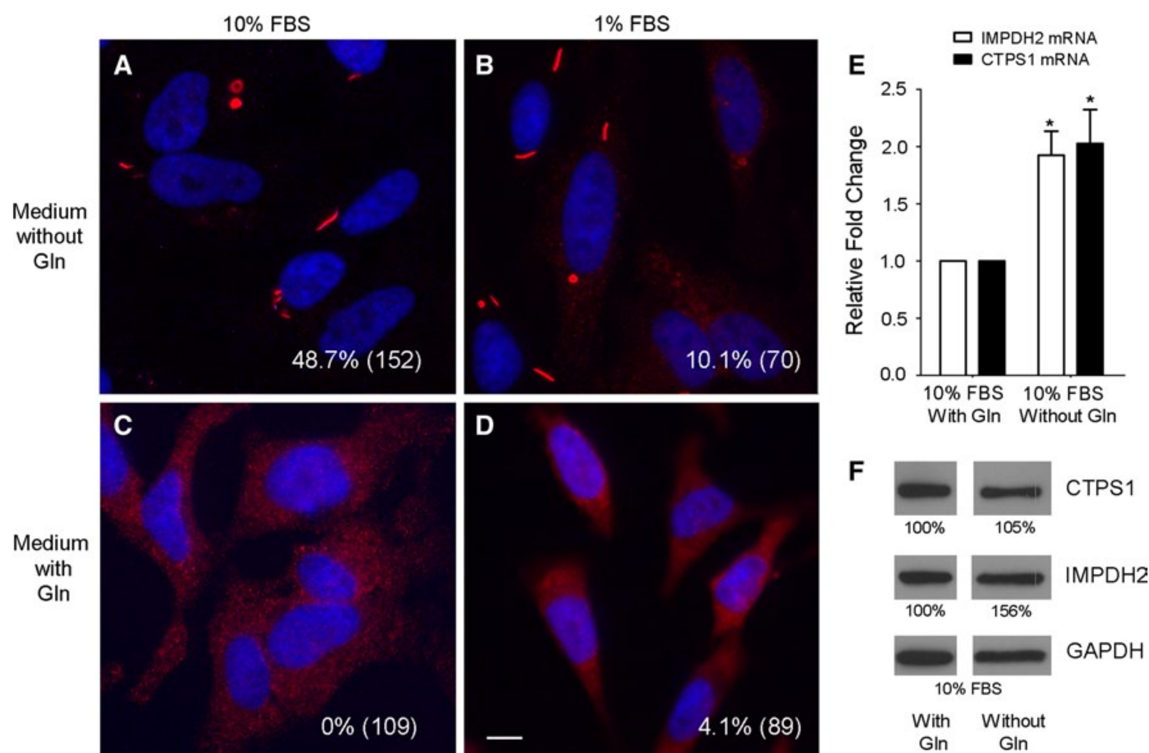
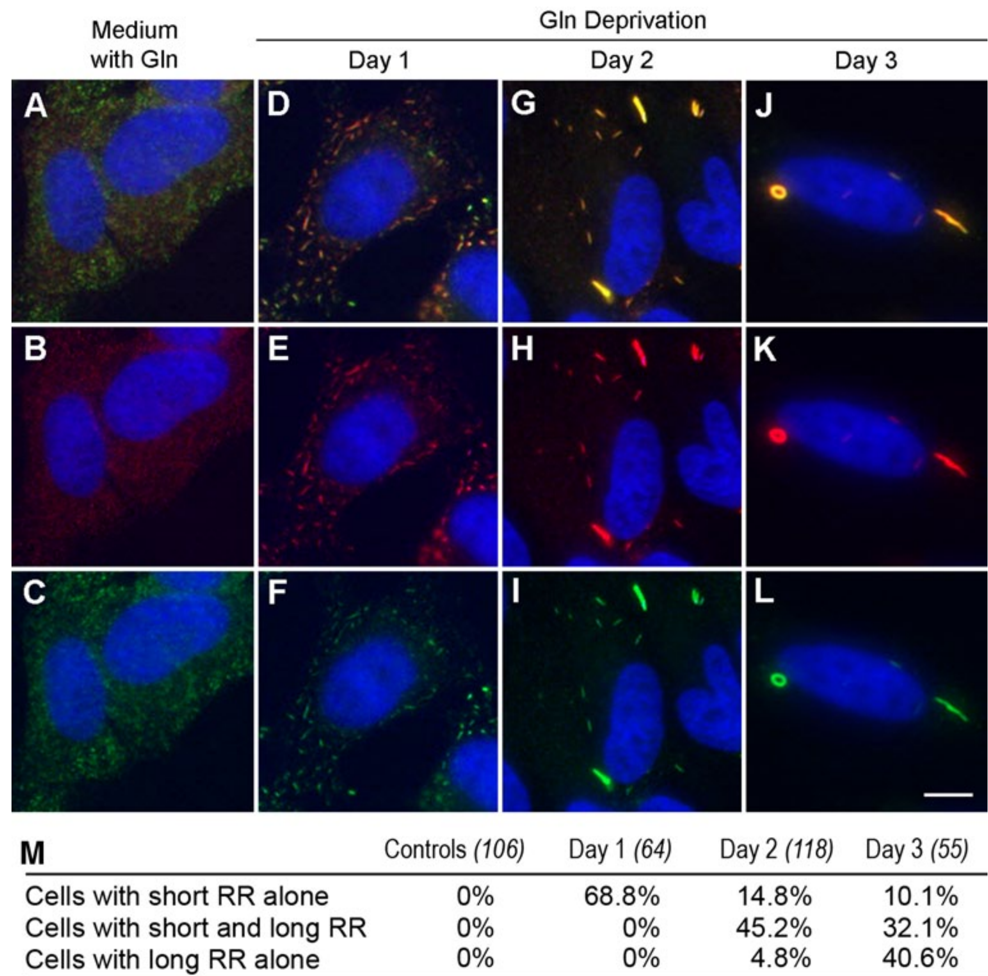


