Supporting Information

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SI Methods

Chemicals. MKT-077, 4,5,6,7-tetrabromobenzimidazole (TBI), geranylgeranylacetone, celastrol methotrexate, and methylene blue were purchased from Sigma-Aldrich; dithiobis(succinimidyl propionate) (DSP) was purchased from Thermo Scientific; 4,5,6,7-tetrabromobenzotriazole (TBB) was purchased from Calbiochem (EMD biosciences); 17-*N*-allylamino-17-demethoxygeldanamycin (17-AAG) and NVP-AUY922 were purchased from Selleck Chemicals; and pifithrin-μ (2-phenylethynesulfonamide) was purchased from Tocris Bioscience.

Plasmids. The constructs of six human enzymes involved in the de novo purine biosynthetic pathway and the tetrahydrofolate(H_4F)-using enzyme (hC1THF) studied in this work were previously used by our laboratory; the plasmids are hFGAMS-EGFP, hFGAMS-OFP, hTrifGART-GFP, hPPAT-EGFP, hPAICS-EGFP, hASL-EGFP, GFP-hATIC, and hC1THF-EGFP. The fluorescent protein fusion vectors pmEGFP-N1 and pmOFP-N1 were modified from pEGFP-N1 (Clontech) and pRSET_mOrange (a gift from R. Y. Tsien, University of California, San Diego, CA) as described previously (1). G3BP-GFP was the gift from the Jamal Tazi group (Institut de Genetique Moleculaire de Montpellier, Montpellier, France). GFP170* and GFP250 were gifts from Elizabeth Sztul (University of Alabama at Birmingham, Birmingham, AL).

The *SHMT1* gene was a gift from Patrick Stover (Cornell University, Ithaca, New York), the *BAG5* gene was purchased from ATCC, and cDNAs of other enzymes were obtained from the Arizona State University Biodesign Institute Plasmid Repository (DNASU). All genes were amplified by PCR with primers containing two restriction sites. NheI and BamHI were used for heat shock protein 90 (HSP90; Swiss-Prot accession no. P08107); NheI and KpnI were used for heat shock protein 70 (HSP70; P07900); XhoI and BamHI were used for DnaJC7 (Q99615); EcoRI and XhoI were used for BAG5 (Q9UL15); and NheI and EcoRI were used for p23 (Q15185), BAG2 (O95816), Stip1(P31948), and SHMT1. The PCR product was introduced into the pmEGFP-N1 or pmOFP-N1 to obtain the green fluorescent protein (GFP) or orange fluorescent protein (OFP) fused protein construct. The formylglycinamidine ribonucleotide synthase (FGAMS)-9xcMyc used for the immunoprecipitation (IP) experiment contains a –9xMyc-6xHis tag (subcloned from pYL436 vector from ABRC) and was constructed by inserting the *FGAMS* gene using NheI and EcoRI sites and the tag region using the EcoRI and NotI sites into the mEGFP vector. The HSP90G97D and HSP70K71E mutants were made by site-directed mutagenesis. All of the gene inserts were confirmed by DNA sequencing. All plasmids were transformed into the XL1-Blue or DH5α competent cells and isolated by the QIAprep Spin Miniprep Kit (Qiagen).

Antibodies and siRNA. The anti-Hsp70 antibody (ab74082), anti-Hsp90 (ab13492), and anti-FGAMS (anti-PFAS from abcam, ab82755) were from Abcam, and anti–β-actin (sc-47778) was from Santa Cruz Biotechnology. The antibody for DnaJC7 (sc-100716) and DnaJA1(sc-47051) were from Santa Cruz Biotechnology, and the DnaJC14 (ab129398) antibody was from Abcam. siRNA against DnaJC7, DnaJA1(HSP 40–4) siRNA, DnaJC14 siRNA, and control siRNA was purchased from Santa Cruz Biotechnology.

Mammalian Cell Culture. Three human cancer cell lines, HeLa, C3A, and A431, were obtained from the American Type Culture Collection. Cells were maintained in MEM with Earle's salts and L-glutamine (Mediatech) supplemented with 10% FBS (Atlanta Biologicals). In the purine-depleted condition, cells were maintained in RPMI 1640 with L-glutamine (Mediatech) supplemented with 5% dialyzed FBS. Purines in FBS were removed by membrane (25 kDa molecular weight cutoff) dialysis against 0.9% NaCl at 4 °C for 2 d.

