Bacterial encapsulins as orthogonal compartments for mammalian cell engineering

Felix Sigmund1,2,3, Christoph Massner1,2,3, Philipp Erdmann4, Anja Stelzl1,2, Hannes Rolbieski1,2, Mitul Desai5, Sarah Bricault5, Tobias P. Wörner6, Joost Snijder6,7, Arie Geerlof8, Helmut Fuchs9, Martin Hrabě de Angelis9, Albert J.R. Heck6, Alan Jasanoff5,10,11, Vasilis Ntziachristos1,12, Jürgen Plitzko4 & Gil G. Westmeyer1,2,3

We genetically controlled compartmentalization in eukaryotic cells by heterologous expression of bacterial encapsulin shell and cargo proteins to engineer enclosed enzymatic reactions and size-constrained metal biomineralization. The shell protein (EncA) from Myxococcus xanthus auto-assembles into nanocompartments inside mammalian cells to which sets of native (EncB,C,D) and engineered cargo proteins self-target enabling localized bimolecular fluorescence and enzyme complementation. Encapsulation of the enzyme tyrosinase leads to the confinement of toxic melanin production for robust detection via multispectral optoacoustic tomography (MSOT). Co-expression of ferritin-like native cargo (EncB,C) results in efficient iron sequestration producing substantial contrast by magnetic resonance imaging (MRI) and allowing for magnetic cell sorting. The monodisperse, spherical, and iron-loading nanoshells are also excellent genetically encoded reporters for electron microscopy (EM). In general, eukaryotically expressed encapsulins enable cellular engineering of spatially confined multicomponent processes with versatile applications in multiscale molecular imaging, as well as intriguing implications for metabolic engineering and cellular therapy.

1 Institute of Biological and Medical Imaging, Helmholtz Zentrum München, Ingolstädter Landstraße 1, Neuherberg 85764, Germany. 2 Institute of Developmental Genetics, Helmholtz Zentrum München, Ingolstädter Landstraße 1, Neuherberg 85764, Germany. 3 Department of Nuclear Medicine, Technical University of Munich, Ismaninger Straße 22, Munich 81675, Germany. 4 Department of Structural Biology, Max Planck Institute of Biochemistry, Am Klopferspitz 18, Martinsried 82152, Germany. 5 Department of Biological Engineering, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge 02139 Massachusetts, USA. 6 Biomolecular Mass Spectrometry and Proteomics Group, Bijvoet Center for Biomolecular Research and Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Padualaan 8, Utrecht 3584CH, The Netherlands. 7 Snijder Bioscience, Spijkerstraat 114-4, Arnhem 6828 DN, The Netherlands. 8 Institute of Structural Biology, Helmholtz Zentrum München, Ingolstädter Landstraße 1, Neuherberg 85764, Germany. 9 Institute of Experimental Genetics, Helmholtz Zentrum München, Ingolstädter Landstraße 1, Neuherberg 85764, Germany. 10 Department of Brain & Cognitive Sciences, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge 02139 Massachusetts, USA. 11 Department of Nuclear Science & Engineering, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge 02139 Massachusetts, USA. 12 Chair for Biological Imaging, Technical University of Munich, Ismaninger Straße 22, Munich 81675, Germany. Correspondence and requests for materials should be addressed to G.G.W. (email: gil.westmeyer@tum.de)
C ompartmentalization, the spatial separation of processes into closed subspaces, is an important principle that has evolved on several biological scales: multi-enzyme complexes that channel substrates, nanocompartments built entirely from proteins, as well as membrane-enclosed organelles, cells, and organs. Compartments make it possible to generate and maintain specific local conditions that can facilitate interactions and reactions in confined environments, such that they can isolate toxic reaction products, protect labile intermediate products from degradation, or separate anabolic from catabolic processes. Whereas eukaryotes possess many membrane-enclosed organelles, membranous compartments are not known in bacteria with a notable exception of magnetosomes in magnetotactic bacteria, in which specific reaction conditions are maintained which enable magnetic biomineralization. However, nanocompartment shells built entirely from protein complexes can serve functions in prokaryotes that are analogous to eukaryotic organelles.