Fig. 2 Glutamine deprivation induces rods and rings. HeLa cells maintained in DMEM medium with 10 % FBS without glutamine (**a**), DMEM medium with 1 % FBS without glutamine (**b**), DMEM medium with 10 % FBS (**c**), and DMEM medium with 1 % FBS (**d**) for 48 h were stained with rabbit anti-IMPDH2 (*red*). Nuclei were counterstained with DAPI (*blue*). The average percentage of cells with RR are displayed in the *lower right corner*, with total number of cells counted in *parentheses*. **e** mRNA levels of CTPS1 and IMPDH2 are significantly elevated in cells depleted of glutamine. mRNA levels were normalized to GAPDH. **f** Western blot of CTPS1 and IMPDH2 protein levels using GAPDH as a loading control in HeLa cells maintained in 10 % FBS medium with glutamine or without glutamine. There was an increase (56 %) in IMPDH2 protein levels after glutamine deprivation in 10 % FBS compared with cells maintained in DMEM medium with 10 % FBS. Data are from three independent experiments (**a–d** mean, **e** mean ± SD). * $p < 0.05$ (two-tailed unpaired t test). *Scale bar* 10 μm



	Controls *(106)*	Day 1 *(64)*	Day 2 *(118)*	Day 3 *(55)*
Cells with short RR alone	0%	68.8%	14.8%	10.1%
Cells with short and long RR	0%	0%	45.2%	32.1%
Cells with long RR alone	0%	0%	4.8%	40.6%

Fig. 3 Time-course of RR formation during glutamine starvation. HeLa cells were maintained in normal DMEM medium with 10 % FBS (**a**–**c**), or in glutamine-depleted medium with 10 % FBS for 1 day (**d**–**f**), 2 days (**g**–**i**), or 3 days (**j**–**l**). Cells were co-stained with human anti-RR serum It2006 (*green* **c**, **f**, **i**, **l**) and rabbit anti-IMPDH2 (*red* **b**, **e**, **h**, **k**). Merged images are shown in **a**, **d**, **g**, and **j**. Nuclei were counterstained with DAPI (*blue*). **m** Summary of the percentage of cells with short RR alone, short and long RR, and long RR alone for each day of glutamine starvation and control. Total number of cells counted are shown in *parentheses*

deprived of glutamine when compared to cells in normal medium (Fig. 2f).

