Eukaryotic cells have evolved highly orchestrated protein catabolic machineries responsible for the timely and selective disposal of proteins and organelles, thereby ensuring amino acid recycling. However, how protein degradation is coordinated with amino acid supply and protein synthesis has remained largely elusive. Here we show that the mammalian proteasome undergoes liquid-liquid phase separation in the nucleus upon amino acid deprivation. We termed these proteasome condensates SIPAN (Starvation-Induced Proteasome Assemblies in the Nucleus) and show that these are a common response of mammalian cells to amino acid deprivation. SIPAN undergo fusion events, rapidly exchange proteasome particles with the surrounding milieu and quickly dissolve following amino acid replenishment. We further show that: (i) SIPAN contain K48-conjugated ubiquitin, (ii) proteasome inhibition accelerates SIPAN formation, (iii) deubiquitinase inhibition prevents SIPAN resolution and (iv) RAD23B proteasome shuttling factor is required for SIPAN formation. Finally, SIPAN formation is associated with decreased cell survival and p53-mediated apoptosis, which might contribute to tissue fitness in diverse pathophysiological conditions.
Protein degradation and subsequent recycling of amino acids is fundamental for normal cell physiology. The autophagy system targets large macromolecule complexes, protein aggregates and organelles, all of which are first engulfed within double membrane-delimited structures and subsequently delivered to the lysosome for degradation\(^\text{1-3}\). On the other hand, the proteasome catalyzes the degradation of proteins that are in surplus, improperly folded or unwanted at a given time or in a specific subcellular location\(^\text{4-6}\).

The proteasome is an evolutionarily conserved protein degradation machinery that generally recognizes substrates that are poly-ubiquitinated through the concerted action of E2 ubiquitin-conjugating enzymes and E3 ubiquitin ligases. The 26S proteasome is composed of two sub-complexes, the 20S cylinder-like catalytic particle (CP) and the 19S regulatory particle (RP) (Fig. 1a). The CP contains the proteases with CASPASE-like, trypsin-like and chymotrypsin-like activities that are responsible for substrate degradation into small peptides\(^\text{7,8}\). This particle is the target of the widely used proteasome inhibitors (e.g., MG132 and Bortezomib)\(^\text{7,8}\). It was also shown to increase proteasome-mediated proteolysis\(^\text{15}\). The RP is also a large multi-protein complex that binds the CP to assemble a competent proteasome. The RP is responsible for the recognition and unfolding of polyubiquitinated proteins, as well as their translocation inside the CP. The CP can also associate with other regulatory complexes including the homoeo-meric ring-shaped 11S complex composed of PSME3 (PA28\(\gamma\) or REG\(\gamma\)), which targets proteins for ubiquitin-independent degradation (Fig. 1a)\(^\text{9}\).

While a large body of findings have elucidated how the proteasome regulates diverse cellular processes, including the identification of a wide spectrum of its substrates, much less is known about how the proteasome is regulated. Nonetheless, ample evidence now indicates that highly conserved mechanisms, e.g., transcriptional feedback mechanisms and post-translational modifications, regulate the abundance of proteasome subunits and their assembly into a degradation competent complex (recently reviewed by Rousseau et al.\(^\text{6}\)).

Several studies have also enquired to determine how protein degradation by the proteasome is coordinated with protein synthesis and metabolic demands. It was originally found that, following amino acid deprivation in mammalian cells, proteasome activity is required to replenish the cellular amino acid pool, thus ensuring protein synthesis\(^\text{10}\). Indeed, proteasome inhibition in yeasts and mammalian cells results in amino acid depletion, which triggers cell death, indicating the critical role of the proteasome in maintaining amino acid homeostasis\(^\text{11}\). On the other hand, ostensibly conflicting results exist on the link between the mechanistic target of rapamycin (mTOR) kinase, which promotes anabolism and protein synthesis, and the signaling pathways that coordinate proteasome function. TOR signaling appears to play an important role in negatively regulating proteasome abundance in yeast and mammalians\(^\text{12,13}\). Inhibition of TORC1 induces a transient increase in the abundance of proteasome subunits and their assembly\(^\text{12}\). Interestingly, the activation of proteasomal degradation upon mTOR inhibition is accompanied by a rapid increase of K48-linked ubiquitination and the degradation of long-lived proteins\(^\text{13,14}\). However, overactivated mTOR signaling was also shown to increase proteasome-mediated proteolysis\(^\text{15}\). While the reasons behind these discrepancies remain unclear, these studies provided evidence for the intricate relationships between cell growth signaling and protein degradation.

Another potentially important determinant in amino acid homeostasis is how the subcellular localization of the proteasome is modulated under conditions of nutrient deprivation. In the budding yeast, **Saccharomyces cerevisiae**, the 26S proteasome is relocated from the nucleus to the cytoplasm during quiescence or carbon starvation\(^\text{16}\). The cytoplasmic proteasome assembles a large condensate termed Proteasome Storage Granule (PSG), and these structures are rapidly dissipated when cells resume proliferation\(^\text{16}\). A genetic screen in yeast revealed that several signaling pathways directly or indirectly contribute to the assembly of PSG\(^\text{17}\). Interestingly, while nitrogen starvation induces proteasome degradation by autophagy, a process termed proteaphagy, glucose starvation induces the formation of cytoplasmic PSG. It was concluded that PSG protects the proteasome from autophagy, thus promoting yeast cell viability following periods of carbon starvation\(^\text{18}\). Remarkably, carbon deprivation also induces the formation of large cytoplasmic PSG-like structures in **Arabidopsis Thaliana**\(^\text{18}\). In contrast, nitrogen starvation in this organism induces proteaphagy, a process that requires a multivalent binding of RPN10 subunit of the proteasome with ubiquitin and the ubiquitin-like protein, Autophagy-related protein 8 (ATG8)\(^\text{19}\). These findings further highlight the evolutionary conservation and functional importance of proteasome delocalization and regulation mechanisms.

In animal species, including mammals, the mechanisms that coordinate the subcellular localization of the proteasome in response to nutrient deprivation are incompletely understood. The cytoplasmic proteasome was shown to be partly degraded by the autophagy machinery following nutrient starvation\(^\text{20}\). Indeed, amino acid starvation induces proteasome recognition by the ubiquitin-associated domain of p62/SQSTM1 and subsequent engulfment by the autophagy system in the cytoplasm\(^\text{29}\). However, it remained unclear how the nuclear proteasome is regulated in response to nutrient deprivation. In this study, we show that the mammalian proteasome undergoes liquid–liquid phase separation (LLPS) in the nucleus in response to amino acid starvation. We present a characterization of these proteasome condensates, which we termed SIPAN (starvation-induced proteasome assemblies in the nucleus). We show that these structures are associated with amino acid deprivation-mediated cell death.

**Results**

**Nutrient deprivation in mammalian cells results in the localization of proteasome components into nuclear foci.** In yeast, the 26S proteasome particle translocate to the cytoplasm and form proteasome storage granules (PSG), in response to carbon starvation or quiescence entry\(^\text{16,18}\), but whether the mammalian proteasome is also subjected to a similar regulation has remained unknown. We used primary human fetal lung fibroblasts IMR90 as a model for normal diploid mammalian cells which were incubated in Hank’s Balanced Salt Solution (HBSS), containing 1 g/L of glucose as the only nutrient (HBSS hereafter). While, nutrient-deprived IMR90 cells showed a rapid loss of 4EBP1 phosphorylation\(^\text{21}\), which is indicative of cell starvation, no major changes were observed on the abundance of proteasome subunits or accessory factors (Fig. 1a, b). However, a noticeable decrease could be observed, at later time points, for certain proteasomal factors, e.g., PSMB7 and PSMD14, possibly reflecting autophagy consumption of the cytoplasmic proteasome, as previously reported\(^\text{20}\). In addition, no signs of cell death were detected after 12 hrs of treatment, as indicated by the normal appearance of PARP1 and CASPASE-3 (Supplementary Fig. 1c).

Similar cellular response to nutrient deprivation was observed in HCT116 colon carcinoma cells with no apparent apoptotic cleavage of PARP1 and CASPASE-3 (Supplementary Fig. 1c). Using these two cell models, we initially conducted immunostaining at 6 or 8 hrs post-nutrient starvation and revealed that PSMD4 subunit of the RP localizes in spherical nuclear foci in IMR90 and HCT116, respectively (Fig. 1c). Importantly, hyper-osmotic stress was recently shown to induce proteasome foci
formation in the nucleus. However, HBSS osmolality was around 280 mOsm/kg, corresponding to normal values of cell culture media. In addition, no significant changes of HBSS osmolality was observed under conditions of HBSS treatment for both cell types used (Supplementary Fig. 1e, f).

