Identification of a multienzyme complex for glucose metabolism in living cells

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Sequential metabolic enzymes in glucose metabolism have long been hypothesized to form multienzyme complexes that regulate glucose flux in living cells. However, it has been challenging to directly observe these complexes and their functional roles in living systems. In this work, we have used wide-field and confocal fluorescence microscopy to investigate the spatial organization of metabolic enzymes participating in glucose metabolism in human cells. We provide compelling evidence that human liver-type phosphofructokinase 1 (PFKL), which catalyzes a bottleneck step of glycolysis, forms various sizes of cytoplasmic clusters in human cancer cells, independent of protein expression levels and of the choice of fluorescent tags. We also report that these PFKL clusters colocalize with other rate-limiting enzymes in both glycolysis and gluconeogenesis, supporting the formation of multienzyme complexes. Subsequent biophysical characterizations with fluorescence recovery after photobleaching and FRET corroborate the formation of multienzyme metabolic complexes in living cells, which appears to be controlled by post-translational acetylation on PFKL. Importantly, quantitative high-content imaging assays indicated that the direction of glucose flux between glycolysis, the pentose phosphate pathway, and serine biosynthesis seems to be spatially regulated by the multienzyme complexes in a cluster-size-dependent manner. Collectively, our results reveal a functionally relevant, multienzyme metabolic complex for glucose metabolism in living human cells.

Glucose metabolism involves two reciprocal pathways: glycolysis and gluconeogenesis, during which glucose flux partitions between energy metabolism and anabolic biosynthetic pathways (supplemental Fig. S1). To regulate both the flux and allocation of glucose-derived pathway intermediates, the cell needs mechanisms to coordinate the activities of the pathway enzymes. Specifically, cancer cells have evolved to control their metabolic activity by genetic alterations of isozyme expression and/or post-translational modifications of the rate-limiting enzymes of glucose metabolism, thus preferentially directing pathway intermediates into building block biosynthesis (1–3). However, spatiotemporal mechanisms controlling the direction of pathway intermediates at various metabolic nodes in single cells have not been proposed yet. Hence, the lack of such fundamental mechanisms regulating the direction of glucose flux inside a cell prevents us from comprehending glucose metabolism in normal cells and thus its metabolic alterations in human disease cells.

Meanwhile, the sequential metabolic enzymes of glucose metabolism have long been proposed to form multienzyme complexes in a variety of organisms. To date, various in vitro studies (4–15) have suggested that glycolytic enzymes in Escherichia coli, Arabidopsis, Drosophila, yeast, and protists form metabolic complexes in cells. These studies have been mainly supported by measuring individual enzyme activities from chromatographically fractionated pools of cell lysates or biochemically semipurified subcellular fractions. For instance, extensive in vitro biochemical analysis of mitochondrial fractions of plant cells demonstrated that glycolytic enzymes were associated with mitochondria in a cellular respiration-dependent manner (5, 7). In addition to such in vitro investigations, immunofluorescence imaging has demonstrated that various glycolytic enzymes in mammalian erythrocytes form a glycolytic complex on the inner surface of the erythrocyte membrane in the presence of the anion transporter band 3 protein (16–18). The assembly and disassembly of this complex was dependent on both the phosphorylation state of the band 3 protein and the oxygenation state of hemoglobin (16). The interactions between glycolytic enzymes and the band 3 protein were further supported by FRET and chemical cross-linking techniques (18, 19). Furthermore, colocalization and direct interaction between fructose-1,6-bisphosphatase (FBPase)3 and aldolase

3 The abbreviations used are: FBPase, fructose-1,6-bisphosphatase; FRAP, fluorescence recovery after photobleaching; PFKL, liver-type phosphofructokinase 1; mEGFP, monomeric form of enhanced green fluorescent protein; dFBS, dialyzed FBS; TC, tetracycline; PKM2, pyruvate kinase M2; PEPCK1, phosphoenolpyruvate carboxykinase 1; mOFP, monomeric orange fluorescent protein; RATS, robust automatic threshold selection.
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have been studied both in vitro and in myocytes (8, 9, 20, 21), proposing the formation of metabolic complexes with α-actinin on the Z-line of vertebrate myocytes. Therefore, these studies have supported the formation of multienzyme metabolic complexes in nature.

However, there are still many challenges ahead when exploring new dimensions of glycolytic enzymes and their complexes, particularly in living human cells. Given the tissue specificity of the band 3 protein in erythrocytes or the unique Z-line structure of myocytes, the observed metabolic complexes in these cells do not fully provide mechanistic insights of how such enzyme complexes are organized in other human cell types absent their reported scaffolds. Importantly, the metabolic influence of these complexes on cells remains to be further elucidated. Therefore, we sought to identify such complexes in living human cancer cells and their functional contributions to cellular metabolism.