Coimmunoprecipitation. hFGAMS-9xcMyc was transiently transfected into HeLa cells grown in purine-rich and purine-depleted media. Formation of purinosomes in the transfected cells grown in purine-depleted medium was enriched by incubating the cells with 23 μM (10 μg/mL) TBI at room temperature for 1 h (2). To capture the cellular interaction network within the purinosome, DSP cross-linking was performed before harvesting the cells. The commercially available c-Myc monoclonal antibody cross-linked to protein A that is immobilized to agarose beads (Clontech) was applied to immunoprecipitate c-Myc–tagged hFGAMS and its interacting partners. HeLa cells growing in the purine-depleted medium (samples A, B, and C) and purine-rich medium (samples D, E, and F) were compared. Mock transfection controls (A, B, D, and E) and controls without TBI treatment (A) or without TBB treatment (D) were carried out to rule out any possibilities of nonspecific interactions. The presence of hFGAMS-9xcMyc (~162 kDa) only in the final IP eluate samples C and F was confirmed by Western blot using the anti-cMyc antibody (Abcam ab9132; Fig. S1). A further tandem MS analysis was used to identify protein components of the purinosome (Table S1 and Dataset S1).

Analysis of MS Data. RAW files were generated from mass spectra using XCalibur version 1.4, and MS/MS spectra data were extracted using RAW Xtractor (version 1.9.1), which is publicly available (http://fields.scripps.edu/?q=content/download). MS/MS spectral data were searched using the Prolucid algorithm (version 3.0) against a custom-made database containing 22,935 human sequences [longest entry for the International Protein Index (IPI) database for each protein] that were concatenated to a decoy database in which the sequences for each entry in the original database were reversed. In total, the search database contained 45,870 protein sequence entries (22,935 real sequences and 22,935 decoy sequences). SEQUEST searches allowed for oxidation of methionine residues (16.0 Da), DSP modification (145.0 Da K, R, and M), static modification of cysteine residues (57.0 Da due to alkylation), no enzyme specificity, and a mass tolerance set to ±1.5 Da for precursor mass and ±0.5 Da for product ion masses. The resulting MS/MS spectra matches were assembled and filtered using DTASelect2 (version 2.0.27). The validity of peptide/spectrum matches was assessed using DTASelect2 (version 2.0.27) and two SEQUEST-defined parameters: the cross-correlation score (XCorr) and normalized difference in cross-correlation scores (DeltaCN). The search results were grouped by charge state (+1, +2, +3), tryptic status, and modification status (modified and unmodified peptides), resulting in 18 distinct subgroups. In each of these subgroups, the distribution of Xcorr and DeltaCN values for the direct and decoy database hits was obtained, and then the direct and decoy subsets were separated by discriminant analysis. Outlier points in the two distributions were discarded. Full separation of the direct and decoy subsets is not generally possible, so the discriminant score was set such that a false discovery rate of <1% was determined based on the number of accepted decoy database peptides (number of decoy database hits/number

of filtered peptides identified × 100). In addition, a minimum peptide length of seven amino acids residues was imposed, and protein identification required the matching of at least two peptides per protein. Such criteria resulted in the elimination of most decoy database hits.

Fluorescence Live Cell Imaging. Cells were imaged at ambient temperature (~25 °C) under a Nikon TE-2000E inverted microscope equipped with a 60× 1.49 numerical aperture objective and a photometrics CoolSnap ES^2 CCD detector. GFP detection was accomplished by using an S484/15× excitation filter, S517/30m emission filter, and Q505LP/HQ510LP dichroic (Chroma Technology). The OFP signal was obtained by using an S555/25× excitation filter, S605/40m emission filter, and Q575LP/HQ585LP dichroic (Chroma Technology). Nikon NIS-Elements (3.0) was used for collecting images samples, which were viewed using a mercury fiber illuminator. Each sample was washed three times for 5-min incubations with buffered saline solution [BSS: 20 mM Hepes (pH 7.4), 135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 5.6 mM glucose] before imaging. In the reversibility imaging experiment, cells were incubated in the 37 °C, 5% CO_2 incubator between images captured at different time points. All images were created using the ImageJ program and were in some cases cropped, inverted, or shown in color for clarity, but were otherwise unmodified. Colocalization analyses were performed with the JACoP plugin (3) in ImageJ. The threshold for each image was created using the Auto Local Threshold tool using the Sauvola method. The resulting images were used for colocalization analyses. To investigate the effect of HSP inhibitors/activators on purinosome formation, the small molecule was added into the growth medium, and the cells were incubated for an additional 1–2 h inside the incubator; images were acquired before and after the incubation.