Intense work has been invested in engineering compartments in prokaryotic systems and yeast to realize features such as substrate channelling for biotechnological production processes. In contrast, no orthogonal compartments with self-targeting cargo molecules exist to date for use in mammalian cells. Such a system could, for instance, enable cellular engineering of reaction chambers that would endow genetically modified mammalian cells with new metabolic pathways that may include labile intermediate products or spatially confined toxic compounds. Engineered orthogonal compartments in eukaryotic cells may also enable size-constrained synthesis of biomaterials via, e.g., metal biomineralization processes occurring under specific localized environmental conditions.

With regards to protein complexes as building blocks for addressable nanocompartments, viruses and virus-like particles have been expressed in bacterial hosts to encase fluorescent proteins, enzymes, and even multi-enzymatic processes. Similarly, bacterial microcompartments (BMC) such as Eut microcompartments and carboxysomes have been genetically engineered to load foreign cargo proteins such as fluorescent proteins. In mammalian systems, vault proteins (vaults) have been explored, which are ribonucleoprotein complexes enclosed by ~60 nm large envelope structures into which foreign cargo proteins such as fluorescent proteins or enzymes can be packaged. However, vaults have openings on both ends and are endogenously expressed by many eukaryotic cells. With respect to protein shell structures that can incorporate iron, the iron storage protein ferritin has been overexpressed to generate MRI contrast under certain conditions although its core size is only ~6 nm containing only ~2000 iron atoms on average per core, which can result in only low magnetization.

Viral capsids such as the ones from cowpea chlorotic mottle virus (CCMV) have also been equipped with iron-binding sites that lead to accumulation of iron. Expression, assembly, and iron loading in mammalian cells, however, have not yet been demonstrated.

In search of a versatile nanocompartment-cargo system for heterologous expression in eukaryotic cells, we were intrigued by the recently discovered class of prokaryotic proteinaceous shell proteins called encapsulins because they possess a set of attractive features: (1) A single shell protein—without the need for protolytic processing—is sufficient to form comparably large shell-like architectures (~18 nm or ~32 nm) auto-assembled from 60, or 180 identical subunits with a triangulation number (T) of one (T = 1) or three (T = 3), respectively. (2) The assembled shells are pH resistant and temperature stable. (3) A versatile set of native cargo molecules including enzymes exist that are packaged into shell structures via specific encapsulation signals defined by a short terminal peptide sequence. (4) The pore size of ~5 Å allows channeling small molecular substrates through the shell. (5) Myxococcus xanthus (M. xanthus) encapsulin was also shown to possess cargo proteins B and C, both containing ruberythrin/ferritin-like domains as well as highly conserved iron-binding ExxH motifs enabling import and sequestration of iron inside the nanoshell. Ferritin-like cargo proteins adopt an “open ferritin structure” and possess ferroxidase activity. A model based on structural data from Thermotoga maritima encapsulins (with T = 1) assumes that the ferritin-like protein docks into the shell where it obtains ferrous iron through the pores which it then oxidizes for deposition of up to an estimated 30,000 iron atoms per shell in the case of M. xanthus. This amount is an order of magnitude more than can be contained inside a ferritin core expressed in eukaryotic cells. The function of the cargo protein D, on the other hand, has so far not been understood. (6) The termini of the shell protein extend to the inner and outer surface, respectively, such that surface functionalizations are conveniently possible. The outer surface can, for instance, be functionalized to install specific targeting motifs. Encapsulin variants were also purified when secreted from HEK293 cells to present glycosylated epitopes for an innovative vaccination approach. Recently, the inner surface of the shell from T. maritima was also modified with silver-binding peptides to cause local silver precipitation in Escherichia coli, but the ferritin-like cargo was deleted to achieve this feature. (7) Non-native cargo proteins including enzymes can be addressed to the inside of the nanocompartment via a short encapsulation signal.

This excellent set of studies showed the feasibility and utility of biotechnological production of encapsulins as biomolecular scaffolds and targetable vehicles and probes.

We here introduce engineered encapsulins modified from M. xanthus in the context of genetic programming of orthogonal and addressable cellular compartments in mammalian cells. We demonstrate that eukaryotically expressed encapsulins not only auto-assemble at high density and without toxic effects but that self-targeting and encapsulation of cargo molecules still efficiently occur in mammalian cells. We furthermore show localized enzymatic reactions in the nanocompartment useful for optical and optoacoustic imaging, as well as confined iron accumulation within the nanocompartments that labels cells for detection by MRI. Importantly, we also show that encapsulins can serve as excellent gene reporters for electron microscopy due their spherical shape and their ability to load iron. These data demonstrate the value of encapsulins as genetic markers across modalities. In addition, the iron sequestration inside the nanoshells affords magnetic manipulation of cells genetically labeled with encapsulins.