In this work, we provide several lines of compelling evidence that every cytoplasmic, rate-limiting enzyme involved in glycolysis, as well as gluconeogenesis, is spatially compartmentalized into three different sizes of cytoplasmic clusters in human cervical adenocarcinoma HeLa and human breast carcinoma Hs578T cells. As controls, we validate that the varying sizes of the enzyme cluster observed in HeLa and Hs578T cells are independent of the expression levels of tagged enzymes, as well as the tagging method. Subsequent biophysical analyses using FRET and fluorescence recovery after photobleaching (FRAP) techniques corroborate the formation of multienzyme metabolic complexes in live cells. We further demonstrate that the multienzyme complex for glucose metabolism is a spatially distinct cellular entity from other cytoplasmic cellular bodies, including stress granules (22), aggresomes (23, 24), and purinosomes (25, 26). Importantly, we provide evidence to support the cluster-size-dependent functional roles of the multienzyme metabolic assemblies at single-cell levels. Collectively, we demonstrate the existence of a multienzyme metabolic complex for glucose metabolism in living human cells, providing new mechanistic insights regarding how a cell regulates the direction of glucose flux between energy metabolism and anabolic biosynthetic pathways at single-cell levels.

**Results**

**Formation of cytoplasmic PFKL clusters in human cancer cells**

We first investigated subcellular locations of the metabolic enzymes of glucose metabolism using fluorescent protein tags under fluorescence live-cell microscopy. We found that human liver-type phosphofructokinase 1, tagged with a monomeric form of enhanced green fluorescent protein (PFKL-mEGFP), forms discrete cytoplasmic clusters of varying sizes in transfected HeLa cells (Fig. 1, A–C). This clustering pattern of PFKL-mEGFP was also identified in human breast carcinoma Hs578T cells (Fig. 1, D–F) and human pancreatic adenocarcinoma Pa04C and Pa18C cells (supplemental Fig. S2, A and B). Moreover, the PFKL clusters were formed when mEGFP was alternatively tagged at the N terminus of PFKL (mEGFP-PFKL) (supplemental Fig. S2C).

To better characterize the diverse clustering phenomena, we analyzed the size distribution of PFKL-mEGFP clusters at single-cell levels when Hs578T cells were grown in RPMI 1640 supplemented with 10% dialyzed FBS (dFBS) (described under “Experimental procedures”). Based on the entirety of the data presented in this work, we have categorized the varying sizes of PFKL-mEGFP clusters into three distinguishable subgroups for clarification (Fig. 1, A–G). In the first subgroup, which represents 58.3 ± 4.7% of transfected Hs578T cells (Fig. 1G), PFKL-mEGFP proteins assembled throughout the cytoplasm into a number of small clusters. The small-sized clusters are defined as having less than 0.1 μm² (Fig. 1, A and D) based on the calculated area of the point spread function for the mEGFP emission (i.e. 0.1 μm²) (27). Line scan fluorescent intensity analysis across the cell also supports that this phenomena was clearly distinguishable from the diffusive pattern exhibited by other mEGFP-tagged metabolic enzymes including, but not limited to, hypoxanthine-guanine phosphoribosyltransferase and C1-tetrahydrofolate synthase (25, 28) (supplemental Fig. S3). In the second subgroup, ~97% of PFKL-mEGFP clusters in 13.4 ± 3.3% transfected cells displayed medium-sized clusters, ranging from 0.1 to 3 μm² in size (Fig. 1G and supplemental Fig. S4A). However, relative to the second subgroup, the third subgroup of transfected cells exhibited an increased fraction of large-sized clusters (i.e. ~16% versus 3%) (supplemental Fig. S4B), ranging between 3 and 8 μm², which were randomly distributed in the cytoplasm in the presence of smaller clusters (Fig. 1, C and F). 26.7 ± 3.6% of transfected Hs578T cells are assigned to the third subgroup (Fig. 1G). In summary, we have defined three differently sized PFKL-mEGFP clusters at single-cell levels in HeLa and Hs578T cells, namely small-, medium-, and large-sized clusters.

**Large-sized clusters are found in various cancer cells, but not in non-cancerous human breast tissue cells (Hs578Bst)**

We then investigated whether the clustering formation of PFKL-mEGFP would be relevant for human cancer cells. Because three different sizes of PFKL-mEGFP clusters were observed in human breast cancer Hs578T cells, we selected to test the formation of PFKL-mEGFP clusters in a non-cancerous human normal breast cell line, Hs578Bst, because this cancer/non-cancerous pair of breast tissue cell lines were derived from the same patient (29). From our cluster size analysis of non-cancerous breast Hs578Bst cells, we found that PFKL-mEGFP formed a number of small clusters (i.e. ~0.1 μm²) throughout the cytoplasm (Fig. 2A), as well as medium-sized clusters (< 3 μm²) (Fig. 2B). However, non-cancerous Hs578Bst cells did not induce large-sized fluorescent clusters. Although additional non-cancerous normal cells may need to be evaluated, we hypothesize that large-sized clusters of PFKL-mEGFP in Hs578T cells are a cancer-relevant cellular phenomenon.

We further evaluated the formation of large-sized PFKL clusters in the cancerous Hs578T cells when they were maintained in the non-cancerous Hs578Bst-supporting medium. Please note that although cancerous Hs578T cells were cultured in the RPMI 1640 and 10% dFBS, the non-cancerous Hs578Bst cells were cultured in Hybri-Care medium (ATCC) supplemented with 1.5 g/liter NaHCO₃, 10% FBS, and 30 ng/ml mouse EGF.
However, in the Hs578Bst-supporting medium, 23.4 ± 4.5% of Hs578T cells showed the formation of large-sized clusters, which is similar to the percentage of cells (26.7 ± 3.6%) showing large-sized clusters in Fig. 1G. Therefore, we conclude that large-sized clusters in cancerous Hs578T cells are induced regardless of the differences of the two cell culture conditions.