Luciferase Reporter Assay. For the transfection for this protocol, HeLa cells suspended in purine-rich or purine-depleted medium without antibiotics were inoculated on a 24-well cell culture plate (BD) at 30,000 or 40,000 cells per well densities. The following day, the cells were transfected with XtremeGene transfection reagent (Roche), according to the manufacturer's protocol. Briefly, 100 μL of cell culture medium containing no serum was mixed with plasmids and XtremeGene reagent and incubated at room temperature for 15 min before being added into a well on the 24-well cell culture plate. For each well, 100 ng of TRE-TIGHT firefly luciferase construct, 100 ng of tobacco etch virus (TEV) cleavage site-tTA fusion construct, 100 ng TEV only or TEV fusion construct, and 3 μL XtremeGene reagent were used. When measuring luciferase signal induced by tTA fusion protein in the absence of TEV or TEV fusion protein, 100 ng pEGFP-N1 plasmid was used in place of TEV or TEV fusion plasmid. When measuring normalized luciferase expression, an additional 10 ng of renillar luciferase expression plasmid (Promega) was added to each well. After adding the transfection reagent, the plate was returned to the cell culture incubator and incubated for 2 h before being rinsed and replaced with fresh medium. The plates of transfected cells were then incubated for 2 d before being using for the luciferase assay.

For the assay, cells were harvested, and luciferase expression was measured with a luciferase assay kit (Biotium), according to the manufacturer's protocol. Briefly, after removing cell culture media, cells in 24-well plates were rinsed with PBS and kept on ice. Cell lysis buffer (100 μL) was added to each well. The plates, on ice, were placed on a rocking platform with gentle rocking for 15 min before beginning the luciferase assay. Luciferase assay buffer (100 μL) was mixed with 20 μL cell lysate from each well, and luminescence was measured on a Lumat LB 9501 luminometer (Berthold).

1. An S, Kumar R, Sheets ED, Benkovic SJ (2008) Reversible compartmentalization of de novo purine biosynthetic complexes in living cells. *Science* 320(5872):103–106.
2. An S, Kyoung M, Allen JJ, Shokat KM, Benkovic SJ (2010) Dynamic regulation of a metabolic multi-enzyme complex by protein kinase CK2. *J Biol Chem* 285(15): 11093–11099.
3. Bolte S, Cordelieres FP (2006) A guided tour into subcellular colocalization analysis in light microscopy. *J Microsc* 224(Pt 3):1365–2818.

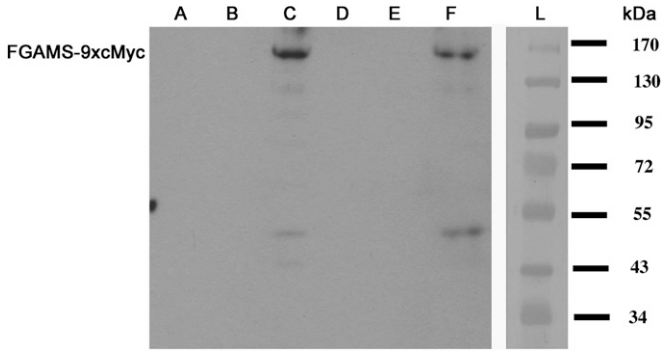

Fig. S1. Western blot showing FGAMS-9xcMyc (162 kDa) was pulled down by anti-cMyc antibody in the co-IP. Lanes A, B, C, D, E, and F are co-IP eluates from HeLa cells growing or treating under different conditions: only C (purine-depleted medium) and F (purine-rich medium) are transfected with hFGAMS-9xcMyc. Lane L is protein standard.