Next, we isolated cytoplasmic and nuclear fractions of IMR90 and HCT116 cells, following incubation in HBSS, and found that, in contrast to yeast, no manifest translocation of proteasome components from the nucleus to the cytoplasm was noticed (Fig. 1d, e). Similar observations were also made in IMR90 cells stably expressing PSMB4-GFP, which showed foci formation following HBSS with no manifest change in its nucleocytoplasmic distribution (Fig. 1f). Thus, in mammalian cells, proteasome components are localized into nuclear foci in response to nutrient limitation.
deprivation. Moreover, the nuclear and cytoplasmic proteasomes appear to be subjected to distinct regulatory events.

Interdependency of catalytic and regulatory particles in proteasome nuclear foci formation upon nutrient starvation. We first probed multiple components of the proteasome and found that several subunits of the CP, e.g., PSMB1, PSMB2, PSMB4, PSMB6, and PSMB7, co-localize with PSMD11 or PSMD4, components of the RP, following incubation of IMR90 in HBSS (Fig. 2a, Supplementary Fig. 2a). C-terminal GFP-tagged versions of PSMB4, PSMB5, PSMD12, or PSMD14, all localize in nuclear foci, following incubation of IMR90 cells in HBSS (Fig.1f and Supplementary Fig. 2b). These results suggest that proteasome nuclear foci formed in response to nutrient deprivation contain fully assembled 26S proteasome particles.

Next, we sought to test whether CP and RP are independently assembled in proteasome foci upon nutrient starvation. Following siRNA-mediated depletion of PSMB5, PSMB6 or PSMB7 components of the CP, we observed reduced accumulation of the RP components, PSMD4, PSMD7, or the CP component PSMB4, in nuclear foci (Fig. 2b, c). Conversely, depletion of PSMD4, PSMD7, PSMD11, or PSMD14 components of RP results in reduced assembly of PSMB4 in nuclear foci. Similar results were obtained following HBSS treatment of IMR90 cells stably expressing PSMB4-GFP (Supplementary Fig. 2c). Efficient depletion of proteasomal proteins was validated by immunoblotting (Supplementary Fig. 2d). Interestingly, PSME3 proteasome activator complex also localizes in nuclear foci with PSMD7 following incubation of IMR90 cells in HBSS (Fig. 2d).

Additionally, we found that while depletion of components of the CP or the 19S RP results in the loss of PSME3 foci (Fig. 2e), no significant effect was observed on either PSMD4 or PSMB4 foci following PSME3 depletion (Fig. 2f, Supplementary Fig. 2e). Thus, following nutrient deprivation, both 20S CP and 19S RP are required for the assembly of intact proteasome particles in nuclear foci.

Starvation-induced proteasome assemblies in the nucleus (SIPAN) are a general response of mammalian cells to nutrient deprivation. Nuclear proteasome foci induced by nutrient deprivation, as detected with PSMD14 or PSMD4 antibodies, do not correspond to known nuclear foci, condensates or bodies such as PML bodies (PML staining), nucleoli (Fibrillarin staining), nuclear speckles (SC35 staining) or DNA double strand break foci (53BP1 staining) (Fig. 3a). Confocal microscopy indicated that PSMB4-GFP foci are preferentially located in low-density chromatin regions (Fig. 3b). This was confirmed by transmission electronic microscopy, which also revealed that proteasome foci are membrane-less nuclear structures (Fig. 3c).

It was recently shown that, under hyperosmotic stress conditions, proteasome undergoes LLPS and form nuclear foci that target ribosomal proteins. However, under conditions of nutrient deprivation, the stress-sensitive nucleolar protein NPM1 is not re-localized to the nucleoplasm (Supplementary Fig. 3a). Moreover, neither endogenous nor overexpressed ribosomal proteins are enriched in nutrient deprivation-associated proteasome foci (Fig. 3d, Supplementary Fig 3b–d). Thus, in contrast to hyperosmotic stress-induced proteasome foci, nucleolar stress is not associated with proteasome foci formation upon nutrient deprivation. Next, we found that PSMB4 foci are not induced when IMR90 cells are exposed to oxidative stress (H2O2), heat shock (45°C), genotoxic stress (ionizing radiation or UVC light) or hypoxia (1% O2) (Fig. 3e). However, some proteasome inhibitors consistently provoked the formation of nuclear PSMD4 foci, but in a very small percentage of cells. Relevant signaling factors and cell survival were assessed to control for treatment efficacy (Fig. 3f, Supplementary Fig. 3e).

We then sought to evaluate the presence of proteasome foci in diverse mammalian cell types upon nutrient deprivation. We found that nuclear foci of PSMD4 or PSMD7 are found, with different frequencies, in several other normal, immortalized or tumoral mammalian cells (Fig. 4a and Supplementary Fig. 4). Proteasome foci are also observed in mouse embryonic stem cells (mESC) and differentiated 3T3L1 mouse adipocytes upon nutrient starvation (Fig. 4b, c). Interestingly, we noted that several cancer cells, i.e., T47D, PC3, MIA PaCa-2, have reduced ability to form proteasome foci following nutrient deprivation (Supplementary Fig. 4). Thus, we concluded that proteasome foci are a general response of mammalian cells to nutrient deprivation and might be modulated during oncogenic transformation. We termed the above-described nuclear structures: Starvation-Induced Proteasome Assemblies in the Nucleus (SIPAN).

SIPAN are highly dynamic, undergo fusion events, and are reversible. To investigate the dynamics of SIPAN, we surveyed SIPAN formation in IMR90 cells and found that these structures form as rapidly as 2–3 hrs with a maximum of cells harboring PSMD4 foci at 6–10 hrs post-nutrient deprivation (Fig. 5a). The percentage of cells with SIPAN, their signal intensity and number per cell all progressively increased during nutrient starvation (Fig. 5a–c). While signal intensity of SIPAN reaches a plateau, their average number per cell decrease at later time points, suggesting SIPAN fusion or resorption (Fig. 5b–c). To further investigate this, we conducted live imaging using IMR90 cells stably expressing PSMB4-GFP (Supplementary Movie 1, 2). Live-cell SIPAN have an apparent average size of 0.3–0.4 µm2 (Fig. 5d) and can indeed undergo fusion events (Fig. 5e–g, Supplementary Movie 3). Further analysis of SIPAN mobility showed that these structures have a mean square displacement of 0.2 µm2/min.
(Fig. 5h–j), three-fold higher for that reported for 53BP1 DNA damage foci.

To further determine whether SIPAN are reversible, we first induced their formation by depriving cells of nutrients and then replenished the cells with fresh culture medium. We observed that SIPAN, detected by PSMD4 immunostaining, dissipate within 60 min after the addition of complete culture medium (Fig. 5k–m and Supplementary Fig. 5a). Similar results were obtained for GFP-tagged PSMB4 component of the CP (Supplementary Fig. 5b). PSME3 proteasome activator particle also dissipates following addition of culture medium (Fig. 5n, Supplementary Fig. 5c). As disassembly of multi-protein complexes might involve the AAA+ type ATPase VCP/p97, we blocked this enzyme and determined the impact on SIPAN resolution. Nutrient-deprived IMR90 cells, with preformed SIPAN, were treated with various VCP/p97 inhibitors in normal conditions, which led to the observation that SIPAN resolution was significantly affected by the presence of these inhibitors.
SIPAN result from liquid–liquid phase separation (LLPS). We sought to determine how SIPAN respond to abrupt physico-chemical changes of the cellular environment. SIPAN were assembled by incubation of IMR90 cells in HBSS and were subsequently subjected to other treatments. Interestingly, while SIPAN dissipate within 2 min upon incubation in 0.01% of Triton X-100, DNA damage-induced 53BP1 foci are resistant to concentrations of this detergent of up to 1% (Fig. 6a and Supplementary Fig. 6a, b). The nuclear staining of PSMD4 does not decrease during the initial time of detergent treatment, suggesting that SIPAN are dissipated in the nucleus as opposed to being expelled from this compartment (Fig. 6a and Supplementary Fig. 6a). Similar results are obtained with digitonin (Supplementary Fig. 6c), a mild detergent that permeabilizes cellular membranes with minimal effects on nuclear membrane. Moreover, within the same period post-HBSS treatment, we did not observe a noticeable diminution in the signal of RNA Polymerase II following incubation with low concentration of digitonin (Supplementary Fig. 6d). Next, we conducted live-cell imaging of PSMB4-GFP and found that, upon treatment with 0.03% Triton X-100, SIPAN fluorescence signals become diffuse before reduction of PSMB4-GFP overall intensity and entry of propidium iodide (P.I.) into the nucleus (Supplementary Fig. 6e, Supplementary Movie 4). These data suggest that SIPAN continuously depend on physico-chemical determinants of the nucleus and cytoplasm that quickly dissipate upon discrete changes in the composition of the nucleocyptoplasm.