Results

Encapsulin expression and self-assembly. Based on the favorable set of features introduced above, we chose to heterologously overexpress the encapsulin shell protein from M. xanthus in HEK293T cells. We tagged the nanoshell with an outward facing FLAG epitope (\textsuperscript{A}\textsuperscript{FLAG}) and found it to express strongly without and with the native cargo molecules from M. xanthus, denoted encapsulins B, C, and D.

Co-expression of Myc-tagged B, C, or D alone, or a combination of all three non-tagged proteins (via co-transfection or a P2A construct, Fig. 1b), co-immunoprecipitated with \textsuperscript{A}\textsuperscript{FLAG} as visualized on silver-stained SDS-PAGE (Fig. 1c, middle panel). A corresponding western blot against the FLAG (Fig. 1c, upper panel) or Myc-epitope (Fig. 1c, lower panel) confirmed the identities of the protein bands (A\textsuperscript{FLAG}: 32.9 kDa, B\textsuperscript{Myc}: 18.5 kDa, C\textsuperscript{Myc}: 15.4 kDa, D\textsuperscript{Myc}: 12.5 kDa).
Furthermore, a corresponding Blue Native PAGE (BN-PAGE) of immunoprecipitated FLAG-tagged material from cells expressing AFLAG together with BCDP2A revealed a band with an apparent molecular weight of above 1.2 MDa indicating self-assembly of encapsulin protein complexes and self-targeting of all native cargo proteins (Fig. 1d).

The strong expression of AFLAG without or with loaded cargo did not result in a reduction of cell viability when compared to cells overexpressing a fluorescent protein as assessed by a viability assay based on lactate dehydrogenase (LDH) release (Fig. 1e). We also generated a construct for a StrepTagII-labeled variant of the shell that co-expresses the ferritin-like Myc-tagged C as cargo protein via a scarless P2A site (MycC-IntP2A-ASTII, Fig. 2a).

Material from HEK293T cells, conveniently purified via StrepTactin affinity chromatography, showed assembled nanospheres of 32.4 ± 1.7 nm as the major component in single particle cryo-electron microscopy (cryo-EM) (Fig. 2b, Supplementary Fig. 1a, b), corresponding to the single band >1.2 MDa in size on BN-PAGE (Fig. 2c, right panel). Again, no effect on cell viability was detected for this construct tested by a luciferase-based viability assay compared to AFLAG with and without cargo (BCDP2A), as well as controls without expression of encapsulins (EYFP and untransfected HEK293T) (Fig. 2d). Furthermore, N-terminal addition of the human BM40 (osteonectin SPARC) secretory signal peptide (SP) to the StrepTagII-modified encapsulin shell protein resulted in entry into the secretory pathway and robust secretion.

**Fig. 1** Assembly of encapsulins and targeting of cargo in HEK293T cells. a Schematic of the heterologous expression of surface-modified encapsulin variants loaded with endogenous cargo proteins. b Genetic constructs encoding the shell protein A (light blue) with a FLAG-tag as C-terminal surface modification as well as individual Myc-tagged cargo proteins (red) B, C, and D that can also be combined in a multi-gene expression construct (BCDP2A). c Co-immunoprecipitation of AFLAG and silver-stained SDS-PAGE from cells co-expressing just B, C, or D, or a combination of these three proteins expressed either via a mixture of individual DNA constructs (MycB + MycC + MycD), or by a multi-gene expression construct (BCDP2A). The top panel shows a western blot (WB) against the exterior FLAG-tag in AFLAG. The bottom panel shows the corresponding WB against the Myc epitope. d Coomassie-stained Blue Native PAGE (BN CM) of purified material from HEK293T expressing AFLAG and BCDP2A yielding a band above 1.2 MDa. e Cell viability after 48 h of overexpression of encapsulins (AFLAG) with or without cargo (BCDP2A) assessed by an LDH release assay. A construct expressing the fluorescent protein mEos4b served as a control. The bars represent the mean ± SEM (p = 0.1965, Kruskal–Wallis, n = 4; no significant (ns) differences at α = 0.05 were found in Dunn's multiple comparisons test between mEos4b and AFLAG expressed without or with BCDP2A).
secretion of StreptagII-modified encapsulins from HEK293T cells as shown by Coomassie-stained BN-PAGE of material present in the cell culture supernatant (Fig. 2c).