Expression-independent formation of PFKL-mEGFP clusters in Hs578T cells

To evaluate whether the varying size of PFKL-mEGFP clusters is dependent on the protein expression level, we further quantitated the mean or total fluorescent intensity per cell as an indication of the protein expression level in single cells. However, we did not identify any positive or negative correlation between the expression level of PFKL-mEGFP and the number of clusters per cell (Fig. 1, H and I) or the average size of clusters per cell (J and K).

Figure 2. No large-sized clusters in non-cancerous human breast tissue cells (Hs578Bst). Non-cancerous human breast tissue cells, Hs578Bst, exhibited only two clustering patterns when transfected with PFKL-mEGFP (A and B), which are an absolute scale equivalent to small- (Fig. 1, A and D) and medium-sized (Fig. 1, B and E) clusters observed in Hs578T cells. Scale bars, 10 μm, unless otherwise indicated.

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relationship between the size of individual cells and the average size of clusters per cell or the number of clusters per cell also appears to be random (supplemental Fig. S4, D and E). Collectively, we conclude that neither the formation of the fluorescent clusters by PFKL-mEGFP, nor their size, is governed by the expression levels of PFKL-mEGFP at single-cell levels.

### Cluster-like distribution of endogenous PFKL in fixed HeLa and Hs578T cells

To examine whether endogenous PFKL exhibits similar cytoplasmic clustering patterns in non-transfected cells, we performed immunocytochemistry against endogenous PFKL in HeLa and Hs578T cells. Although chemical manipulation sometimes alters subcellular distribution of endogenous proteins during fixation and permeabilization (30), we observed in >95% of fixed cells that the subcellular distribution of endogenous PFKL appears to be discrete clusters in the cytoplasm (supplemental Fig. S5), much as we observed in transfected cells expressing PFKL-mEGFP. We did not detect immunofluorescent signal absent primary, secondary, or both antibodies in our controls. In addition, our results are consistent with the subcellular distribution of immunostained PFKLs in other fixed human cells, as can be seen at the Human Protein Atlas. Taken together, these data indicate that the subcellular distribution of PFKL-mEGFP in transfected cells mirrors the distribution pattern of endogenous PFKL, setting us to investigate real-time spatial dynamics of the PFKL-mEGFP clusters in live cells.

**The mEGFP tag does not interfere with the subcellular localization of metabolic enzymes, nor does it induce cytoplasmic clusters**

We also demonstrated as controls that other enzymes in glucose metabolism whose subcellular locations are genetically determined were properly localized in their designated organelles when tagged with mEGFP in the presence and absence of monomeric orange fluorescent protein-tagged PFKL (PFKL-mOFP). mEGFP-tagged hexokinase 1 and 2 and pyruvate carboxylase were explicitly associated with mitochondria in HeLa and Hs578T cells (supplemental Fig. S6A) (28), whereas mEGFP-tagged glucose-6-phosphatase 3 stained the endoplasmic reticulum in HeLa and Hs578T cells (supplemental Fig. S6B). The ectopic expression of mEGFP alone did not induce any manner of fluorescent foci with or without PFKL-mOFP in our experimental conditions in either HeLa or Hs578T cells. We conclude that along with other enzymes in glucose metabolism, Fourteen enzymes participate in glycolysis and/or gluconeogenesis. Half of these, including PFKL, catalyze irreversible reactions and thus control the rate-limiting steps in either glycolysis or gluconeogenesis. Fourteen enzymes include glycolysis and/or gluconeogenesis. This FRAP analysis strongly supports the possibility that the PFKL-mEGFP clusters are composed of “mobile” enzymes rather than insoluble protein aggregates, regardless of their sizes in Hs578T cells.

**Colocalization of PFKL with the other cytoplasmic, rate-limiting enzymes involved in glucose metabolism**

From our FRAP analysis, we anticipated that PFKL-mEGFP would be involved in specific or non-specific protein-protein interactions in cluster-positive Hs578T cells. To identify potential protein partners of PFKL in the spatially distinct compartments, we first carried out dual-color colocalization microscopy with other enzymes in glucose metabolism. Fourteen enzymes include glycolysis and/or gluconeogenesis. Our live-cell imaging revealed that these cytoplasmic, rate-limiting enzymes in either glycolysis or gluconeogenesis (i.e. mEGFP-tagged liver-type fructose-1,6-bisphosphatase (FBPase-mEGFP), pyruvate kinase M2 (mEGFP-PKM2), and phosphoenolpyruvate carboxykinase 1 (PEPCK1-mEGFP)) formed coclusters with PFKL-mOFP in both HeLa and Hs578T cell lines (Fig. 3, A–J). The co-clustering efficiency between FBPase-mEGFP and PFKL-mOFP among cotransfected Hs578T cells was ~85%. Importantly, we observed co-clusters having various sizes between ~0.5 and ~8 μm² in Hs578T cells, indicating that medium- and large-sized clusters are multienzyme compartments. Colocalization of PFKL-mEGFP with FBPase-mOFP was also monitored after we reversed the fusion of the...
fluorescent tags in HeLa cells (supplemental Fig. S9). Collectively, this indicates that all four cytoplasmic, rate-limiting enzymes in glucose metabolism (i.e., PFKL, FBPase, PKM2, and PEPCK1) are spatially organized into multienzyme complexes in live human cells.