The assembly of RR in response to glutamine deprivation was further examined in cells treated for 1, 2, and 3 days (Fig. 3). RR were not detected in cells maintained with glutamine-containing medium. Diffuse cytoplasmic staining was observed when cells were co-stained with human serum It2006 and rabbit anti-IMPDH2 antibodies (Fig. 3a–c). However, when the glutamine-containing medium was replaced with medium deprived of glutamine, RR formation was evident by day 2 (Fig. 3g–i). At day 1, short structures resembling rods were discernable in the cytoplasm (Fig. 3d–f); these short rods remained among the "mature" long RR at day 2 (Fig. 3g–i). By day 3, however, the majority of short rods disappeared and mature long RR were predominant (Fig. 3j–l). Counting of short and long RR showed the reduction of the short structures from 68.8 % at day 1 to 10.1 % at day 3 (Fig. 3m). This is consistent with the hypothesis that mature RR in later time points are assembled at the expense of shorter structures that formed at earlier time points. Induction of RR by glutamine deprivation was also confirmed to work with different brands of medium (Cellgro and Sigma) to rule out unknown brand-specific manufacturing variables (data not shown).

Glutamine replenishment disassembles RR induced in response to glutamine deprivation

RR formed in response to glutamine deprivation for 72 h rapidly disassembled within 15 min after the addition of 2.5 mM glutamine (Fig. 4). After glutamine replenishment, cells had the same diffuse cytoplasmic staining (Fig. 4g–i) that was observed in cells in normal medium with glutamine (Fig. 4a–c). Elevated glutamine (as high as 125 mM) resulted in the complete disassembly of RR (data not shown). Culture media typically contain 4 mM glutamine, and the 2.5 mM level was below that value. When glutamine was maintained at normal culture medium levels, no RR formation was observed (data not shown). Interestingly, we observed that increased CTPS1 and IMPDH2 mRNA levels in glutamine-depleted cells were reduced when cells were replenished with 2.5 mM glutamine (Fig. 4j). On further analyses, supplement with 2.5 mM