In vivo expression of encapsulins. To achieve in vivo expression of encapsulins, we generated a coexpression construct that encoded both the nanoshell AFLAG and the ferritin-like protein B from a single plasmid that was small enough to be packaged into an Adeno-associated virus (AAV) (Supplementary Fig. 1c). After transduction of murine brains via intracranial injections of this viral vector co-expressing AFLAG and B\textsuperscript{M7} by a P2A peptide, we observed robust neuronal expression of the shell protein (Supplementary Fig. 1f, i). Silver-stained BN-PAGE and SDS-PAGE of immunoprecipitated (anti-FLAG) proteins extracted from murine brain showed that the nanocompartments assembled in vivo and that the cargo B\textsuperscript{M7} was associated with the shell (Supplementary Fig. 1g, j). Similar in vivo results could be obtained by co-expressing the nanoshell and ferritin-like B cargo via an IRES site (Supplementary Fig. 1h).

**Encapsulation of engineered cargo.** We next tested whether non-natural cargo molecules could be efficiently targeted into the nanocompartments. We thus C-terminally appended a minimal encapsulation signal, which we found to only necessitate eight amino acids (EncSig), to the photoactivatable fluorescent protein mEos4b\textsuperscript{42}, coexpressed it with AFLAG and found by co-immunoprecipitation and BN-PAGE analysis under an UV imager that the cargo readily associated with the encapsulin shell (Fig. 3b).

Selective degradation of non-encapsulated cargo proteins. Importantly, we could also selectively enrich cargo proteins to the encapsulin lumen by fusing an FKBP12-derived destabilizing domain (DD) that labels the cargo for rapid degradation unless it is shielded from proteasomal machinery\textsuperscript{43}. We show that coexpressing AFLAG and DD-mEos4b-EncSig in HEK293T yielded significantly higher mean fluorescence values than DD-mEos4b-EncSig alone, indicating that cargos inside the encapsulin are protected from proteolytic degradation (Fig. 3c, d). Confocal microscopy revealed that coexpression of DD-mEos4b-EncSig with AFLAG shows green fluorescence throughout the cytosol but not in the nucleus, whereas the absence of the encapsulin shell ablated the fluorescence signal. In a positive control in which DD-mEos4b-EncSig was stabilized by adding a small molecule instead of encapsulating it, fluorescence was observed throughout the cell including the nucleus (Fig. 3e).

We then purified encapsulins co-expressed with and without DD-mEos4b-EncSig as cargo to determine their native mass and found that in the absence of cargo, also smaller nanospheres assembled consistent with the known configuration as 60-mers with \( T = 1 \) symmetry (Supplementary Fig. 2). We estimated that on average \( \sim 60 \) fluorescent proteins per nanoshell were enclosed as confirmed by gel densitometry (Supplementary Fig. 3a, b). We furthermore found that the FLAG-tagged encapsulin shell is phosphorylated (Supplementary Fig. 3c-e).

Simultaneous encapsulation of sets of engineered cargo. We next wanted to assess whether multiple engineered cargo molecules could be encapsulated together. We thus fused the two halves of split PAmCherry1 (PA-s1, PA-s2) to either B or C (B-PA-s2: 27.0 kDa, C-PA-s1: 33.1 kDa) and tested for bimolecular fluorescence complementation (BiFC) within the nanocompartment\textsuperscript{44} (Fig. 4a, b). Either of these components could be co-immunoprecipitated with AFLAG as shown by silver-stained SDS-PAGE (Fig. 4b, Supplementary Fig. 4a). The photoactivation of the complemented split PAmCherry1 inside the encapsulins could also be detected via fluorescence imaging of the corresponding BN-PAGE (Fig. 4b, right panel, Supplementary Fig. 4a). Co-expression of both split halves together with AFLAG lead to a strong increase of photoactivatable fluorescent signal throughout the cytosol of HEK293T cells as quantified by confocal microscopy compared to cells that did not express AFLAG (Fig. 4c).
Compartmentalized enzymatic reactions. To showcase the use of the eukaryotically expressed encapsulins as bioengineered reaction chambers with pores that can constrain passage of reactants and reaction products, we targeted several enzymes to the nanocapsules. In the presence of AFLAG, the split luciferase parts LgBit and SmBit fused to C and B (C-LgBit: 32.7 kDa, B-SmBit: 19.6 kDa) were complemented to functional enzymes as demonstrated by bioluminescence detection from BN-PAGE (Fig. 4d, left) and from total lysate (Fig. 4d, bar graph on the right). Importantly, only very low luminescence signals were detected when the encapsulins shell was not present indicating that using split protein approaches can also ensure confined enzyme activity inside the capsules, in addition to the strategy for selective enrichment of cargo inside the nanocompartment as shown in Fig. 3.