A fundamental characteristic of LLPS is a thermodynamic equilibrium that can shift towards assembly or disassembly depending on the cellular environment. First, we treated IMR90 cells with 1,6-hexanediol, an aliphatic alcohol, which disrupts weak hydrophobic interactions and LLPS, and found that this treatment causes rapid SIPAN dissolution (Fig. 6b, Supplementary Movie 5). Next, we wanted to determine whether SIPAN assembly/disassembly could be influenced by salt concentration, without plasma membrane permeabilization. We induced SIPAN in IMR90 cells, which were subsequently incubated in various detergent-free solutions for 2 min. We found that hypotonic treatments induce quick SIPAN dissipation (Fig. 6c and Supplementary Fig. 6f). Changing the pH of the hypotonic buffer from 6.8 to 8.8 had no impact on SIPAN dissolution, while supplementing this minimal buffer with 100 mM or 400 mM of NaCl maintained SIPAN assembled (Fig. 6c, Supplementary Fig. 6g). Interestingly, live-cell imaging indicated that SIPAN dissipation is followed by quick recovery in the original locations, when hypotonic buffer is supplemented with 200 mM NaCl (Fig. 6d, Supplementary Fig. 6h and Supplementary Movie 6). Importantly, no staining of DNA with P.I. was observed, indicating that plasma membrane integrity is not compromised during the course of these treatments (Fig. 6d). Of note, a similar behavior of foci dissipation upon incubation in hypotonic buffer conditions is also observed for PML bodies, which also result from LLPS (Supplementary Fig. 6g). Overall, these results indicate that SIPAN are highly sensitive to the physico-chemical environment of the cells and their assembly is governed, at least partly, by hydrophobic interactions.

To further investigate SIPAN dynamics, we conducted fluorescence recovery after photobleaching (FRAP) experiments in HCT116 cells stably expressing PSMB4-GFP. 53BP1-GFP and GFP-H2A, which are tightly associated to DNA, were included for comparison with DNA damage foci or high-density chromatin domains, respectively. We found that PSMB4-GFP fluorescence recovery is very rapid, while little or no apparent recovery of fluorescence was detected for 53BP1-GFP or histone GFP-H2A, respectively (Fig. 6e, Supplementary Movie 7–9). Taken altogether, these results indicate that SIPAN result from LLPS, and that these structures can be dynamically modulated in response to extracellular cues.

Ubiquitin dynamics drive SIPAN formation. We sought to determine whether ubiquitin signaling is involved in SIPAN formation. No manifest accumulation of ubiquitin conjugates is observed over time by immunoblotting in IMR90 or HCT116 following incubation in HBSS (Fig. 7a, Supplementary Fig. 7a). Interestingly, SIPAN are enriched with K48-conjugated ubiquitin indicating the presence of ubiquitinated proteins (Fig. 7b, c and Supplementary Fig. 7b). In contrast, while SUMO formed foci in untreated cells that likely correspond to PML bodies, we did not observe accumulation of this protein in SIPAN (Supplementary Fig. 7c). These results suggest that discrete ubiquitination events might play a role in SIPAN dynamics. This prompted us to determine whether increasing the pool of ubiquitinated proteins promotes SIPAN formation. We treated IMR90 with HBSS and MG132 for 3 hrs, time at which, little foci are formed following nutrient deprivation only. Indeed, inhibition of proteasome activity with MG132 accelerates SIPAN formation, as both foci number and intensity were increased upon nutrient starvation (Fig. 7d–f). In addition, inhibition of UAE1 ubiquitin-
activating enzyme with TAK-243 prevents SIPAN formation (Fig. 7g, h, Supplementary Fig. 7d). Interestingly, continuous ubiquitination is required for SIPAN maintenance, as UAE1 inhibition results in SIPAN dissolution under conditions of nutrient deprivation (Fig. 7g, i). As expected, UAE1 inhibition does not impact SIPAN dissolution following replenishment of nutrient-deprived IMR90 cells with normal culture medium (Fig. 7g, j). Next, we reasoned that ubiquitin removal from SIPAN might, however, be required for SIPAN resolution upon incubation in normal culture medium. Inhibition of the proteasome-associated deubiquitinases, ubiquitin C-terminal hydrolase 5 (UCHL5) and ubiquitin-specific peptidase 14 (USP14), using the small molecule b-AP1533, prevents SIPAN resolution (Fig. 7g, k). On the other hand, proteasome inhibition does not impact
SIPAN resolution following replenishment of normal culture medium (Fig. 7g, k). Thus, we concluded that continuous ubiquitination and deubiquitination cycles are critical for SIPAN dynamics.

RAD23B is required for SIPAN formation. Based on the data described above, we rationalized that ubiquitin-binding proteins, and notably proteasome shuttling factors, might be involved in SIPAN formation. We found that siRNA depletion of several ubiquitin-binding proteins and shutting factors, notably RAD23B, inhibit SIPAN formation (Fig. 7f, Supplementary Fig. 7e). We validated the effect of RAD23B depletion using additional siRNAs targeting other regions of RAD23B mRNA (Fig. 7m). As expected, depletion of RAD23B does not affect the levels of proteasome components (Supplementary Fig. 7f). Endogenous RAD23B localizes in SIPAN (Fig. 7f). We also expressed RAD23B by lentiviral transduction in IMR90 and found that this factor localizes in SIPAN (Supplementary Fig. 7g). Of note, while depletion of RAD23A does not prevent SIPAN formation, this factor also localized in SIPAN (Fig. 7f, Supplementary Fig. 7h). Interestingly, deletion of the gene encoding the RAD23B orthologue in yeast, RAD23, compromises the assembly of RPN5-GFP (PSMD12) into PSG, suggesting that the role of RAD23B in proteasome phase separation might be conserved throughout evolution (Fig. 7o, p).

Multivalent interactions ensured by intrinsically disordered regions and/or structured domains are key determinants in LLPS. RAD23B is known to interact with the proteasome and contains an ubiquitin-like domain (UBL) and two ubiquitin-binding motifs, UBA1 and UBA2, which can engage in multivalent interactions. Bioinformatics analysis of human RAD23B and yeast RAD23 showed that intrinsically disordered regions are mainly located between functional domains, while maximal hydrophobicity is found within UBL and UBA domains. As SIPAN appear to depend on hydrophobic interactions, we tested the requirement of the above-mentioned domains for their formation. We expressed several mutants lacking key domains of RAD23B and found that the UBL and UBA domains are required for SIPAN formation (Fig. 8b, c and Supplementary Fig. 8b, c). We subsequently purified human RAD23B from bacteria and found that this factor undergoes LLPS in the presence of Ficoll 400 molecular crowding agent, as the protein mixture became turbid and liquid droplets could be readily observed (Fig. 8d–f). In contrast, BSA did not undergo LLPS in the same conditions. RAD23B LLPS depends on protein and crowder concentration, and is induced by other molecular crowding agents (Fig. 8g, h and Supplementary 8d). Of note, removal of N-terminal His tag on RAD23B has little or no impact on protein droplet formation (Supplementary Fig. 8e). Consistent with its ability to undergo phase separation in vitro, RAD23B droplets are disrupted by 1,6-hexanediol (Fig. 8i, Supplementary Fig 8f). Significantly, live imaging indicates that RAD23B droplets undergo fusion events in vitro (Fig. 8j, Supplementary Movie 10). Finally, we found that deletion of RAD23B domains, notably UBA2 reduced RAD23B ability to undergo phase separation (Fig. 8k, l).