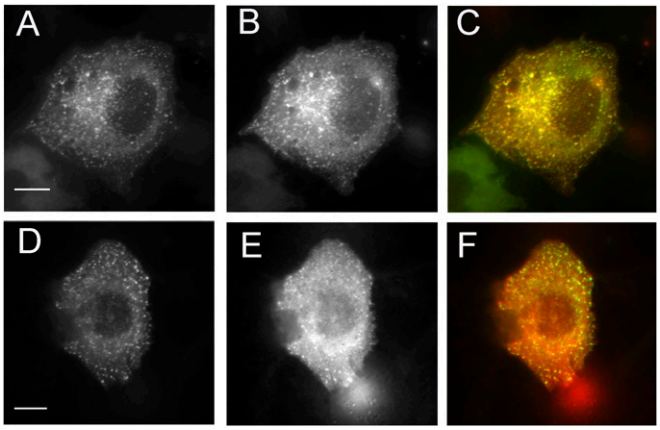

Fig. S2. Cellular colocalization of FGAMS-GFP and Hsp90-OFP in C3A and A431 cells. Human liver cancer cell line, C3A (*A*–*C*), and human skin cancer cell line, A431 (*D*–*F*), were grown in purine-depleted conditions. FGAMS-GFP (*A*, *D*, and green in *C* and *F*) colocalized with Hsp90-OFP (*B*, *E*, and red in *C* and *F*) in both cells. *C* and *F* show merged images of *A* and *B*, and *D* and *E*, respectively. (Scale bar, 10 μm.)