Bioengineered melanosomes as gene reporters for MSOT. We subsequently sought to utilize selective passage of small substrates through the nanoshell to load the compartments with tyrosinase as cargo which is the sole enzyme generating the photoabsorbing polymer melanin from the amino acid tyrosine. Because of these attractive features, tyrosinase has been used as a gene reporter for optoacoustic tomography, an imaging modality that maps the distribution of photoabsorbing molecules in tissue by locating the ultrasonic waves that they emit in response to local heating upon laser absorption. However, melanin production is toxic to cells if not confined in melanosomes, which are membranous compartments of specialized cells. We thus chose a soluble tyrosinase from Bacillus megaterium that we thought could still be functional as a fusion protein to the native cargo D serving as targeting moiety (Fig. 5a). Indeed we could observe generation of melanin on the BN-PAGE band corresponding to the assembled nanocompartment (Fig. 5b). In cells expressing the encapsulin-targeted tyrosinase and the shell AFLAG, we observed robust melanin formation by bright-field microscopy without the strong toxicity apparent in the morphology of control cells expressing just the tyrosinase (Fig. 5c, white arrows). Encapsulation of the tyrosinase also led to a significant increase in cell viability as assessed by a luciferase-based viability assay (Fig. 5d). Cells expressing melanin-producing encapsulins were dark in color (Fig. 5e, inset) and thus generated intense photoacoustic signal even when referenced against strongly absorbing synthetic ink with an optical density of 0.2.

Similarly, we showed that the engineered peroxidase APEX can polymerize Diaminobenzidine (DAB) when targeted to the nanocompartment (APEX2-EncSig; 31.0 kDa) as indicated by the generation of photoabsorbing DAB polymers associated with the BN-PAGE band corresponding to the assembled nanosphere.
Since the electrophoretic mobility of protein complexes on BN-PAGE also depends on their hydrodynamic size and shape, cargo-loading could be confirmed by observing an identical migration behavior of loaded as compared to unloaded capsules (Supplementary Fig. 4c).

Moreover, we targeted the putative cystathionine γ-lyase (SmCSE) to the nanospheres via an EncSig (smCSE-EncSig: 43.9 kDa) as shown by Co-IP with AFLAG (Supplementary Fig. 4d). In the presence of 1-cysteine, this enzyme was reported to catalyze a conversion of cadmium acetate in aqueous solution into cadmium sulfide (CdS) nanocrystals such that they would generate a photoluminescence signal under UV illumination characteristic for crystal formation at quantum confined sizes. Indeed, we could detect a photoluminescence signal from the BN-PAGE band corresponding to encapsulin compartments. The bar graph (right panel) shows the corresponding total luminescence signals from a co-immunoprecipitation (Co-IP) of AFLAG co-expressed with photoactivatable mCherry1 with EncSig (PAmCherry1-EncSig) or with either one of the halves of split PAmCherry1 fused to C or B, or a combination of both (C-PA-s1 + B-PA-s2). Fluorescence originating from complemented split PAmCherry1 inside the encapsulins was detected on BN-PAGE loaded with whole cell lysates of cells expressing AFLAG and C-PA-s1 + B-PA-s2 after 2 min of photoactivation (PA) on an UV imager. Live cell confocal microscopy images (scale bar represents 20 μm) of HEK293T cells expressing B-PA-s2 and C-PA-s2 with or without the shell-protein AFLAG before and after 60 s of photoactivation (PA) with 405 nm (upper panel) demonstrating efficient bimolecular fluorescence complementation inside encapsulin compartments. Fluorescence of photoactivated split PAmCherry1 was excited using a 561 nm laser. Fluorescence signals of the sample without and with AFLAG were quantified by calculating the ratio of the mean signal after PA divided by the signal before PA. The bars in the lower panel represent the mean fluorescence intensity ratios averaged over independent transfection experiments ± SEM (p = 0.0123, unpaired t-test, n = 3).