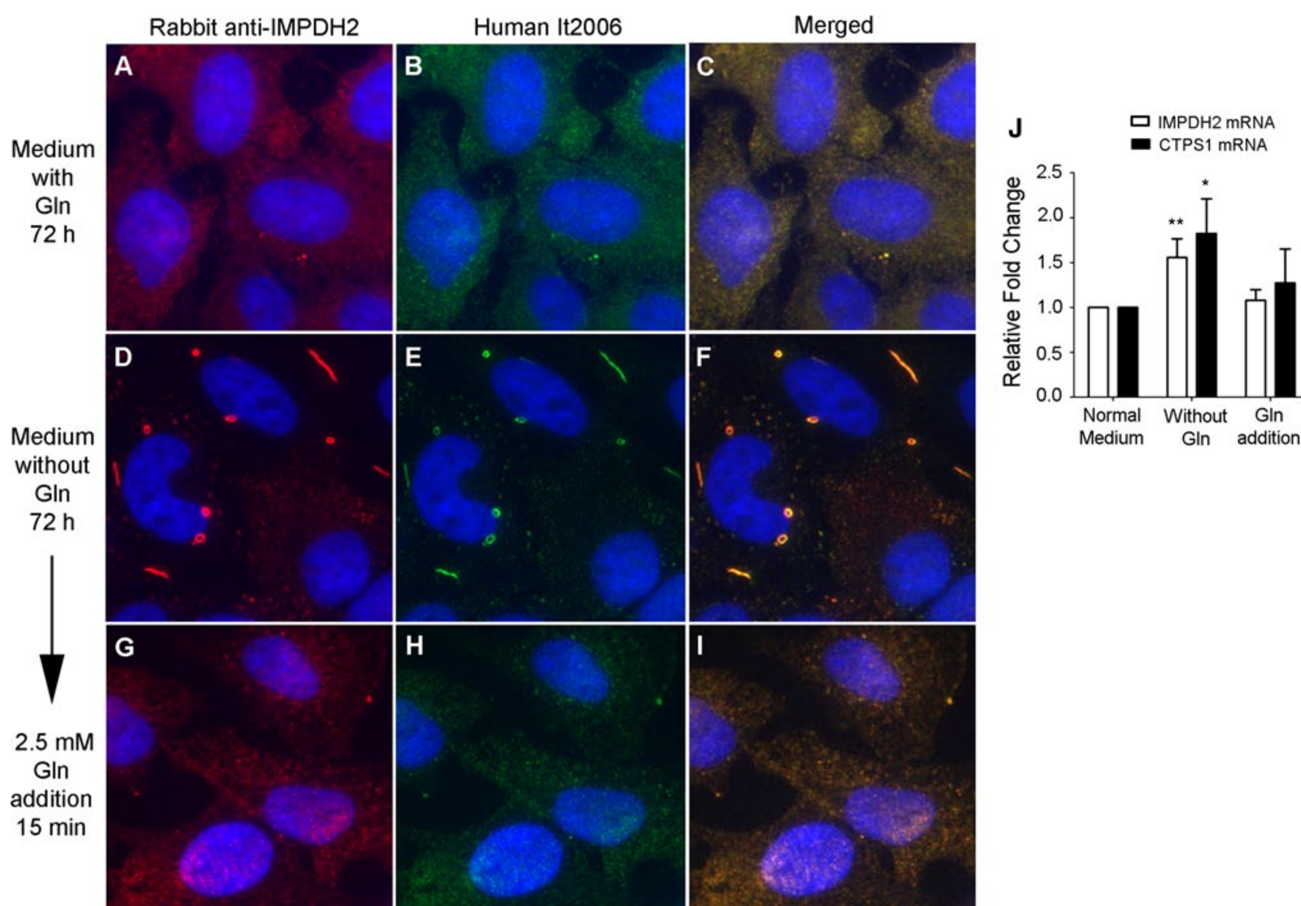


Fig. 4 Glutamine replenishment results in disassembly of RR previously formed in response to glutamine deprivation. **a–c** RR were not detected in HeLa cells maintained in normal DMEM medium with 10 % FBS. **d–f** RR formed in cells maintained in glutamine-deprived medium for 72 h. **g–i** Glutamine deprivation-induced RR disassembled when cells were supplemented with 2.5 mM glutamine for 15 min. *Scale bar* 10 µm. **j** mRNA levels of CTPS1 and IMPDH2 were elevated in glutamine-deprived cells and returned to apparently lower levels in cells supplemented with 2.5 mM glutamine after 24 h. mRNA levels were normalized to GAPDH

glutamine significantly reduced the level of IMPDH2, but not CTPS1, and concentrations of glutamine below 2.5 mM did not significantly reduce mRNA levels (Supplementary Fig. 1). The RR structures that were induced in response to glutamine deprivation could be co-stained with human prototype anti-RR serum and rabbit anti-IMPDH2, indicating that these RR are likely to be identical to previously reported RR induced by IMPDH2/CTPS inhibitors [1].

Guanosine supplementation induces disassembly of RR formed in response to glutamine deprivation

Glutamine is involved in several pathways, and its requirement in the purine and pyrimidine biosynthesis pathways (Fig. 1a) is notable for this discussion. Glutamine is required in the first and fourth step of the de novo pathway of purine biosynthesis. In addition, glutamine is required for the synthesis of GMP from IMP. In the pyrimidine biosynthesis pathway, glutamine is utilized by CTPS1 in the conversion of UTP to CTP. Thus, depletion of glutamine prevents the formation of both GTP and CTP.

Intracellular levels of CTP and GTP can be increased by adding cytosine and guanosine, respectively, to culture medium [31, 32]. Since previous publications have shown that guanosine can prevent or reverse RR structures induced by mycophenolic acid [7, 8], we decided to test the effects of guanosine addition on RR induced by glutamine deprivation. However, since guanosine is a ribonucleoside whereas cytosine is a nucleobase, the ribonucleoside cytidine was also examined. RR induced in response to glutamine deprivation disassembled in a concentration-dependent manner upon the addition of guanosine, but not cytosine or cytidine. HeLa cells deprived of glutamine for 72 h showed abundant RR (Fig. 5a). Addition of 1 mM guanosine for 24 h, however, resulted in the complete disassembly of RR induced by glutamine deprivation (Fig. 5b); 24 h treatment with 1 mM cytosine, cytidine, uridine, or thymidine had no impact on disassembly of RR (Fig. 5c–e and data