Deprivation of non-essential amino acids is responsible for SIPAN formation. To further dissect the mechanism that governs SIPAN formation, we supplemented the nutrient-free HBSS solution with specific nutrients and found that addition or removal of glucose or pyruvate does not significantly impact SIPAN formation in IMR90 cells (Fig. 9a–c). In contrast, addition of fetal bovine serum (FBS) or amino acid mixture inhibit SIPAN formation (Fig. 9a–c). Preserving amino acid pools by inhibiting residual protein synthesis with cycloheximide (CHX), during incubation in HBSS, also blocks SIPAN formation (Fig. 9d–f). Conversely, preventing amino acid recycling by blocking autophagy with chloroquine or 3-methyladenine accelerates SIPAN formation (Fig. 9d–f, Supplementary Movie 11). Of note, mTOR is inhibited during nutrient deprivation and further blocking of this pathway with rapamycin or torin1, does not affect SIPAN formation following nutrient deprivation (Fig. 9d, e). Moreover, no formation of SIPAN was detected following mTOR inhibition in complete culture medium (Supplementary Fig. 9a, b). These results also suggest that SIPAN formation is either independent of mTOR signaling or that inhibition of this pathway is not sufficient to licence their formation. Next, we deconvoluted the amino acid mixture and treated IMR90 cells with HBSS supplemented with 1 mM of individual amino acids. Interestingly, we found that most non-essential amino acids (NEAA), rather than essential amino acids (EAA), strongly prevent SIPAN formation (Fig. 9g). Similar results were observed on SIPAN resolution, following amino acid replenishment in IMR90 cells (Fig. 9h). More pronounced effects were noticed with higher concentrations of amino acids (Supplementary Fig. 9c, d). Live-cell imaging of IMR90 expressing PSMB4-GFP further confirmed the rapid resolution of SIPAN following addition of NEAA (Supplementary Movie 12). We concluded that deprivation of amino acids, and especially non-essential amino acid, is a major determinant of SIPAN formation and resolution in mammalian cells.

RAD23B and PSME3 provide a link between amino acid supply, SIPAN formation, and apoptosis. In yeast, PSG promote viability and cell fitness following carbon starvation. To provide insights into the significance of SIPAN formation in mammalian cells, we first sought to determine the state of proteasome activity upon amino acid deprivation. We were unable to extract SIPAN to conduct in vitro activity studies, as these foci are quickly dissipated in the nucleus upon detergent treatment or hypotonic cell lysis (Fig. 6a, c, d). We, therefore, preformed SIPAN in IMR90...
Fig. 4 SIPAN are a general phenomenon common to many mammalian cell types. 

**a** Immunostaining of endogenous PSMD4 or PSMD7 in diverse cell types, showing that these proteins localize in nuclear foci following incubation in HBSS. SIPAN are observed in normal primary cells (e.g., HDLF: primary human lung fibroblasts, HUVEC: human endothelial cells, mESC: mouse embryonic stem cell); immortalized cells (e.g., NIH3T3: mouse embryonic fibroblasts, C2C12: mouse myoblast cell line, 3T3L1: mouse preadipocytes); transformed (e.g., Cos7: Transformed monkey kidney cells, RAW264.7: Abelson murine leukemia virus transformed macrophage) and tumoral cells (MCF7: human breast cancer, H1299: human non-small cell lung cancer).

**b** Immunostaining of PSMD7 in mouse pre-adipocytes 3T3L1 and differentiated adipocytes showing that this protein localizes in nuclear foci following incubation in HBSS. Right panel, Oil Red O staining was conducted to control for adipocyte differentiation (representative images from 3 independent experiments).
cells by incubation in HBSS for 8 hrs followed by treatment with MG132 in the same solution for 1 hr. Following proteasome inhibition, we observed increased levels of several short-lived nuclear stress-associated transcription factors, including p53, C-FOS, and C-JUN, suggesting that nuclear proteasome is still active under conditions of SIPAN formation (Fig. 10a). Next, we used a Me4BodipyFL-Ahx3Leu3VS (Proteasome Activity Probe) which accumulates in SIPAN after hydrolysis, and this also suggested that the proteasome is active in SIPAN (Fig. 10b).

To further define the biological significance of SIPAN, we investigated the link between SIPAN formation and cell viability. Treatment of IMR90 cells with chloroquine or 3-MA, which promotes SIPAN formation, diminishes cell survival following incubation in HBSS (Supplementary Fig. 9e, f). In contrast, treatment with CHX, which dampens SIPAN formation, protects cells from loss of viability relative to treatment with HBSS alone (Supplementary Fig. 9e, f). As expected, the CASPASE inhibitor Z-VAD protects IMR90 cells from undergoing apoptosis following...
Fig. 5 Rapid dynamics of SIPAN assembly and resolution. a–c Kinetics of SIPAN formation in IMR90 fibroblasts. a Cells were incubated in HBSS and harvested for immunostaining with PSMD4 antibody. Cells with more than 10 foci were counted (n = 3 independent experiments). b Signal intensity of SIPAN. Images from control and starved cells were used to estimate SIPAN signals (representative from 3 independent experiments). Arbitrary units (arb. units). c Quantification of the number of SIPAN per cell at different time points post-starvation (representative from 3 independent experiments). d Quantification of the size of the traced PSMB4-GFP foci (n = 2 independent experiments). e Time lapse from live-cell imaging indicating SIPAN fusion in vivo. IMR90 cells expressing PSMB4-GFP were incubated in HBSS and used for live imaging. Two fusion events are indicated by arrow of different colors (representative from 3 independent experiments). f Determination of the frequency of PSMB4-GFP foci fusion events over 1 hr (representative from 2 independent experiments). g Comparison of the average intensity of PSMB4-GFP foci in the corresponding cells, before and after fusion events. Note the increase in average intensity after fusion (representative from 2 independent experiments). h A representative image of a 10-min mobility trace of a PSMB4-GFP focus (representative from 2 independent experiments). i A mean-square displacement measurement plot of PSMB4-GFP foci is represented (representative from 2 independent experiments). j Quantification of the distance traveled by PSMB4-GFP foci over 10 min in two independent experiments. Each data point in the distribution plot represents a traced focus (n = 2 independent experiments). k–m SIPAN are reversible. SIPAN formation is induced for 8 hrs and then IMR90 cells were replenished with normal culture medium and harvested for immunostaining. k Cells with more than 10 foci were counted (n = 3 independent experiments). l Signal intensity of SIPAN from nutrient-starved cells and nutrient-replenished cells (representative from 3 independent experiments). m Cell nucleus showing dissipation of PSMD4 signals after medium replenishment (representative from 3 independent experiments). n Resolution of PSME3 foci after nutrient replenishment. IMR90 were incubated 6 hrs with HBSS and then incubated with culture media for 1 hr (representative from 3 independent experiments). o VCP chaperone is not required for SIPAN resolution. IMR90 cells were treated with HBSS solution for 6 hrs and then treated with various inhibitors at the indicated concentrations in complete medium for 1 hr (n = 3 independent experiments). Cells with more than 10 foci are counted. Data represent mean ± SD (a, d, f, g, i, j, k, o) or median with interquartile range for one representative experiment (b, c, l). *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; ns: not significant; one-way ANOVA with Holm-Sidak’s (a, k) or Kruskal–Wallis test with Dunn’s test (b, c, l) or 2-sided unpaired Student t-test (g, o). Source data are provided as a Source data file.

Supplementary Fig. 4, we rationalized that oncogenic transformation and/or tumor environment, both of which induce profound metabolic changes46, might further select for cells with reduced ability to form SIPAN. Thus, we induced oncogenic transformation of IMR90 cells by inhibiting p53, through MDM2 overexpression, with concomitant expression of two collaborating oncogenes, E1A and RAS<sup>G12V</sup> 47. The resulting transformed cells were further injected into immunodeficient mice and the tumors were isolated, and cells dissociated and cultured (Fig. 10k). Using normal, transformed (IMR90<sub>Trans</sub>) and tumoral (IMR90<sub>Tumor</sub>) cells, we then analyzed SIPAN formation and survival upon amino acid deprivation. We found that oncogenic transformation results in reduced ability of the cells to form SIPAN (Fig. 10l). Additionally, passage of transformed cells through tumors further decreased the ability of the cells to form SIPAN (Fig. 10l, Supplementary Fig. 10l). Moreover, propidium iodide incorporation-based cell viability assay indicated that transformed and tumoral IMR90 cells have increased cell survival ability in response to nutrient deprivation (Fig. 10m, n). These results provide further evidence for a possible role for SIPAN as a defense mechanism against malignant transformation.

Discussion

In this study, we demonstrate that the mammalian proteasome undergoes LLPS upon amino acid deprivation, resulting in the formation of nuclear membrane-less organelles we termed SIPAN. SIPAN assemble in nuclear regions of low chromatin density and the proteasome within these structures is labeled with a fluorescent activity probe, suggesting that SIPAN are subnuclear sites of active proteolysis. SIPAN undergo fusion events, can be dissipated and reassembled, and rapidly exchange components with the nuclear milieu, reflecting their fast turnover rates and highly dynamic nature. Moreover, SIPAN are nuclear substructures common to mammalian cells of various origins. Recently, it was also shown that, under hyperosmotic stress conditions, the proteasome undergoes LLPS and forms nuclear foci that target ribosomal proteins22. While, no ribosomal proteins were observed in SIPAN, these data altogether indicate that LLPS-mediated proteasome foci formation is a major determinant of mammalian stress responses. Indeed, SIPAN are associated with nutrient deprivation-induced cell death (Fig. 10o).