**Direct interaction between PFKL and FBPase upon colocalization**

We further investigated whether the colocalization of these enzymes indicates the occurrence of direct protein-protein associations within the cluster site. Particularly, PFKL and FBPase catalyze the same step of glucose metabolism, though in opposite directions. The activities of both enzymes are reciprocally regulated by a set of allosteric metabolites, and thus it has been proposed that they might directly interact as a means to further this reciprocal control mechanism. However, evidence of this proposed protein-protein interaction had been circumstantial in the 1970s (32–36), only to be overlooked afterward (37). Consequently, we took advantage of our colocalization event of these two enzymes in live Hs578T cells to reexamine this hypothesis. To study their direct interaction in live cells, we measured FRET signals by employing an acceptor photobleaching method under confocal fluorescence microscopy. In this technique, we detect the increased emission of the donor signals upon the photobleaching of the acceptors because of the loss of FRET. Indeed, a direct protein-protein interaction between FBPase-mEGFP and PFKL-mOFP was detected upon their colocalization within the various sizes of the clusters (i.e., 0.3 to 8 \( \mu \text{m}^2 \); \( N_{\text{FRET}} = 30 \)) in live Hs578T cells (Fig. 3J, black line with closed diamonds). As a negative control, FRET signals were not detected in the absence of colocalization (Fig. 3J, gray line with open squares). Therefore, our intracellular FRET sig-
Post-translational acetylation of PFKL for the formation of the multienzyme complex

Since a proteomic investigation catalogued the existence of a multitude of acetylated metabolic enzymes in human cells (38), lysine acetylation on pyruvate kinase, phosphoglycerate mutase, and phosphoenolpyruvate carboxykinase has been reported to influence their enzymatic activities or expression levels in human cells (39–43). However, the role of acetylation on PFK in cells remains to be elucidated. We have focused on protein acetylation as a potential regulator of the PFKL clustering events in cells (3), the S529A mutation did not influence its colocalization efficiency with PFKL-mOFP. Neither did single mutants of mEGFP-PKM2 containing K305A, K305R, K305Q, K62A, or K62Q. We conclude that their acetylation status is not the determinant for their complexation with PFKL. Collectively, we deduce that the acetylation of PFKL is required for the formation of multienzyme metabolic complexes.

Functional characterization of the multienzyme complexes in Hs578T cells

Considering the protein contents and their interaction within medium- and large-sized clusters of multienzyme complexes (Fig. 3), we hypothesized that the different sizes of protein clusters might play functionally different roles in cells. To characterize subcellular functions of spatially resolved multienzyme assemblies in live cells, we have treated PFKL-mEGFP-expressing Hs578T cells with glucose flux regulators and subsequently quantified the number of cells displaying the three differently sized clusters.

First, we have promoted the pentose phosphate pathway in Hs578T cells with two small molecules. Methylene blue has been known to effectively deplete the pool of NADH/NADPH in cells because of its reduction potential, resulting in the promotion of the pentose phosphate pathway (44–46). In the presence of methylene blue (5 μM), we have detected the promotion of medium-sized clusters from small-sized clusters at single-cell levels. Indeed, our quantitative high-content imaging analysis has revealed that 12.3% more cells displayed medium-sized clusters in the presence of methylene blue, whereas cells showing small-sized clusters decreased 15.4% (Fig. 5, A and C). In addition, we have treated Hs578T cells with fructose 1,6-bisphosphate (15 mM), which allosterically inhibits the metabolic activity of PFK in cells while activating FBPase. Such metabolic perturbation is known to shunt glucose flux into the pentose phosphate pathway (47, 48). Excitingly, we have detected that 15.9% more cells formed medium-sized clusters in the presence of fructose 1,6-bisphosphate, whereas cells showing small-sized clusters decreased 12.9% (Fig. 5, A and C). We also confirmed that as a control, the percentage of cells displaying various sized clusters was not changed by treatment of vehicles. These data imply that the ensemble-level promotion of the pentose phosphate shunts by methylene blue and fructose 1,6-bisphosphate in the literature (44–48) appears to be the consequence of the increased population of cells showing medium-sized clusters. Collectively, we conclude that medium-sized clusters are met-
abolically responsible for shunting glucose flux into the pentose phosphate pathway in cells.

Second, we have diverted glucose flux into serine biosynthesis by treatment of EGF. According to the literature (49–52), the EGF treatment up-regulates the activities of PFK and phosphoglycerate dehydrogenase, which catalyzes the first step of serine biosynthesis while simultaneously down-regulating PKM2 activity in cancer cells. The mechanism of EGF action was indeed demonstrated to divert glucose flux into serine biosynthesis (53). When we supplemented 30 ng/ml EGF to Hs578T cells that had been cultured in the RPMI 1640 medium with 10% dFBS, we were able to promote the cell population displaying large-sized clusters from 26.7 to 38.9% while decreasing the number of cells showing medium-sized clusters from 13.4 to 7.7% (Fig. 5, B and C). However, we note here that when Hs578T cells were cultured in the Hybri-Care medium containing 30 ng/ml EGF and 10% non-dialyzed FBS, the population of Hs578T cells showing large-sized clusters was barely changed (i.e. 26.7% in the RPMI1640 versus 23.4% in the Hybri-Care). In addition, non-cancerous Hs578Bst cells did not form large-sized clusters in the Hybri-Care medium in the presence of EGF (Fig. 2). These seemingly contrasting observations can be explained by the fact that the chemical formulations of RPMI 1640 and Hybri-Care are significantly different from each other with respect to the appended compounds of amino acids, vitamins, inorganic salts, metabolites, and co-factors and also that the different levels of nutrients between dialyzed and non-dialyzed FBS are also substantial. Collectively, although we need more in-depth studies with EGF along with these components, we conclude that to increase the net flux of serine biosynthesis at ensemble levels, the cells with large-sized clusters preferentially divert glucose flux into serine biosynthesis.