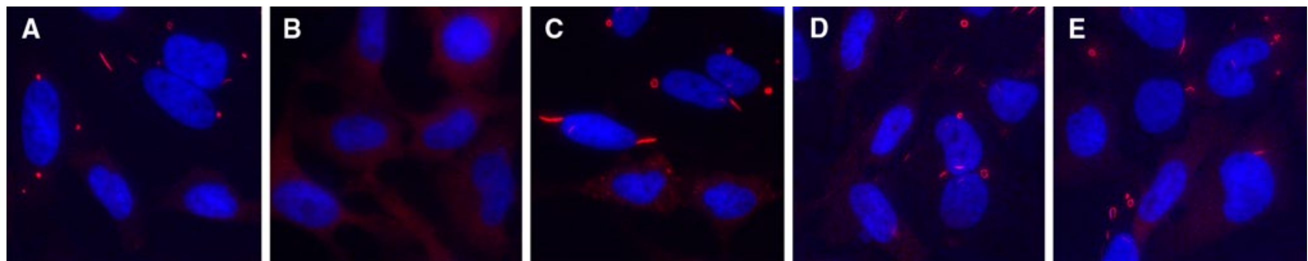


Fig. 5 Supplement of guanosine induces the disassembly of RR formed in response to glutamine deprivation. RR are detected in HeLa cells maintained in medium with glutamine for 72 h (**a**). Glutamine-deprived cells are treated with either 1 mM guanosine for 15 min (**b**) or 1 mM cytosine (**c**), 1 mM cytidine (**d**), or 1 mM uridine (**e**) for 24 h. The addition of guanosine was able to completely disassemble RR structures at various time points from 15 min to 24 h (0 % of cells contained RR after treatment with guanosine), while cytosine, cytidine, and uridine did not affect RR at any time point examined. *Scale bar* 10 μm