We uncovered a previously unappreciated link between proteasome dynamics and mammalian cell metabolism. Interestingly,
our results highlight significant evolutionary divergence in the responses of mammalian and yeast proteasomes to nutrient deprivation. (i) SIPAN form in the nucleus, whereas yeast proteasome is exported from the nucleus and form PSG structures in the cytoplasm\textsuperscript{16,18}. (ii) SIPAN are formed following amino acid, but not glucose deprivation, while the opposite situation occurs in yeast\textsuperscript{18}. (iii) Our data suggest that proteasome foci promote cell death in mammalian cells; however, the reverse outcome occurs in yeast, i.e., PSG improve cell fitness and viability\textsuperscript{18}. (iv) Finally, while SIPAN contain conjugated ubiquitin, PSG contain free ubiquitin\textsuperscript{17}. The reasons that can explain these differences between mammalian and yeast proteasome structures are currently unknown, but we emphasize that yeast and high-order metazoan present both common (conserved) and distinct metabolic responses to nutrient deprivation\textsuperscript{48}. Wild yeast can face dramatic changes in their environment and need to adapt quickly.
to maintain survival. In contrast, most of the cells of high-order multicellular organisms are in stable tissue microenvironments, with a generally stable and constant supply of nutrients. In addition, the utilization of nutrients in higher eukaryotes, such as mammals, depends on growth factor signaling. Moreover, in these organisms, nutrient deprivation is usually a condition of tissue or organ stress. On the other hand, common features unify the responses of the yeast and mammalian proteasomes to nutrient deprivation. The formation of SIPAN or PSG is reversible, as these structures dissipate quickly when nutrients are replenished. SIPAN and PSG contain ubiquitin, and the mammalian ubiquitin-binding factor RAD23B and its yeast orthologue RAD23 are important regulators of their formation. Thus, RAD23B or RAD23 provide a link between ubiquitin signaling pathways and the dynamics of SIPAN or PSG. Nonetheless, we cannot exclude, at this point, that PSG and SIPAN are unrelated structures in terms of composition, dynamics and functional significance.

One explanation of SIPAN formation is that, under conditions of nutrient deprivation, increase of E3 ubiquitin ligase activity and/or decreased deubiquitinase activity results in the accumulation of a subset of ubiquitinated substrates. This, in turn, triggers proteasome LLPS, through interaction with RAD23B and other ubiquitin receptors. Consistent with this notion, we found that (i) proteasome inhibition accelerates SIPAN formation, (ii) inhibition of ubiquitination, using UAEl inhibitor, prevents SIPAN formation and maintenance without altering their resolution upon amino acid replenishment, and (iii) proteasome deubiquitinase inhibition prevents SIPAN resolution. Thus, our studies altogether support a model whereby K48 chain linked ubiquitination promotes LLPS of the mammalian proteasome. Interestingly, a parallel can be made with K63-linked polyubiquitin chains which drives LLPS of the p62 scaffold protein resulting in autophagosome formation and selective autophagy.

Mechanistically, we found that the multiple RAD23B domains are required for SIPAN formation. The multivalent interactions ensured by UBL and UBA domains might link ubiquitinated substrates to the proteasome and thereby drive LLPS in the nucleus. Nonetheless, we cannot exclude that a combination of domain-mediated interactions and weak interactions involving non-organized regions act in a concerted manner to promote proteasome LLPS. It is noteworthy that another proteasome shuttling factor, UBQLN2, with similarities to RAD23B, also uses its multiple domains to undergo LLPS and association with stress granules in the cytoplasm. However, in this case, ubiquitin was found to inhibit LLPS mediated by UBQLN2. Further studies are required to determine how ubiquitin promotes or inhibits LLPS.

Protein ubiquitination per se is not sufficient to trigger SIPAN formation, as proteasome inhibition alone, under conditions of nutrient availability, barely induces SIPAN formation. On the other hand, PSME3, which activates proteasome in ubiquitin-independent manner, is also recruited and assembled in SIPAN. Thus, ubiquitin-independent signals are also needed to liences SIPAN formation. Importantly, our results indicate that the cellular levels of amino acids, and notably NEAA, are key determinants in SIPAN assembly and disassembly. Interestingly, NEAA are actively involved in intermediate carbon metabolism, nucleotide metabolism and signaling processes, and their presence might favor the formation of metabolic intermediates that might act as hydrotropes, thereby preventing LLPS.

What is the biological significance of LLPS-mediated nuclear proteasome in mammalian cells? Our data suggest that SIPAN are disadvantageous for the fitness of individual cells. It is possible that, in multicellular organisms, this response has evolved for the benefit of tissues and organs rather than that of individual cells. In multiple pathological conditions including wound, organ injury, and tissue ischemia, cells experience drastic nutrient deprivation that could trigger SIPAN formation and eventually cell death. For instance, SIPAN, by promoting cell death upon nutrient deprivation, might contribute to tissue and organ homeostasis by decreasing competition between cells for nutrients. In addition, release of constituents by dying cells might be beneficial for surrounding cells during periods of nutrient deprivation. This response might further contribute to tissue repair and homeostasis.

Our data also suggest a link between SIPAN and tumor development/progression. First, certain cancer cells including T47D, PC3, MIA PaCa-2, have reduced ability to form SIPAN. Second, nutrient deprivation induces p53-dependent apoptosis with a notable upregulation of the p53 target gene NOXA; and inhibition of RAD23B and PSME3 prevents p53/NOXA upregulation and apoptosis. Third, we also found that oncogenic transformation of normal human fibroblasts results in reduced cell ability to form SIPAN and resistance to apoptosis induced by nutrient deprivation. Based on these results, SIPAN formation might ensure tumor suppression by preventing the survival and propagation of cells that have been exposed to extreme metabolic changes, which, in turn, can induce genetic or epigenetic changes that might promote oncogenic transformation. Interestingly, transformed cells that were subjected to tumor formation have a highly reduced ability to form SIPAN, suggesting that further cell selection might take place during tumor progression. Indeed, nutrient scarcity within the tumor microenvironment, i.e., poorly vascularized regions, is known to impose a selective pressure that can further impact on cancer cell progression. For instance, in pancreatic ductal adenocarcinoma, a poorly vascularized tumor type, it was revealed that certain NEAA, and particularly glutamine and serine, are depleted in tumors, while EAA levels are increased. Glutamine, which has the strongest effect on SIPAN formation, is often depleted in the central region of solid tumors compared to other amino acids. Clearly, further manipulation of metabolic and signaling pathways that link proteasome LLPS and amino acid sensing is expected to provide additional insights into the role of SIPAN in physiology and human disease.
**Methods**

**Plasmids.** The cDNAs of human *PSMB4*, *PSMB5*, *PSMD14*, and *PSMD12* genes were generated in a modified pBluescript using gene synthesis (Biobasic). The constructs were then subcloned as a fusion with the GFP gene and recombined using gene LR-clonase in lentiviral expression vectors as previously described58. SiRNA-resistant constructs for human *RAD23B* and its corresponding mutants *RAD23B*ΔUBL, *RAD23B*ΔUBA1, *RAD23B*ΔUBA2, *RAD23B*ΔUBA1/UBA2, and *RAD23B*ΔSTI were generated using gene synthesis (Biobasic) and then recombined into lentiviral expression constructs. PAX2 (#35002) and pMD2G (#12259) lentiviral packaging plasmids were from Addgene. Human histone *H2A* was generated by gene synthesis in pENTR gateway plasmid and then recombined into pDEST GFP plasmid. For bacterial expression of RAD23B, His-tagged human *RAD23B* was generated by recombination of pBluescript into pDEST-His expression vector. pcDNA5-FRT/TO-eGFP-53BP1 (Addgene #60813) is used to express GFP-53BP1.
were cultured in EndoGRO basal medium (Millipore) supplemented with 1000 U/ml of leukemia inhibitory factor (LIF) (Life technologies). HUVEC cells captoethanol, 0.1 mM Non-essential amino acids, 1 mM sodium pyruvate and containing 15% FBS, 1% L-glutamine, penicillin/streptomycin, 0.1 mM monkey kidney cells Cos-7, human embryonic kidney HEK293FT cells were cul-