Lastly, we compared the PFKL-mediated multienzyme assemblies with other cellular bodies identified in cultured human cells. Specifically, we included aggresomes formed by chimeric GFP170* and GFP250 proteins (23, 24), stress granules by their scaffold protein, EGFP-tagged the RasGAP-associated endoribonuclease G3BP protein (EGFP-G3BP) (22, 54), and purinosomes (25, 28). As we previously demonstrated with FRAP in Hs578T cells (28), the protein markers for aggresomes

Figure 5. Functional contributions of medium- and large-sized clusters to cell metabolism. The population (%) of Hs578T cells displaying each size of PFKL-mEGFP cluster was analyzed in the presence of glucose flux regulators. The pentose phosphate shunt was promoted by methylene blue (MB, 5 nm) and fructose-1,6-bisphosphate (F16P, 15 mM), respectively (A and C). In addition, EGF (30 ng/ml) was incubated with Hs578T cells to divert glucose flux into serine biosynthesis (B and C). The error bars indicate the standard deviations of at least three independent experiments. C lists the average percentages (%) of cells displaying the given sized clusters along with their standard deviations (±). Statistical analyses were performed using two-sample two-tailed t test. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001. N.S., not statistically significant.
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Importantly, we demonstrate that PFKL-mOFP did not colocalize with a purinosome marker in Hs578T and HeLa cells (Fig. 6, J and K). Collectively, confocal FRAP analysis and dual-color colocalization microscopy support that PFKL clusters are spatially distinguishable from aggresomes, stress granules, and purinosomes.

Discussion

In this work, we provide compelling evidence that the cytoplasmic, rate-limiting enzymes of glucose metabolism (total of four enzymes; PFKL, FBPase, PKM2, and PEPCK1) are spatially organized into multienzyme complexes in living cells. In addition, the large size of the compartments (i.e., displaying >3 μm²) was detected in various human cancer cells but not in the non-cancerous human breast cell line Hs578Bst. Importantly, the formation of medium- and large-sized clusters at single-cell levels corresponds to the metabolic shunts of glucose flux into the pentose phosphate pathway and serine biosynthesis, respectively, supporting potential functional contributions of the metabolic complexes to cellular metabolism. Collectively, we report the identification of a multienzyme metabolic complex for human glucose metabolism in living cells, which we propose function to regulate the direction of glucose flux in a cluster-size-dependent manner.

At the same time, all the presented experimental evidence strongly indicates that the PFKL-mEGFP clusters are neither insoluble protein aggregates nor fluorescent protein-mediated artifacts. First, all the mEGFP-tagged metabolic enzymes were properly localized either in the cytoplasm or into the organelles based on current understanding of their functional roles in a cell (Fig. 1 and supplemental Figs. S2, S3, and S6) (25, 28). Second, our cluster size analysis with the mean and total fluorescence intensities of transfected PFKL-mEGFP confirmed that PFKL clustering was not governed by the expression level of the tagged protein (Fig. 1 and supplemental Fig. S4, C–E). Third, endogenous PFKL displayed a similar distribution pattern (supplemental Fig. S5) as that observed in transfected cells expressing PFKL-mEGFP (Fig. 1), as well as in other fixed human cells from the Human Protein Atlas database. Fourth, the mEGFP tag itself did not promote the formation of fluorescent clusters in Hs578T cells (supplemental Fig. S7). Fifth, FRAP measurements demonstrated that PFKL-mEGFP, whether located within clusters or not, is mobile in transfected live cells (supplemental Fig. S8). Sixth, FRET signals in live cells revealed that PFKL and FBPase directly interacted upon their colocalization (Fig. 4). Lastly, the multienzyme complexes were biophysically differentiated from aggresomes, stress granules, and purinosomes (Fig. 6) (28). Collectively, all the evidence provided here strongly support that these PFKL-mediated multienzyme assemblies are not technique-mediated artifacts but rather bona fide compartments utilized in human cells.

The results described above also represent a significant advance upon what has been gleaned to date. Although there are many articles reporting glycolytic enzyme complexes in var-

and stress granules did not show gradual fluorescent recoveries as a function of time in our conditions. Based on the recovery curve of PFKL-mEGFP in this work (supplemental Fig. S8), it is clear that PFKL-mEGFP clusters are different from aggresomes and stress granules in live cells. However, the fluorescent recovery curve analysis does not differentiate the PFKL-mediated multienzyme clusters from another metabolic complex, the purinosome (25, 28).

To further validate the authenticity of the PFKL clusters, we performed dual-color colocalization microscopy. To begin with, we confirmed that aggresomes and stress granules were spatially different cellular bodies in dually transfected cells with PFKL-mOFP (Fig. 6, A–I). It is important to note that their observed sizes in mammalian cells are comparable with the medium- and large-sized clusters of PFKL-mediated multienzyme assemblies. Also, stress granules were detected in less than 10% of transfected cells, which indicates that our culture conditions do not promote cellular stress. These data imply that PFKL clusters are not a by-product of either protein aggregation or cellular stress in our imaging conditions.