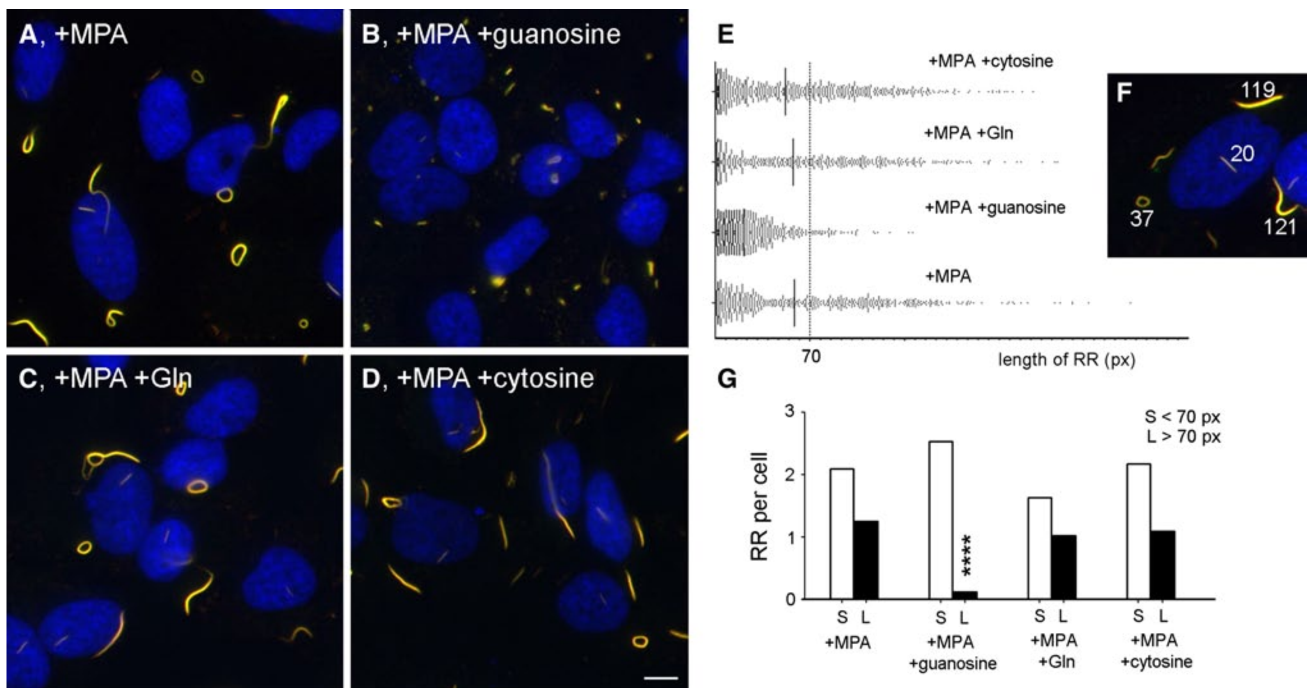


Fig. 6 RR induced with MPA disassemble after replenishing growth media with guanosine. HeLa cells were treated with 1 mM MPA for 24 h to induce RR formation (**a**). Replicate wells were treated with 1 mM guanosine (**b**), 2.5 mM glutamine (**c**), or 1 mM cytosine (**d**). *Scale bar* 10 μm. Length of rods defined in pixel density (px) were quantitated by Mayachitra Imago and plotted for each condition (**e** >300 cells analyzed for each). Px values for typical RR structures are shown (**f**). By defining short rods (S) as <70 px and long rods (L) as >70 px, the number of short and long RR structures are plotted for each condition (**g**). It is acknowledged that this rough quantitation may not absolutely differentiate length vs. thickness of rods

not shown). In a separate experiment to determine the time points of guanosine-induced disassembly of glutamine deprivation-induced RR, guanosine was able to disassemble all RR in as little as 15 min (Supplementary Fig. 2).

MPA-induced RR also disassemble after replenishing with guanosine

Mycophenolic acid (MPA) is a reversible IMPDH2 inhibitor that also induces RR formation in a concentration-dependent manner (Fig. 6a). MPA can induce RR in as little as 30 min when present at concentrations as low as 2 μM. Addition of guanosine to cells treated with MPA for 24 h led to a decrease in the size of RR (Fig. 6b). However, MPA-induced RR in cells treated with glutamine or cytosine were unaffected (Fig. 6c, d). Cells treated with MPA had longer RR and contained more long RR compared to cells treated with guanosine (Fig. 6e–g; p value = 0.0001). Long rods were taken to be those structures having more than 70 pixels. Guanosine-treated cells

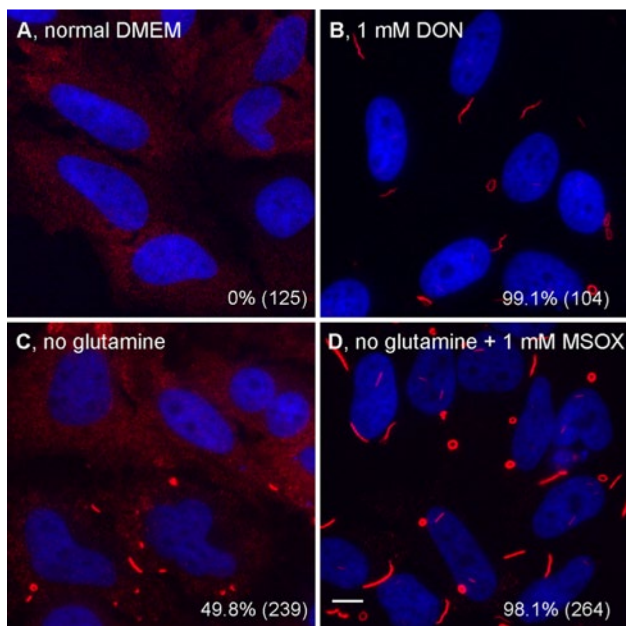


Fig. 7 Inhibition of glutamine synthetase increases RR formation in glutamine-deprived HeLa cells. RR were not detected in cells maintained in normal DMEM medium with 10 % FBS (**a**). HeLa cells that were treated with 1 mM DON (**b**), deprived of glutamine (**c**), or deprived of glutamine and then treated with 1 mM methionine sulfoximine (MSOX) for 24 h (**d**) induced RR formation. The average percentage of cells with RR are displayed in the *lower right corner* with total number of cells counted in *parentheses*. *Scale bar* 10 µm

typically had two or more short rods per cell on average, and only one out of ten cells contained noticeably longer RR structures. In this analysis, rings were scored as linearized rods.