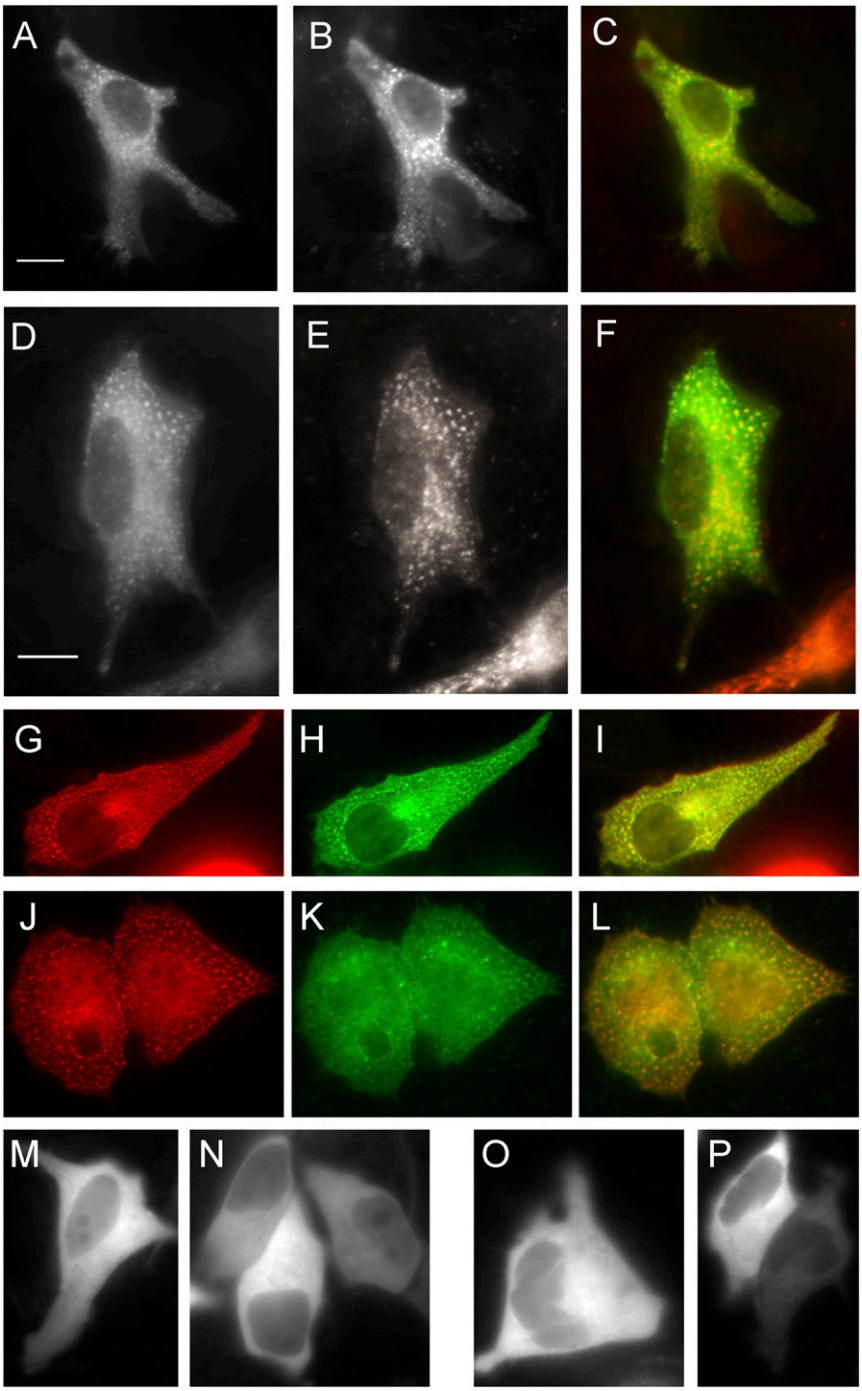

Fig. S3. Cellular colocalization of phosphoribosylpyrophosphate amidotransferase (PPAT)-GFP and Hsp90-OFP, PAICS-GFP, and Hsp90-OFP in HeLa cells grown in purine-depleted conditions. PPAT (*A*–*C*) is the enzyme that catalyzes the first step in the purine de novo biosynthetic pathway, whereas PAICS (*D*–*F*) is the sixth step. Both PPAT-GFP (*A* and green in *C*) and PAICS (*D* and green in *F*) are colocalized with Hsp90-OFP (*B*, *E*, and red in *C* and *F*) in HeLa cells. *G*–*L* show the cellular colocalization of Hsp70-OFP and PPAT-GFP or PAICS-GFP in HeLa cells grown in purine-depleted conditions. Both PPAT-GFP (*H* and green in merged image *I*) and PAICS (*K* and green in merged image *L*) are colocalized with Hsp70-OFP (*G*, *J*, and red in *I* and *L*) in HeLa cells. *M*–*P* show Hsp70 and Hsp90 in the absence of purinosome proteins. Hsp90-GFP (*M* and *N*) and Hsp70-GFP (*O* and *P*) show a diffuse staining pattern when expressed in HeLa cells grown in purine-depleted media. (Scale bar, 10 μm.)

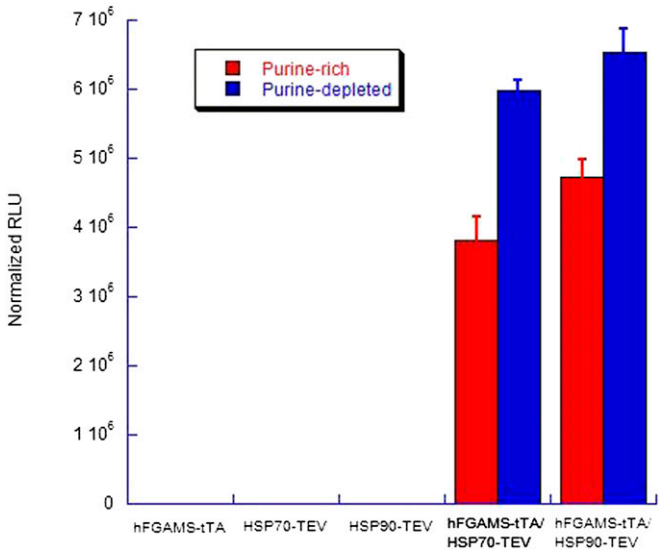

Fig. S4. Luciferase reporter assay of the interaction between FGAMS and HSP70 or HSP90. In this method, a transcriptional reporter is tagged to the C terminus of FGAMS, whereas the sequence intervening the protein and the tag contains a TEV protease recognition site. The Hsp70 or Hsp90 is fused to a modified form of TEV protease, which, if the two proteins (FGAMS-tTa and Hsp70 or 90-TEV) interact closely, will cleave the transcription factor. This factor is targeted to the nucleus where it turns on the production of firefly luciferase. The details of the assay have been previously reported (1). The interaction between FGAMS and HSP79 or HSP90 was measured by firefly luciferase activity (RLU) and normalized by renilla luciferase activity. When the FGAMS-tTA and the reporter constructs were introduced into HeLa cells in the absence of the Hsp70-TEV or Hsp90-TEV construct, the expression of the firefly reporter gene was at a very low level. When either Hsp70-TEV or Hsp90-TEV was introduced with FGAMS-tTA and the reporter constructs, the expression of firefly luciferase was greatly increased, indicating that FGAMS and HSP70/HSP90 associate closely in vivo. Note that for the FGAMS-tTA, HSP70-TEV, and HSP90-TEV controls, the normalized RLU values are 1,382/2,182, 4,977/5,525, and 2,404/3,115 for purine-rich/purine-depleted media, respectively, and thus do not show up on Fig. S4 above, given the scale used.