Cell culture

Human primary lung fibroblasts IMR90 and HDL cells, MCF7 human breast cancer cell line, human non-small cell lung carcinoma NCI-H1299, A3 mouse androgen-independent prostate cancer cells, RAW 264.7 murine macrophage cell line, HCT116 human colon cancer cell line, HeLa human cervical cancer cell line, T47D human breast cancer cell line, MDA-MA-231 human breast cancer cell line, PCA PaCa-2 human pancreatic cancer cell line, LLC mouse Lewis lung carcinoma cell line, murrine C2C12 myoblasts, 3T3-L1 mouse preadipocytes, NIH/3T3 mouse fibroblasts were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine and 1% penicillin/streptomycin. Transformed monkey kidney cells Cos-7, human embryonic kidney HEK299FT cells were cul-

Deubiquitination is required for SIPAN formation. Cells were incubated in HBSS solution for 6 hrs and then treated with b-AP15 deubiquitinase inhibitor, MG132, or BTZ in normal culture medium for 1 hr (n = 3 independent experiments). I RAD23B and other ubiquitin receptors are required for SIPAN formation. Following siRNA depletion of several ubiquitin-binding proteins and shuttling factors, IMR90 cells were incubated in HBSS for 6 hrs and harvested for immunostaining for SIPAN formation (n = 3 independent experiments). m Validation of RAD23B using additional siRNAs. Following siRNA transfection, IMR90 cells were incubated in HBSS for 6 hrs and harvested for immunostaining for SIPAN formation (representative from 3 independent experiments). n RAD23B is assembled in SIPAN following nutrient starvation. IMR90 cells were incubated in HBSS for 6 hrs and harvested for immunostaining for endogenous RAD23B, and RAD234 (representative from 3 independent experiments). o, p in yeast, RAD23 is important for PSGs formation under conditions of carbon depletion (n = 3 independent experiments). Data in graphs h, i, j, k, l, p represent the mean ± SD. 2-sided unpaired Student’s t-test (h, i, l, p). Data in e, f represent the median with interquartile range for one representative experiment. 2-sided Mann–Whitney test (e, f). *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; ns: not significant. Source data are provided as a Source data file.

For the bacteria production of RAD23B without tag, 6His-TEV-TEAD23B construct was generated by inserting the DNA sequence for the TEV cleavage site into pDEST-RAD23B. Site-directed mutagenesis was performed using primers TEV_RAD23B_F with TEV_RAD23B_R for RAD23B. Successful insertion of the TEV site was confirmed using DNA sequencing.

Cell culture

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For the electroporation of IMR90 cells, 2 million cells were resuspended in electroporation buffer (5 mM KC1; 15 mM MgCl2; 120 mM Na2HPO4/NaHPO4 pH 7.2; 25 mM sodium succinate; 25 mM manitol) and electroporation was done using X-100 program on Amaxa™ Nucleofector® II Device (Lonza).

Immunoblotting and antibodies. Total cell extracts were prepared following cell lysis in 25 mM Tris pH 7.3 and 2% dodecylmaltoside (SDS). Cell extracts were boiled at 95 °C for 10 min and then sonicated. Total protein concentration was determined using the bichinchoninic acid (BCA) protein quantification assay. The samples were diluted in Laemmli buffer. SDS-PAGE and the immunoblotting were done as we previously described59. The chemiluminescence band signals were measured and represented by violin plot (representative from 3 independent experiments). For the electroporation of IMR90 cells, 2 million cells were resuspended in electroporation buffer (5 mM KC1; 15 mM MgCl2; 120 mM Na2HPO4/NaHPO4 pH 7.2; 25 mM sodium succinate; 25 mM manitol) and electroporation was done using X-100 program on Amaxa™ Nucleofector® II Device (Lonza).

Preparation of nuclear and cytoplasmic cell fractions. IMR90 cells were incubated in HBSS and fractionated with a hypotonic lysis buffer as previously described, but with some modifications62. Briefly, IMR90 cells were washed once with cold PBS and then twice with hypotonic buffer (10 mM KC1, 10 mM Tris-HCl pH 7.4). Nuclei were isolated by centrifugation at 1000 × g. Nuclear fractions were suspended in hypotonic buffer supplemented with 10% FBS, 1% glutamine, 1% penicillin/streptomycin, and 1 μg/ml insulin. The cells were resuspended in electroporation buffer (5 mM KC1; 15 mM MgCl2; 120 mM Na2HPO4/NaHPO4 pH 7.2; 25 mM sodium succinate; 25 mM manitol) and electroporation was done using X-100 program on Amaxa™ Nucleofector® II Device (Lonza).

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Preparation of nuclear and cytoplasmic cell fractions. IMR90 cells were incubated in HBSS and fractionated with a hypotonic lysis buffer as previously described, but with some modifications62. Briefly, IMR90 cells were washed once with cold PBS and then twice with hypotonic buffer (10 mM KC1, 10 mM Tris-HCl pH 7.4) or Digitonin (Sigma, D-5628) at the indicated concentrations and then resolved for the indicated time points for fixation and immunostaining. For live-

Cell transfection and lentivirus transduction. HCT116 cells were transected with expression plasmids using lipofectamine 2000. Two to three days after the transfection, cells were harvested for immunoblotting or treated with HBSS and served as indicated. For lentivirus production, expression constructs were transfected in HEK293FT cells, in combination with lentivirus packaging constructs and lentivirus particles were harvested several times post-transfection. IMR90 or HCT116 cells were infected once or multiple times with lentivirus suspension. Two days later, cells were selected with puromycin for 2 days and then used as indicated. siRNA oligonucleotides and short interfering RNA oligonucleotides used in Supplementary Table 2.

For the cell transfection, cells were treated as indicated or directly harvested for immunoblotting or immunostaining. siRNA oligonucleotides used are listed in Supplementary Table 2.

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is resuspended in a volume of hypotonic buffer corresponding to that of the cytoplasmic fraction, and then mixed with 2% SDS. All samples were then used for immunoblotting. HCT116 cells were treated with HBSS and nuclear and cytoplasmic fractions were prepared by incubating the cells for 1 min in 10 mM Tris pH 7.3 containing 100 mM KCl, 1 mM β-mercaptoethanol, 1 mM PMSF, and 0.1% NP-40. Cells were centrifuged at 3,500 × g for 1 min and the pellet fraction was washed once in detergent-free buffer. All samples were then used for immunoblotting.

**Colloidal gold-based immunodetection and transmission electronic microscopy.** This procedure was performed based on previous protocols with the following modifications. Cells were fixed for 30 min in 4% paraformaldehyde (PFA) in 0.1 M cacodylate buffer pH 7.2 (Tcaco), washed twice in Tcaco buffer and then once in PBS. Cells were permeabilized in PBS containing 0.2% de NP-40 for 10 min and non-specific sites were saturated with PBS containing 0.04% NP-40 and 10% FBS for 30 min (blocking buffer). Cells were then incubated for 3 hrs at RT with anti-PSMD4 primary antibody in blocking buffer. Cells were then washed 6 times, 5 min each, with the blocking buffer and incubated with anti-mouse IgG nanogold antibody (Nanoprobes, NY, USA), diluted 1/100 in the blocking buffer, followed by several washes in the blocking buffer. Coverslips were then incubated in 2% glutaraldehyde in PBS for 10 min followed by a 10 min incubation in silver enhancement solution (HQ Silver enhancement kit, Nanoprobes, NY, USA) at room temperature. Samples were post-fixed with 1% OsO4 in Tcaco buffer for 1 hour.
10 min, stained en bloc with 1% uranyl acetate for 5 min. Cells were dehydrated in graded series of ethanol and scrapped off the plates in ethanol and pelleted. The pellets were embedded in Epon®46. Ultrathin sections of the samples were obtained using a Reichert Ultracut ultramicrotome and mounted on naked nickel grids. Sections were stained with lead citrate and examination was performed with a Philips CM100 transmission electron microscope. Electron micrographs were captured using an AMT XR80 digital camera (Advanced Microscopy Techniques, Corp. MA, USA).

Immunofluorescence. The immunostaining was conducted as previously described60. Briefly, culture medium was removed and cells were directly fixed in PBS containing 3% PFA for 20 min. For antigen retrieval, the samples were incubated in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) and heated for 30 s in the microwave. The cells were then washed three times to remove sodium citrate buffer. Cells were permeabilized by incubation in PBS 0.5% Triton. Non-specific sites were blocked for 1 hr using PBS containing 0.1% Triton X-100 and 10% PBS. The coverslips were then incubated with primary antibodies for 3 hrs at room temperature or overnight at 4°C. After three washes, cells were incubated with secondary anti-mouse Alexa Fluor® 594 (1/1,000), anti-mouse Alexa Fluor® 488 (1/1,000), anti-rabbit Alexa Fluor® 488 (1/1,000) or anti-rabbit Alexa Fluor® 594 (1/1,000) antibodies for 1 hr. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) during secondary antibodies incubation. Cell samples were mounted on a stage of an Olympus RX53 (Olympus Corp., Japan) upright microscope equipped with an Olympus UPlan SAP0.45× NA 0.15 immersion objective. The corresponding fluorescence cubes (DAPI-1160B, GFP-3035C and Texas-4040C; Semrock Inc. USA) were used to efficiently filter the excitation wavelengths and pass the emission wavelengths into the CCD camera detection channel. Images were acquired by confocal microscopy and by 1.4 megapixel CCD Olympus XM10 (Olympus Corp., Japan) CCD camera controlled by the Olympus CellSens software or using a DeltaVision Elite system (GE Healthcare) with z-stacking, Gamma, brightness, and contrast are adjusted on displayed images using the CellSens software. The collected EPI-fluorescence images were processed using WCIF-ImageJ program (NIH).