Inhibition of glutamine synthetase increases RR formation

While manipulation of exogenously added glutamine doubtlessly affects both cell growth and RR formation, HeLa cells contain glutamine synthetase and therefore can produce glutamine to make up for the lack of glutamine in the growth medium. Intracellular levels of glutamine are thus the sum of glutamine supplied by extracellular supplementation and that formed by cytoplasmic glutamine synthetase. We therefore carried out experiments to determine whether an inhibitor of de novo glutamine synthesis would affect the formation of RR. DON is a glutamine analogue that functions as an inhibitor of both CTPS1 and glutamine synthetase. It was previously shown that DON can induce RR formation in a concentration-dependent manner [1]. As expected, treatment with 1 mM DON for 24 h induced RR formation (Fig. 7b). Another inhibitor, methionine sulfoximine (MSOX), is specific for glutamine synthetase, forming nearly irreversibly-bound methionine sulfoximine-phosphate upon ATP-dependent phosphorylation in the active site of mammalian glutamine synthetase [17]. MSOX was therefore used to determine if specific inhibition of glutamine synthetase could induce RR assembly. We found that treatment of HeLa cells with 1 mM methionine sulfoximine alone did not induce RR assembly, presumably because uptake of exogenous glutamine could bypass any requirement for endogenous glutamine synthesis. However, when cells were maintained in glutamine-deficient DMEM and then treated with 1 mM methionine sulfoximine, RR formed in ~98.1 % of cells, a much higher percentage of cells than in the absence of this inhibitor (~49.8 %, Fig. 7c, d). Methionine sulfoximine showed similar effects at a range of concentrations from as low as 100 nM up to 4 mM (data not shown). Our findings suggest that blockade of de novo glutamine synthesis, when carried out in combination with deprivation of extracellular glutamine, has a great impact on RR formation.

Discussion

Earlier work demonstrated that glucose deprivation is sufficient to induce the formation of CTP synthetase-containing filaments in yeast [33]. Snake-like structures, or cytoophidia, are RR-like filaments composed of CTPS in human and *Drosophila* cell lines treated with DON or azaserine [3]. These glutamine amidohydrolase inhibitors are known to interfere with biosynthetic pathways requiring glutamine for the synthesis of purines, pyrimidines, amino acids (e.g., histidine, tryptophan, etc.), *N*-acetylglucosamine, *p*-aminobenzoate, as well as other nitrogenous metabolites requiring biosynthetic enzymes that have glutamine-hydrolyzing subunits. Such findings and the new findings presented in this report support the idea that the RR structures accumulate as a nutrient-deprivation stress response. Additionally, glucose deprivation in hamster fibroblasts affected the levels (pool sizes) of intracellular UTP, GTP, and CTP [34], suggesting that imposed limitations of glucose and glutamine availability may reduce cellular CTP and GTP to levels that favor the formation of filaments in yeast and RR in HeLa cells [33].

In our experience, rod and ring assembly induced by glutamine deprivation is a slow, time-dependent process. At the earliest stage, we consistently observe the appearance of diffuse cytoplasmic staining, followed by the assembly of short, immature rods, eventually culminating in the formation of recognizable RR structures. In the present study, RR form in response to glutamine deprivation, requiring a minimum of 24 h for short rods to be visualized. Some visibly shorter (or immature) rods were observed at earlier times and these structures appear to aggregate, reaching full size (mature form) by 72 h after glutamine starvation is imposed. At intermediate times (48 h), we find that there

is a noticeable decline in diffuse staining, suggesting that smaller oligomeric forms are assimilated into maturing RR structures. At 72 h, when fully mature RR are formed, there is almost no diffuse cytoplasmic staining, suggesting that a majority of immature RR have been incorporated into the final RR structures. Interestingly, our experience shows that cell sensitivity to glutamine deprivation appears to vary widely between different cell types, and even between cell batches. We have replicated this work in different batches of HeLa cells, but have occasionally encountered certain batches requiring modifications to culture conditions for optimal visualization of the effects of this process. In our earlier experiments, RR were induced in HeLa cells grown in 10 % FBS and no glutamine, with maximum RR formation occurring as early as 48 h. We subsequently found that some cells required FBS to be reduced to 5 % and 72 h to realize the full effects of the RR assembly. Thus, this system appears to be highly sensitive to various experimental conditions. We hypothesize that the activity level of glutamine synthetase or other metabolic demands for glutamine may also be variables. Another variable is likely to be the glutamine content of multiple lots of FBS used during the course of this work, perhaps explaining the need to reduce FBS from 10 to 5 % for maximum appearance of mature RR.

Mounting evidence suggests that many cell types rely on circulating glutamine to augment marginal levels of cellular glutamine synthesis. This behavior, referred to as "glutamine addiction" by some researchers, suggests that many cells are unable to make enough glutamine to meet their needs during periods of elevated amino acid and nucleotide synthesis [35]. Such behavior must be considered whenever RR formation exhibits variability.