1. Deng Y, et al. (2012) Mapping protein-protein proximity in the purinosome. *J Biol Chem* 287(43):36201–36207.

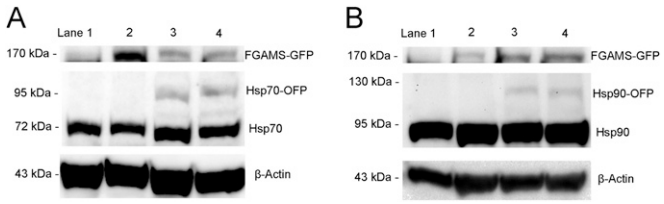

Fig. S5. (*A*) Western blot of HeLa cells cotransfected with FGAMS-GFP and Hsp70-OFP or Hsp70K71E-OFP. Lane 1 is cells with mock transfection, lane 2 is cells transfected with FGAMS-GFP and OFP, lane 3 is cells transfected with FGAMS-GFP and Hsp70-OFP, and lane 4 is cells transfected with FGAMS-GFP and Hsp70K71E-OFP. β-Actin, loading control. (*B*) Western blot of HeLa cells cotransfected with FGAMS-GFP and Hsp90-OFP or Hsp90G97D-OFP. Lane 1 is cells with the mock transfection, lane 2 is cells transfected with FGAMS-GFP and OFP, lane 3 is cells transfected with FGAMS-GFP and Hsp90-OFP, and lane 4 is cells transfected with FGAMS-GFP and Hsp90G97D-OFP. β-Actin, loading control. Note that the low level of Hsp70/90-OFP observed in our Western blots is most likely due to the poor transfection efficiency obtained for these constructs (~30%).