EPI-fluorescence and bright field live-cell microscopy. Cells stably expressing PSMB4-EGFP were grown on Mattek glass bottom petri dishes (Coverslip thick-mounted on a motorized stage of a Zeiss AxioObserver.Z1/7 inverted microscope EPI-images were processed using WCIF-ImageJ program (NIH) and the foci mobility was corrected by any positional shifting due to the movement of the whole cell during the course of image recordings. The number of foci was evaluated using automatic particle counting option in ImageJ. Microsoft Excel and Kaleida Graph (Synergy) were used for data analysis and presentation and Student’s t-test was used for statistical analysis. Mean-square displacement was calculated by the following equation as previously described64:

\[ MSD(\Delta t) = \frac{1}{n} \sum_{i=1}^{n} D(\Delta t) \]

Where

\[ D(\Delta t) = \sqrt{\left(\frac{x_i^t - x_i^{t-\Delta t}}{C_0(\Delta t)}\right)^2 + \left(\frac{y_i^t - y_i^{t-\Delta t}}{C_0(\Delta t)}\right)^2} \]

For image presentations, 2D-maximum intensity projected images were generated using the ZEN blue software.

For the determination of the size and the average intensity of foci, the objects (foci) were identified automatically using MetaMorph (Molecular Devices). The size (area) of the identified objects and the integrated intensity of the identified objects was automatically measured. The foci average intensity was then calculated from their integrated intensity and size as below

\[ \text{Average intensity} = \frac{\text{Integrated intensity of foci}}{\text{Area of foci}} \]

Fluorescence recovery after photobleaching (FRAP) experiments. PSMB4-EGFP expressing HCT116 cells were cultured in 35 mm Mattek chambers and foci formation was induced by incubation in HBSS for 6 hrs prior to the imaging experiments. HCT116 cells were transfected with a plasmid containing 3SIPB1-EGFP were exposed to gamma irradiation for 4 hrs to induce the formation of DNA double-strand breaks/repair 3SIPB1 foci. HCT116 transiently expressing human histone GFP-H2A were
directly used. The samples were then mounted on a Prior ProScan III motorized stage of an Olympus IX81 inverted microscope. The FRAP experiments were performed on an Olympus FluoView FV1000 laser scanning confocal (CLSM) system equipped with spectral detectors. Regions of interest (ROI) from individual cells in the FluoView software were chosen (10 × 10 pixels with 1.03 µm/pixel) for the FRAP experiments. The 488 nm line of the Argon laser was used for both imaging (attenuated by 95%) and photobleaching (full 100% power output) of GFP in combination with a PLAPON ×60/1.4 NA OSC oil immersion lens. The DM405/488 polychroic mirror was used to efficiently reflect the excitation wavelength and pass the emission wavelengths into the corresponding detection channel. Prebleach images (10 frames) were taken prior to FRAP activation for normalization of the data. Photobleaching of EGFP was generated by scanning the selected ROIs for 10 s (dwell time of 2 µs/pixel). Acquisition parameters (laser intensity, pixel dwell time, photomultiplier tube (PMT) voltage/gain, confocal pinhole aperture) were set within the linear range of the PMT detection. The acquired fluorescence recovery
Availability of amino acids regulates SIPAN formation. Cells were incubated with various inhibitors in HBSS solution and harvested after 3 hrs or 6 hrs for immunostaining (d) and count (e). Inhibition of autophagy by chloroquine accelerates SIPAN formation while inhibition of protein synthesis by cycloheximide inhibits SIPAN formation. Blocking mTOR pathway, with Rapamycin or Torin 1, does not affect SIPAN formation following nutrient deprivation (n = 3 independent experiments). f Measured foci intensity is represented as violin plot (representative from 3 independent experiments). g Non-essential amino acids (NEAA) completely prevented SIPAN formation. IMR90 cells were incubated with individual amino acids in HBSS solution and harvested after 6 hrs for immunostaining (n = 3 independent experiments). h NEAA promote SIPAN resolution. SIPAN formation is induced and then cells were replenished with fresh medium containing individual amino acids and harvested after 2 hrs for immunostaining (n = 3 independent experiments). Red arrows represent NEAA (g, h). Data in b, c, e, f, g, h represent mean ± SD. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; ns: not significant; 2-sided unpaired Student’s t-test used in (b) and 2-sided ANOVA in (e). Source data are provided as a Source data file.

Fig. 9 Exhaustion of non-essential amino acid is responsible for SIPAN formation. a-c Addition of amino acids, but not glucose or pyruvate, prevents SIPAN formation in IMR90 cells. Cells were incubated with various nutrients in HBSS solution and harvested after 6 hrs for immunostaining (a) and count (b) (n = 3 independent experiments). d-f Non-essential amino acids (NEAA) completely prevented SIPAN formation. IMR90 cells were incubated with individual amino acids in HBSS solution and harvested after 6 hrs for immunostaining (n = 3 independent experiments). f Measured foci intensity is represented as violin plot (representative from 3 independent experiments). g Non-essential amino acids (NEAA) completely prevented SIPAN formation. IMR90 cells were incubated with individual amino acids in HBSS solution and harvested after 2 hrs for immunostaining (n = 3 independent experiments). h NEAA promote SIPAN resolution. SIPAN formation is induced and then cells were replenished with fresh medium containing individual amino acids and harvested after 2 hrs for immunostaining (n = 3 independent experiments). Red arrows represent NEAA (g, h). Data in b, c, e, f, g, h represent mean ± SD. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; ns: not significant; 2-sided unpaired Student’s t-test used in (b) and 2-sided ANOVA in (e). Source data are provided as a Source data file.

curves were generated using the FluoView software. The values were obtained from the analysis of three independent experiments.

MTT assay. IMR90 and HDLF cells were plated in 24-well culture plates and incubated in HBSS with treatments as indicated in figure legends. For siRNA experiment, the same number of cells (80,000 cells) were plated and treated with HBSS for 4 hrs for IMR90 or 24 hrs for HDLF. Medium was removed and replaced with fresh medium containing 5 μM MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide, Bioshop, MT2222). Cells were then incubated at 37 °C for 3 hrs. Cells were washed once with PBS and DMSO was added to extract the formazan product. The absorbance was measured at 490 nm using a microplate reader (Biotek Instruments).

FACS analysis. Cells were washed with PBS and harvested by trypsinization. Cells were centrifuged, washed once with PBS and fixed with 75% cold ethanol overnight. Cells were centrifuged and resuspended in PBS containing 100 μg/ml RNase A and 10 μg/ml propidium iodide (P.I.) for 20 min at 4 °C. Cells were then washed twice with PBS and stained with 0.5% crystal violet for 30 min and then washed several times with water.

RAD23B and RAD23 protein sequence analysis. Analysis of domains and disorder regions of RAD23 proteins were conducted using PONDRT-FIT algorithm20, PONDRT-VLXT and PONDRT-VSIL20. The value of 0.5 in the Y-axis is considered as a threshold. Residues with a score above and below 0.5 are predicted to be disordered and ordered respectively. Hydrophobicity calculation was assessed using Kyte Doolittle hydrophathy algorithm24. The highest values indicate the hydrophobic amino acids along the sequence.