![Figure 6. No colocalization of PFKL with other cellular bodies in cells. A–I, EGFP-G3BP (A), GFP170* (D), and GFP250 (G) were cotransfected with PFKL-mOFP (B, E, and H, respectively) in Hs578T cells. Green channels correspond to the EGFP-fusion constructs, whereas red channels correspond to PFKL-mOFP in the merged images (C, F, and I). PFKL-mEGFP formed spatially distinct cellular bodies apart from stress granules and aggresomes. J and K, PFKL-mEGFP and mOFP-tagged formylglycinamidine ribonucleotide synthase were cotransfected into HeLa cells. These two proteins, representing the metabolic complexes in glucose metabolism and de novo purine biosynthesis (i.e., the purinosome), respectively, do not colocalize in cells (J). A random representative region in J was zoomed in for clarification (K). Scale bars, 10 μm, unless otherwise indicated.](image-url)
ious organisms over several decades (5–7, 9–12, 14), we have had a very limited understanding of their dynamic properties inside living cells with respect to their metabolic functions. In addition, most of the previous studies have centered on the identification of glycolytic complexes, excluding gluconeogenic enzymes, thus providing limited insights of the interplay between glycolysis and gluconeogenesis upon the protein complexation. Hence, our functional characterization of the multienzyme complex in living cells will advance our understanding of the reversible nature of glucose metabolism and metabolic shunts not only for normal healthy cell metabolism but also for dysregulated cancer cell metabolism.

This multienzyme assembly for glucose metabolism is also analogous to the purinosome assembly, which regulates de novo purine biosynthesis in human cells (25). In terms of their sizes, ~97% of PFKL-mEGFP clusters were ranged from 0.1 to 3 μm² in size (supplemental Fig. S4A), which is in good agreement with the ~97% of purinosome clusters displaying less than 3 μm² in HeLa and Hs578T cells. Also, the average size of PFKL-mEGFP clusters in HeLa cells, ranging from 0.2 to 1 μm² (supplemental Fig. S4A, inset), is comparable with the average size of purinosomes in HeLa cells, ranging from ~0.1 to ~0.8 μm² (55). In addition to their sizes observed under wide-field and confocal fluorescence microscopy, we notice that the diffusion coefficients of purinosome-participating enzymes (i.e. 0.007–0.075 μm²/s in Ref. 28) are also in good agreement with the diffusion coefficients of PFKL-mEGFP (i.e. D_{app} = 0.018 ± 0.009 μm²/s; supplemental Fig. S8). However, these two complexes are spatially independent metabolic granules in cells (Fig. 6, J and K). Although more advanced analysis may be necessary, it seems that their spatial relationship may reflect their biochemical network (supplemental Fig. S1), providing us with novel insights into how biochemically defined metabolic networks are spatially regulated in a coordinated fashion at the single-cell level.

We may identify the cancer cell-relevant size of clusters formed by the rate-limiting enzymes in human glucose metabolism. Our data suggest that cancer cells seem to promote the clustering events in a larger volume to induce large-sized clusters for their altered metabolic needs (Fig. 1, A–F, versus Fig. 2). Indeed, our experimental data support that large-sized clusters primarily shunt metabolic intermediates into serine biosynthesis at subcellular levels, which is considered one of the hallmarks of altered glucose metabolism in various cancer cells (1, 3, 56, 57). Therefore, although extensive functional studies are required, we propose that the varying sizes of this assembly represent various metabolic roles, like traffic signals, to guide the direction of glucose-mediated carbon flux at various metabolic nodes in the cell.

Collectively, we demonstrate the formation of a multienzyme metabolic complex for glucose metabolism in living human cells, namely the “glucosome.” We envision that comprehensive understanding of such multienzyme metabolic assemblies, the “metabolon,” will open new avenues to address their functional and/or regulatory contributions to human metabolic diseases, like cancer and beyond.

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**Experimental procedures**

The cDNAs of the human enzymes involved in glucose metabolism were acquired from the PlasmID Repository, the DNA Resource Core at Harvard Medical School. Most cDNAs, except for the plasmids expressing EGF-tagged hexokinases (Addgene), were amplified by PCR using Pfu DNA polymerase (Stratogene) with pairs of restriction sites on primers. Subsequently, the genes were cloned into either a pmEGFP-N1 plasmid, which possesses an A206K mutation in the EGFP sequence of pEGFP-N1 (Clontech) to produce mEGFP (58) or the pmOFP-N1 (25) plasmid expressing monomeric orange fluorescent protein (mOFP). The resulting cloned plasmids were confirmed by restriction enzyme digestions and DNA sequencing (GeneWiz).

Consequently, we have used the following mEGFP/mOFP-fusion constructs to visualize proteins under fluorescence live-cell microscopy: hexokinase (HK1-EGFP and HK2-EGFP) (59), glucose-6-phosphatase 1 and 3 (G6Pase1/3-mEGFP), liver-type phosphofructokinase 1 (PFKL-mEGFP, mEGFP-PFKL, and PFKL-mOFP), liver-type fructose 1,6-bisphosphatase (FBPase-mEGFP/mOFP), pyruvate kinase M2 (mEGFP/mOFP-PKM2), phosphoenolpyruvate carboxykinase 1 (PEPCK1-mEGFP), EGFP-tagged RasGAP-associated endoribonuclease G3BP protein (EGFP-G3BP), and the internal segment of the Golgi complex protein 170 fused to GFP (GFP170⁰) and the C-terminal fragment of p115 fused to GFP (GFP250) were used as protein markers representing purinosome (25), stress granules (22), and aggresomes (23, 24), respectively. EGFP-G3BP and GFP170⁰/GFP250 were acquired from Drs. J. Tazi (Institut de Genetique Moleculaire de Montpellier, Montpellier, France) and E. S. Sztl (University of Alabama, Birmingham, AL), respectively.