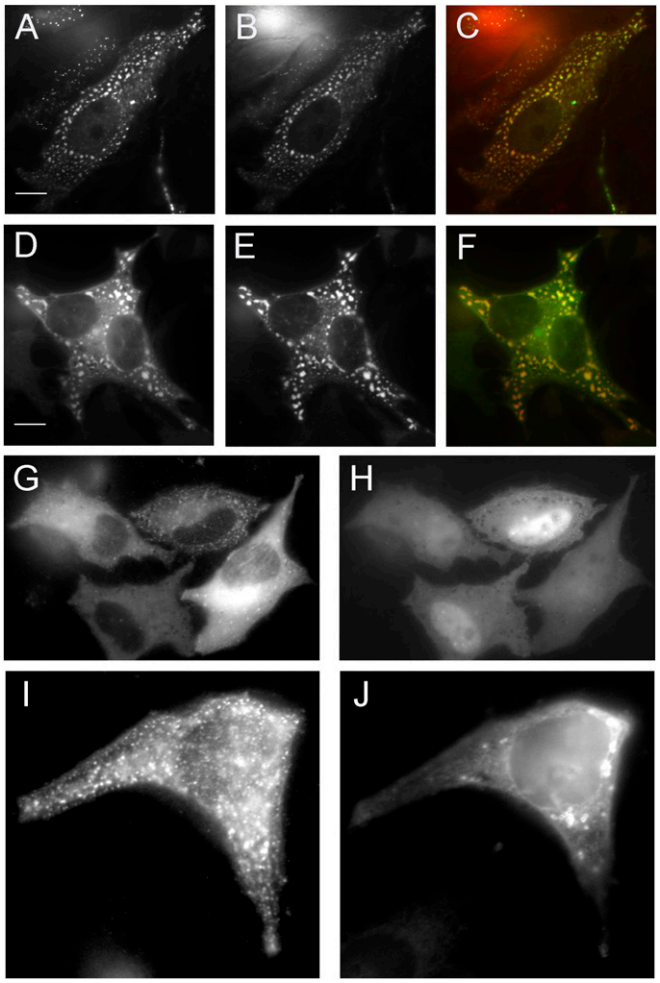

Fig. S6. Cellular colocalization of FGAMS-OFP and cochaperone BAG2-GFP and DnaJA1-GFP in HeLa cells grown in purine-depleted conditions. BAG2-GFP (*A* and green in *C*), DnaJA1-GFP (*D* and green in *F*), and Hsp90-OFP (*B*, *E*, and red in *C* and *F*). *C* and *F* show merged images of *A* and *B*, and *D* and *E*, respectively. *G–J* show DnaJ proteins that were not identified in IP do not colocalize with purinosomes. Fluorescence microscopy of HeLa cells cotransfected with FGAMS-OFP (*G* and *I*) and DnaJ-B1-GFP (*H*) or DnaJ-C14-GFP (*J*) revealed that these J-domain proteins do not colocalize with purinosomes. (Scale bar, 10 μm.)

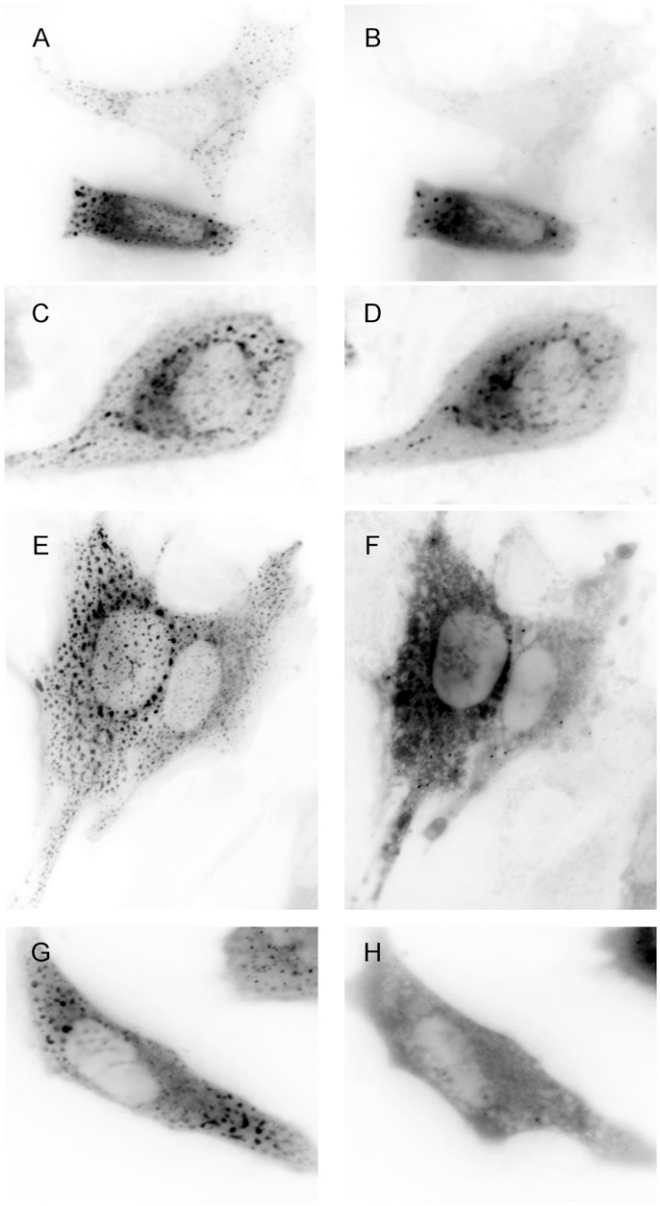

Fig. S7. Disruption of purinosomes by Hsp90 and Hsp70 inhibitors. HeLa cells grown in purine-depleted media before addition of drug (*A, C, E*, and *G*) and 30 min after addition of 250 μM methylene blue (*B*), 100 μM pithrin-μ (*D*), 15 μM MKT-077 (*F*), or 20 μM 17-AAG (*H*) (note: an excess amount of 17-AAG was used due to its poor solubility; the expected actual concentration is ~20 μM).