Purification of human RAD23B. Expression constructs for wild type His-RAD23B and its corresponding mutants were transformed into BL21-CodonPlus-RIL competent cells. Cells were grown at 37 °C and then treated with 400 μM Isopropyl β-D-thiogalactopyranoside (IPTG) to induce RAD23B protein production. Then, cells were harvested and centrifuged at 1,000 × g for 15 min at 4 °C. The bacteria were washed with cold PBS, centrifuged and frozen on dry ice as pellets. The cell pellets were lysed in 50 mM Tris-HCl pH 8.0, 500 mM NaCl, 3 mM β-mercaptoethanol, 1 mM PMSF and 1× protease inhibitors (Sigma-Aldrich). The bacteria suspensions were sonicated and the resulting lysates centrifuged at 27,000 × g for 20 min. Supernatants were incubated with Ni-NTA Agarose resin (Invitrogen, R901-15) overnight at 4 °C. Then, the resin was washed 5 times with 20 volumes of 50 mM Tris-HCl pH 8.0, 500 mM NaCl, 3 mM β-mercaptoethanol, 1 mM PMSF, 1× protease inhibitors, 20 mM imidazole and transferred into a Bio-Spin Disposable Chromatography column (Bio Rad, 731-1550). Proteins were eluted 3 times with 50 mM Tris-HCl pH 8.0, 500 mM NaCl, 3 mM β-mercaptoethanol, 1 mM PMSF, 1× protease inhibitors, 200 mM imidazole and transferred into 1 mL EDTA and proteins were used subsequently used for phase separation or temporarily stored at 4 °C.

For the production of RAD23B without His-tag, the elution of His-TEV-RAD23B was dialyzed in 20 mM Tris-HCl pH 7.5 for 1 hr at 4 °C. The His tag was cleaved using TEV protease (New England Biolabs, P8125S) according to the manufacturer’s protocol. The protein samples were incubated again with Ni-NTA Agarose resin (QIAGEN) for 5 hrs at 4 °C and then transferred into a Bio-Spin disposable chromatography column. The elution was collected and the beads were washed three times with three volumes of the wash buffer. Proteins were immediately used for phase separation or temporarily stored at 4 °C.

RAD23B liquid phase separation and droplet fusion in vitro. His-RAD23B protein was concentrated 5 times and the elution buffer was changed with a phase separation buffer containing 50 mM HEPES pH 7.2 and 100 mM NaCl using Amicon Ultra-3000 Centrifugal Filter Units (Millipore, UFC901024). The MTT assay, an equal volume of His-RAD23B and Ficoll 400 (Sigma, F4375, 300 mg/ml prepared in 50 mM HEPES pH 7.2 and 100 mM NaCl buffer) were mixed at room temperature to initiate droplet formation. The droplets were deposited on a glass slide in a chamber formed of two overlapping cover slips to allow liquid mixing and to observe fusion events. PEG 6000 (Aldrich, 17,451A) or Dextran 60,000–90,000 (ICN, 10,1513) crowding agents prepared in 50 mM HEPES pH 7.2 and 100 mM NaCl buffer were used at 10 % or 5 % final concentration, respectively. Crystal violet (0.5% w/v) prepared in 25% methanol is occasionally used to stain droplets (1 μl of crystal violet for 20 μl of droplets solution).

Yeast strains and growth conditions. Yeast cells were generated and propagated using standard yeast genetics methods. The genotype of the yeast strains used in this study are BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 RPN5-GFP-HIS3MX (from Life Technologies Yeast GFP collection, catalog #: 95702) and BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 RPN5-GFP-HIS3MX Δrad23a::kanMX (generated for this study using standard yeast genetics methods). For carbon starvation, cells were grown to saturation in synthetic complete medium containing 2% glucose. Cells were diluted in the same medium and allowed to grow to the exponential phase overnight. Cells were then washed once in synthetic complete medium without glucose and resuspended in the same medium at a density of 0.1 OD (630 nm). Cells were incubated in this medium for 24 hrs before harvest and flow cytometry microscopy observation as described22. Briefly, yeast cells were fixed for 15 min in 0.1 M of potassium phosphate buffer (pH 6.4) containing 3.7% formaldehyde. Fixed cells are centrifuged at 1,000 × g for 2 min, resuspended in 50 mM Tris pH 7.5 and stored at 4 °C. Prior microscopy analysis, fixed cells were layered on a concanavalin A-coated 15-well slides and incubated with the antifade mounting medium containing DAPI (VECTASHIELD®). Images were taken by fluorescence microscopy using a ×60.

Measurement of osmolality. Osmolality of conditioned HBSS recovered from treated cells was measured with micro-osmometer Fiske® 210 (Advanced Instruments Inc.) according to the manufacturer recommendations. Calibration was done using calibration standard (Fiske 50 mOsm/kg #00A405 and 850 mOsm/kg #00A405).

Oncogenic transformation and tumor generation. Primary IMR90 fibroblasts were generated following transduction by MDM2, E1A and RASV12 by retroviral infection as previously established9. Transformed cells were trypsinized, counted and then resuspended in culture media supplemented with Matrigel. 3 x 10^6 cells were subcutaneously injected (0.1 ml) using a 21-gauge needle in the right flank of 6-week aged athymic nude mice (JAX#02019). All animal studies were approved by the Animal Care Committee of the research center of the Maastricht University Hospital and in agreement with the Canadian Council on Animal Care guidelines.

Tumor size was determined by measuring the length and width of the tumor using a caliper. At the end of the experiment, animals were euthanized with CO2/ isoflurane inuff. Tumors were collected 4–6 weeks post-injection to isolate and culture tumor cells. For cell isolation, tumors are minced into 1–2 mm pieces using a scalpel. Tumor pieces were incubated at 37 °C for 30 min in digestion media composed of DMEM/F12 media (Wisent), 0.5 ml collagenase (Bioshop), 100 μg/ml DNase (NEB), 2 % FBS, 1/100 Penicillin/Streptomycin. After incubation, tumor pieces were digested in prewarmed trypsin for 3–5 min and then with complete F12 media supplemented with DNase. Cell mixture was filtered using 40 μm cell strainer and isolated cells were cultured for subsequent assays.
Analysis of cell death using propidium iodide/Hoechst 33342. After 96 hrs incubation with HBSS, IMR90, and IMR90tumor cells were incubated for 1 hr with 1 µg/ml propidium iodide and 1 µg/ml Hoechst 33342. Cells were washed, fixed with PBS containing 3% PFA and analyzed by fluorescence microscopy as described above.

Real-time quantitative PCR. Expression levels of mRNAs encoding protein shuttling factors siRNA were analyzed as described before. The mRNA levels were normalized to PGK1 expression. The primers used are listed in Supplementary Table 4.

Statistics and reproducibility. Statistical analysis was carried out with Prism 8 (GraphPad). Data are represented as mean ± SD or median with interquartile. When applicable, appropriate statistical tests are used as described in figure legends. Briefly, unpaired 2-sided Student’s t-test with or without Welch’s correction or 2-sided
Fig. 10 Inhibition of SIPAN formation is associated with apoptosis. a Proteasome is active under nutrient starvation. IMR90 cells were incubated in HBSS for 8 hrs and then treated with MG132 for 1 hr and harvested for immunoblotting (representative from 3 independent experiments). b SIPAN are catalytic active. IMR90 cells were treated with HBSS for 6 hrs and then incubated with 1 µM of Me4Bodipy-Ahx3-L3-V5 proteasomal activity probe for 1 hr. Cells were then harvested for immunostaining (representative from 3 independent experiments). c-e Inhibition of RAD23B or PSME3 result in increased cell survival following nutrient deprivation. Three days following siRNA transfection, IMR90 cells were incubated in HBSS for 48 hrs and harvested for MTT viability assay (e), Western blotting (d), phase contrast imaging or FACS analysis (e) (n = 2 independent experiments). Red arrow in (e) represents subG1 apoptotic cell population. f Induction of pro-apoptotic factors during HBSS treatment. IMR90 cells were treated with HBSS and harvested at the indicated times for western blotting (representative from 3 independent experiments). g Depletion of RAD23B or PSME3 cells protects from cell death induced by nutrient starvation. IMR90 cells without RAD23B or PSME3 were treated with HBSS for 48 hrs and harvested for immunoblotting for pro-apoptotic proteins (representative from 3 independent experiments). h-j Depletion of NOXA protects from cell death induced by nutrient deprivation. IMR90 were depleted of NOXA and treated with HBSS for 48 hrs and harvested for western blotting (h), MTT assay (i) and phase contrast (j) (representative from 3 independent experiments). k Schematic representation of the procedure for the generation of IMR90 cells transformed or derived from tumors. l Primary, transformed or tumoral IMR90 cells were treated with HBSS for 8 hrs and harvested for immunostaining for PSMD4 (representative from 3 independent experiments). m, n Cells were treated with HBSS for 96 hrs and stained with Hoechst (DNA) and propidium iodide for imaging (m) and counting of dead cells (n) (n = 3 independent experiments). o Model of SIPAN formation and function. Amino acids exhaustion promotes foci formation in the nucleus with RAD23B as a driver. SIPAN formation is associated with cell death. Data in c, i, l, n represent the mean ± SD. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; ns: not significant; 2-sided unpaired Student’s t-test used in (c, n). Source data are provided as a Source data file.

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