In addition, a TC motif of six amino acids (N-CCPGGC-C) was introduced to PFKL-mEGFP, resulting in PFKL-TC-mEGFP and PFKL-TC. Site-directed mutagenesis was also used to either abolish or mimic reported lysine acetylation sites on human PFKL, FBPase, and PKM2. Single mutants reported here were generated using QuikChange site-directed mutagenesis kits (Agilent).

**Cell culture and transfection**

Human cervix adenocarcinoma HeLa, human breast carcinoma Hs578T (HTB-126), and human breast normal Hs578Bst (HTB-125) cell lines were obtained from the ATCC. HeLa and Hs578T cells were maintained in the RPMI 1640 (Mediatech, catalog no. 10-040-CV) supplemented with 10% FBS (Atlanta Biological, catalog no. S12850) and 50 μg/ml gentamicin sulfate. Hs578T cells were maintained as recommended in Hybri-Care medium (ATCC, catalog no. 46-X) supplemented with 1.5 g/liter NaHCO₃, 10% FBS, 30 ng/ml mouse EGF (Sigma), and 50 μg/ml gentamicin sulfate. In addition, human pancreatic adenocarcinoma Pa04C and Pa18C cell lines were a gift of Dr. Anirban Maitra (Johns Hopkins School of Medicine) and were cultured in minimum essential medium (Mediatech,
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catalog no. 10-010-CV) supplemented with 20% FBS (Atlanta Biological, catalog no. SI1550), 1% L-glutamine (200 mm; Gibco, catalog no. 25030), and 1% penicillin-streptomycin (Gibco, catalog no. 15070-63). The cells were maintained in a HeraCell CO2 incubator (37 °C, 5% CO2, and 95% humidity).

To prepare cells for transfection and subsequent imaging, HeLa, Hs578T, Hs578Bst, Pa04C, and Pa18C cells were gently removed from the culture flask by replacing the culture medium with trypsin-EDTA (Corning, catalog no. 25-053-CL). Fresh, antibiotic-free growth medium was subsequently used to harvest and resuspend cells that were used to plate either glass-bottomed 35-mm Petri dishes (MatTek) or 8-well chambers (LabTek) such that next-day confluency was ~70–90%. The following day, the cells were transfected with either Lipofectamine 2000 (Invitrogen) or Xfect (Clontech). For dual transfection, the two plasmids were used in the same transfection mixture whether using Lipofectamine 2000 or Xfect. When using Lipofectamine 2000, the Opti-MEM-I reduced serum medium (Opti-MEM-I; Gibco, catalog no. 11058) was used for transfection, but the medium was exchanged with fresh antibiotic-free growth medium after a 5-h incubation (37 °C, 5% CO2, and 95% humidity), followed by ~18–24 h of incubation in the incubator. Conversely, Xfect-treated cells in antibiotic-free growth medium did not require a medium exchange and were left in the incubator for ~18–24 h following the initial transfection.

Fluorescence live-cell imaging

On the day of imaging (~18–24 h post-transfection), the cells were washed with buffered-saline solution (20 mm HEPES, pH 7.4, 135 mm NaCl, 5 mm KCl, 1 mm MgCl2, 1.8 mm CaCl2, and 5.6 mm glucose) for three 10-min incubations, followed by a ~1–2 h incubation at ambient temperature. All samples were then imaged at ambient temperature (~25 °C) with a 60× 1.45 NA objective (Nikon CFi Plan Apo TIRF) using a Photometrics CoolSnap EZ monochrome CCD camera on a Nikon Eclipse Ti inverted C2 confocal microscope. Wide-field imaging was carried out using the following filter sets from Chroma Technology: mEGFP detection by a set of Z488/10-HC clean-up, HC TIRF dichroic, and 525/50-HC emission filter; and mOFP detection by a set of Z561/10-HC clean-up, HC TIRF dichroic, and 600/50-HC emission filter.

For cell-based metabolic flux assays, small molecules, including fructose-1,6-bisphosphate (15 mm) and methylene blue (5 nm), were added to cells after washing three times with buffered-saline solution. Images showing PFKL-mEGFP clusters were acquired before and after cells had been incubated with the small molecules at various time points. Control experiments were also carried out with 1–30 μl of vehicle. In addition, to study the effect of EGF, 30 ng/ml mouse EGF (Sigma) was supplemented to the RPMI 1640 medium along with 10% dia lyzed FBS. The EGF effect on PFKL-mEGFP clustering was quantified after cells had been cultured in the EGF-added RPMI 1640 plus dialyzed FBS medium for at least 3 weeks.