Glutamine deprivation prevents the formation of CTP and GTP by affecting enzymes in the purine and pyrimidine biosynthesis pathways [18, 36]. Cytosine and guanosine are known to be transported into HeLa cells, where they are metabolically phosphorylated to form CTP and GTP. The notion that RR formation is a consequence of decreased intracellular levels of GTP is supported by our finding that exogenously supplied guanosine can reverse RR formation, as well as previous work done by other laboratories [7, 8]. The inability of cytosine or cytidine to disassemble the RR structures (Fig. 5c, d), the lack of increase in CTPS protein levels after glutamine deprivation (Fig. 2f), and the fact that antibodies to CTPS were unable to recognize RR structures induced by glutamine deprivation (data not shown) lead us to question whether decreased CTP pools are directly related to RR induced by glutamine deprivation, and thus it is possible that CTPS is not incorporated into mammalian RR under these conditions. As several groups, including us [6, 23, 24], have speculated, it is likely that IMPDH is the major protein involved in polymerization of the mammalian form of these structures, although it is possible that the composition of mature RR structures could vary when intracellular conditions change. Clearly, the assembly of RR is a complex process that will require significant work to elucidate the varying roles that CTP and GTP biosynthesis pathways are playing in this system, especially since CTPS is a well-documented component of RR-like cytoophidia in human and *Drosophila* cell lines, as well as in filaments found in yeast [3, 32, 36]. Our findings also suggest that, when cellular nucleotide pools are replenished by exogenously supplied guanosine, RR structures undergo reversible disassembly. Whatever the mechanism, it is clear that the assembly of RR from the polymerization of IMPDH2 (and potentially CTPS1 under certain conditions) is an adaptive stress response to decreased production of GTP (after glutamine deprivation) or inhibition of CTPS1 or IMPDH2 enzymatic activity (by DON, azaserine, and other small-molecule inhibitors). Our experiments with methionine sulfoximine, a highly selective glutamine synthetase inhibitor, support the conclusion that formation of RR structures is caused by glutamine deficiency. Notably, RR formation is most prominent when this inhibitor is used in combination with glutamine deprivation, occurring in a very high percentage (~98.1 %) of cells (compare with a ~99.1 % value found with DON), although the full effect is not obtained until 72 h. The observed effects of methionine sulfoximine are thus consistent with our hypothesis that decreased intracellular glutamine strongly promotes RR assembly.

A previous study also showed that MPA-induced RR-like filaments could disassemble upon incubation of cells with guanosine [7]. Our data confirmed this finding when MPA-treated HeLa cells were supplemented with guanosine, but not with glutamine or cytosine. MPA targets IMPDH2 enzymatic activity, preventing the formation of xanthosine 5′-monophosphate, an essential precursor in the synthesis of GMP, GDP and GTP. Replenishing GTP by the addition of guanosine after IMPDH2 inhibition leads to RR disassembly, whereas replenishing CTP levels does not. We would therefore suggest that GTP is the most likely metabolite controlling the extent of RR formation.

In summary, the present study demonstrates that glutamine availability plays a major role in the assembly and disassembly of rod and ring structures assembled from IMP dehydrogenase-2 (IMPDH2). Glutamine depletion favors IMPDH2 polymerization to form RR structures, whereas restoration of glutamine results in the depolymerization of these supramolecular structures. Given GTP's central involvement in signal transduction and CTP's multiple roles in pyrimidine and phospholipid synthesis, we are also mindful that cells are likely to be endowed with many options for regulating RR formation. Nutrient deprivation often engages a variety of transcriptional and translational

mechanisms that regulate enzyme synthesis and turnover. Our findings suggest RR assembly/disassembly actively responds to nutrient-related stresses, but the impact of RR formation on cellular metabolism (e.g., nucleotide metabolism, G-protein signaling, and GTP-requiring enzymes) remains to be determined. Such efforts promise further insight about whether RR formation regulates cellular metabolism. Future work must also assess whether RR formation changes the kinetics and/or regulation of IMPDH and CTPS or whether RR assembly/disassembly controls interactions with other cellular components. In any case, our observations already show that RR assembly/disassembly is a highly dynamic process and is likely to be metabolically significant.

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