Size-constrained iron biominalization. Another reason to choose encapsulins from *M. xanthus* was that it was previously reported to deposit iron via the ferritin-like cargo B and C into relatively large compartments (~32 nm, T = 3)31. We thus investigated whether this functionality could also be realized in eukaryotic cells to enable spatially confined iron deposition sequestered away from the complex signaling network controlling mammalian iron homeostasis. We thus generated a stable cell line co-expressing the nanoshell (AFLAG) with all native cargo proteins (B,C,D) via a dual-promoter construct. Transient co-expression of the ferrous iron transporter MmZip14FLAG (Zip14) in the stable cell line resulted in a robust dose-dependent iron loading (with ferrous ammonium sulfate (FAS) at concentrations between 0.25–1.25 mM) already after 48 h of supplementation as detected on BN-PAGE via
DAB-enhanced Prussian Blue staining (DAB PB) (Fig. 6b, right panel).

Efficient iron loading could also be achieved by transient expression of $\text{A}^{\text{FLAG}} + \text{BCD}_{\text{P2A}}$, together with Zip14. Under these conditions, iron supplementation with ~0–3 mM FAS for 48 h led to a substantial dose-dependent iron loading of the nanocompartment that saturated at ~1 mM FAS as shown by Coomassie and DAB-enhanced Prussian Blue BN PAGE (Fig. 6c, upper panel, Supplementary Fig. 5b). Interestingly, when we tested the cargo molecules individually for their ability to load iron into the nanosphere, we found that co-expression of only B or C generated equally intense DAB PB bands as compared to $\text{BCD}_{\text{P2A}}$, indicating that either B or C is sufficient for iron deposition inside the nanocompartment.

In contrast, co-expression of D with $\text{A}^{\text{FLAG}}$ or any of the cargo molecules without the presence of $\text{A}^{\text{FLAG}}$ did not lead to discernable DAB PB signals (Fig. 6c, lower panel, Supplementary Fig. 5c). In a standard cell viability assay, we found no impairment of the cells when Zip14 was co-expressed together with $\text{A}^{\text{FLAG}}$ and $\text{BCD}_{\text{P2A}}$, or just B. However, ~7% of cells showed reduced viability when the cargo $\text{BCD}_{\text{P2A}}$ were expressed without the nanocompartment ($p = 0.0238$, Mann Whitney test, $n = 3$) or when only the fluorescent protein mEos4b-EncSig was expressed (Supplementary Fig. 5d) indicating that in the absence of the nanocompartment the imported iron was not sufficiently sequestered by the endogenous iron homeostasis machinery.

We furthermore tested variants of A with N-terminal fusions with peptide sequences from *Magnetospirillum magneticum* Mms (6 and 7) proteins reported to aid in templating iron mineralization56 but found no additional benefit of these modified inner surfaces over $\text{A}^{\text{FLAG}}$ using our current readout (Supplementary Fig. 5e). In addition, we analyzed several variants of the cargo proteins B and C, fused C-terminally to peptides from Mms proteins (superscripts M6, M7, please see Supplementary Fig. 5f). These data confirmed that either B or C are sufficient to load the nanocompartment with iron and showed that no obvious additional iron loading resulted from the presence of the Mms peptides.