Immunocytochemistry

We have also performed immunocytochemistry against endogenous PFKL in HeLa and Hs578T cells. The cells were fixed with freshly prepared 3% formaldehyde, permeabilized with 0.2% Triton X-100, and blocked with 10% normal donkey serum (Jackson ImmunoResearch Laboratories). The cells were then incubated with a rabbit polyclonal anti-PFKL antibody (Thermo Scientific; PA5-21685) and a Cy3-conjugated goat anti-rabbit IgG (H+L) (Jackson ImmunoResearch Lab). Controls for non-specificity and autofluorescence included the fixed cells incubated with primary only, secondary only, and neither antibody.

Fluorescence recovery after photobleaching

FRAP was performed as described previously (28). Briefly, confocal imaging was performed using a JDSU argon ion 488-nm laser line (50 milliwatt) for mEGFP detection via a 488/561 dichroic mirror with 525/50 emission filter and photomultipliers. To photobleach specific areas of interest in live cells, the argon ion 488-nm laser line was applied at 50–75% power for 0.5 s. Because the equipment has better precision when the bleach diameter is larger than 1 μm, the target bleaching area was maintained at ~3 μm in diameter, regardless of fluorescent cluster size. At least 10 images were obtained before bleaching, and subsequent images were acquired every 0.5 s for at least 50 s. Fluorescence recovery was individually fitted after the degree of background photobleaching was normalized. Apparent diffusion coefficients ($D_{app}$) were then calculated as we have described before (28, 60).

FRET

To measure FRET in live cells, FBPase-mEGFP and PFKL-mOFP were dually transfected into Hs578T cells using Xfect (Clontech). The next day, coclustering Hs578T cells were subjected to measurement of their FRET signals using a confocal microscopy-based acceptor photobleaching method. In this technique, we detected the increased emission of the donors’ signals upon the acceptors’ photobleaching because of the loss of FRET. Briefly, to photobleach the mOFP-tagged acceptor molecules in coclustering areas, a Coherent sapphire 561/20-nm laser was applied at ~40% power for 0.5 s. At least 10 images were obtained before the acceptor bleaching, and subsequent images were acquired every 0.5 s for at least 50 s. After the degree of photobleaching of the mEGFP donor molecules was corrected in each data point, the temporal increase of the emission of the mEGFP-tagged donor molecules from the same areas was graphed to reveal their direct interaction in live cells. Dual-color confocal imaging were achieved via a 488/561/640 dichroic mirror with 525/50 and 600/50 emission filters and photomultipliers. Note that there is sufficient spectral overlap between the donor mEGFP emission and the acceptor mOFP excitation for FRET measurement (61).

Cluster size analysis

Cluster size analysis was accomplished using ImageJ processing software (National Institutes of Health). Prior to analysis, fluorescent wide-field images were edited to isolate in-focus, single, whole cells from an image. This was accomplished by cropping the original image and, in some instances, manually outlining the cell to entirely remove surrounding pixel intensity information. The latter step was necessary when cropping alone could not isolate a single cell in a group. Neither affected the original pixel information of the image. Edited...
images were then processed through ImageJ using a custom script and macro that automates the counting of fluorescent clusters using its built-in module, the so-called robust automatic threshold selection (RATS). Briefly, the images were scaled according to the pixel size of the microscope (i.e. 0.12 μm/pixel) before the RATS segmentation tool was used to automatically identify fluorescent clusters within a cell by outlining. Default parameters for RATS were used in this analysis (i.e. noise threshold = 25, λ factor = 3). Once fluorescent clusters were isolated, the inverse look-up table function was used to generate a mask of the original image that only displayed fluorescent clusters. The module for particle analysis was then applied to this mask to attain both the number and area of fluorescent clusters within an image. This process was repeated for all subsequent cell images. The operator then evaluated the original cell images against the particle mask to eliminate data in which more than one cluster was counted as a single particle. The data were then analyzed and graphed using Microsoft Excel.

**Single-cell fluorescence intensity analysis**

Nikon imaging software and ImageJ processing software were used to compare the fluorescence intensity between cells in fluorescent images, which were captured using a mercury arc lamp at 75% power with 50-ms exposure time. Briefly, the free-hand selection tool was used to manually outline single cells, which were subsequently analyzed for their cluster sizes as described above. In parallel, the cropped raw images were subjected to Nikon imaging software, with which we defined the boundary of cells to quantify the total fluorescent intensities and the size of cells. The mean fluorescent intensities were calculated by dividing the total fluorescent intensities of whole cells with the area of the cells. Of note, the mean or total fluorescent intensities were graphed with the number of clusters and the size of cells. The mean fluorescent intensities were calculated by dividing the total fluorescent intensities of whole cells with the area of the cells. Of note, the mean or total fluorescent intensities were graphed with the number of clusters per cell or the average size of clusters per cell that we obtained from the cluster size analysis.

**Line-scan fluorescence intensity analysis**

Nikon imaging software was used to quantify the fluorescence intensity of cells. Using the 12-bit raw images, we drew a user-defined line across a cell, which included at least 10 pixels of background on each end of the line. The fluorescent intensity was then normalized to arbitrary units by setting the background to 0 and the maximum intensity to 100 before analysis.

**Author contributions**—M. K. and S. A. designed the research; C. L. K., M. K., M. J., D. L. S, E. L. K., S. M. B., and S. A. performed the research; C. L. K., M. J., D. L. S, S. M. B., B. T. L., S. J. R., and S. A. contributed new reagents; C. L. K., M. K., M. J., J. R., and S. A. analyzed the data; and C. L. K., M. K., and S. A. wrote the paper.

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