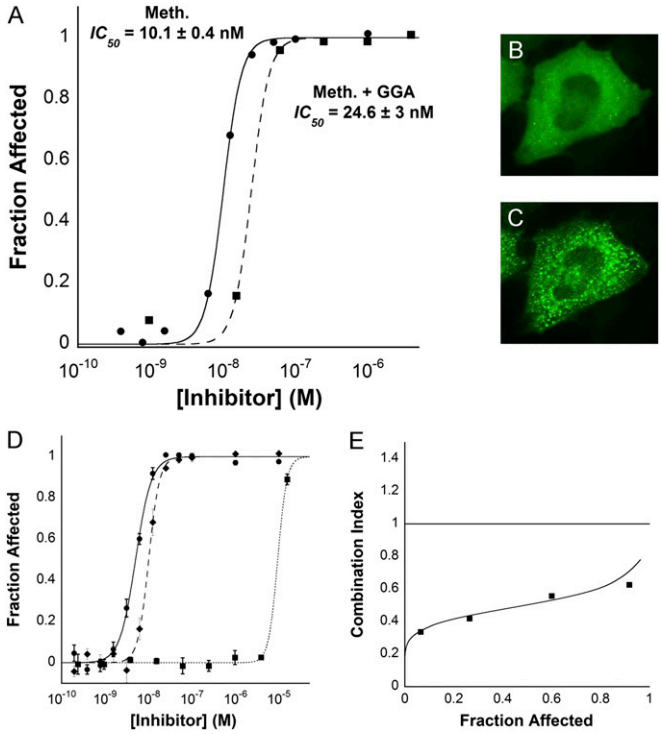

Fig. S8. The Hsp70 inducer, geranylgeranylacetone, has a protective effect on HeLa cells treated with methotrexate. (*A*) Treatment of HeLa cells with methotrexate alone for 72 h kills cells with an IC_{50} of 10.1 ± 0.4 nM (filled circles and solid line), whereas treatment of cells with a 1:1 combination of methotrexate and geranylgeranylacetone (filled squares and dashed line) yields an IC_{50} of 24.6 ± 3 nM. Geranylgeranylacetone treatment alone did not effectively kill cells at the concentrations used in our assay (up to 1 mM). Treatment of HeLa cells with geranylgeranylacetone stimulates purinosome formation. HeLa cells, transfected with FGAMS, grown in purine-depleted conditions before (*B*) and after treatment with geranylgeranylacetone (1 mM) for 85 min (*C*). *D* and *E* show the synergistic effect of the Hsp70 inhibitor 2-phenylethynesulfonamide (Pifithrin-μ) with purine biosynthesis inhibitor methotrexate. (*D*) Treatment of HeLa cells for 72 h with Pifithrin-μ alone (solid squares, dotted line) yields an IC_{50} of 9.4 ± 0.8 μM, methotrexate alone (solid diamonds, dashed line) gives an IC_{50} of 10.1 ± 0.4 nM, and the combination of the two inhibitors (solid circles, solid line) gives a twofold decrease in the IC_{50} to 5.0 ± 0.3 nM. (*E*) The Chou-Talalay plot confirms that the effect is synergistic [combination index (CI) < 1].

Table S1. Summary of selected proteins from the IP experiment described in the text

	Spectral count average of proteins from cells cultured in purine-depleted medium		Spectral count average of proteins from cells cultured in purine-rich medium	
Protein (Swiss-Prot accession no.)	Control cells without transfection	FGAMS-9xcMyc–transfected cells	Control cells without transfection	FGAMS-9xcMyc–transfected cells
FGAMS (O15067)	0	129.3	4.0	177.3
Hsp70-1A/1B (P08107)	4.0	50.0	10.5	38.3
Hsp90-alpha (P07900)	2.3	8.3	15	8.3
Bag2 (O95816)	0	6.3	0	7.3
Bag5 (Q9UL15)	0	1.0	0	3.7
DnaJ A1 (P31689)	0.7	1.3	0.5	4.0
DnaJ C7 (Q99615)	0	1.3	0	1.7
Stip1 (P31948)	0	7.0	2.5	4.3
ATIC (P31939)	0.7	1.7	3.5	1.3
PAICS (P22234)	0	1.0	1.5	0

Spectral count shows the total number of spectra identified for a protein, which suggests the relative abundance of proteins in the proteomic studies. Average number is from three repeated experiments.

Other Supporting Information Files

Dataset S1 (XLSX)