**Encapsulins enable detection via MRI and magnetic sorting.**

Next, we were interested in whether the strong iron accumulation inside eukaryotically expressed encapsulin shells would yield significant contrast by MRI. We thus expressed $\text{A}^{\text{FLAG}}$ alone or together with either all native cargos $\text{BCD}_{\text{P2A}}$ or just MycB, or MycD and Zip14 and subjected cell pellets to relaxometry measurements by MRI. The nanocompartment $\text{A}^{\text{FLAG}}$ co-expressed with all native cargo proteins (BCD) lead to a significant increase in $R_2^*$-relaxation rates as compared to just $\text{A}^{\text{FLAG}}$. The same effect was observed by co-expressing just the ferritin-like B (Fig. 7a, $p = 0.0047$, Kruskal–Wallis with significant differences at $\alpha = 0.05$ from Dunn’s multiple comparisons test vs. $\text{A}^{\text{FLAG}}$,...
Fig. 6 Efficient iron loading of eukaryotically expressed encapsulin nanospheres. a Schematic of a dual-promoter construct used for generation of a stable cell line expressing AFLAG and all native cargos B, C, and D. Also depicted is a construct encoding the iron-transporter MmZip14FLAG (Zip14) used to transport additional amounts of iron into the cell. b Co-immunoprecipitation (Co-IP) against the FLAG epitope from a whole cell lysate of a stable HEK293T clone expressing AFLAG together with B, C, and D analyzed by silver-stained SDS PAGE and the corresponding WB against the FLAG epitope (left panel). The pair of Blue Native (BN) gels visualizes proteins from whole cell lysates via Coomassie staining (CM) (left panel) and iron content via treatment with DAB enhanced Prussian Blue (DAB PB) (right panel) from the same stable cell line. Robust iron loading of the assembled nanocompartments was achieved by transient co-expression of MmZip14FLAG−IRES−ZsGreen1 in which case 0.25 mM ferrous ammonium sulfate (FAS) for 48 h was sufficient to see strong iron loading. BN gel stained with CM or DAB PB loaded with whole cell lysates of HEK293T cells transiently expressing AFLAG and different combinations of native cargo molecules: MycB, MycC, and MycD alone, or all three (BCDP2A) with or without AFLAG. The robust DAB PB stains show that the ferritin-like cargo proteins B or C are sufficient for iron loading into encapsulins. FAS was supplemented at 2.5 mM for 48 h.

Fig. 7 Iron-filled encapsulins enable detection by MRI and magnetic cell separation. a Relaxometry measurements by MRI conducted on cell pellets (~10^7 cells) from HEK293T cells transiently expressing AFLAG + BCDP2A, MycB, or MycD, or AFLAG alone (1 mM FAS for 24 h and expression of Zip14FLAG). Expression of AFLAG with BCDP2A or with MycB showed a significantly enhanced R2* -relaxation rate as compared with AFLAG alone or loaded with MycD; ferritin-like cargo B was sufficient to generate an increase in R2* in the presence of the AFLAG nanocompartment (p = 0.0047, Kruskal–Wallis, n = 7 from four independent experiments, stars indicate significance at α = 0.05 from Dunn’s multiple comparisons test vs. AFLAG, the bars represent the mean ± SEM). The insets show MRI slices (13.5 ms echo time) through test tubes in which cells were pelleted on a layer of agar. b In vivo MRI detection of HEK293T cells transiently co-expressing AFLAG together with ferritin-like BM7 that were xenografted into rat brains. The horizontal lines represent the mean ± SEM; the horizontal lines represent the mean ± SEM (Sec−1). The insets show MRI slices (13.5 ms echo time) through test tubes in which cells were pelleted on a layer of agar. c Bar graph showing sorted cells from a Linker FLAGCMV + Zip14 + AFlag + BCDP2A, MycB, or MycD, or AFLAG alone (1 mM FAS for 24 h and expression of Zip14FLAG) supplemented with different concentrations of FAS (0–3 mM) for 48 h (upper panel). The strong bands, which correspond to the assembled nanoshell, indicate high expression levels of encapsulins and efficient, dose-dependent iron loading. The lower panel shows a CM and DAB PB-stained gel from whole cell lysates of HEK293T cells expressing Zip14FLAG and different combinations of native cargo molecules: MycB, MycC, and MycD alone, or all three (BCDP2A) with or without AFLAG. The robust DAB PB stains show that the ferritin-like cargo proteins B or C are sufficient for iron loading into encapsulins.
n = 7). This indicated again that co-expression of B was sufficient to generate efficient iron deposition inside the nanoshell. We subsequently sought to test whether cells genetically labeled with encapsulins could be detected by MRI in vivo. As an initial assessment, we thus xenografted cells co-expressing AFLAG together with BM7 into rat brains and obtained R2-relaxation maps that showed elevated relaxation rates (p = 0.0078, Wilcoxon matched-pairs signed rank test, n = 9) at the injection site as compared to xenografted cells in which the fluorescent protein mEos4b-EncSig was used as a control cargo (Fig. 7b).

In addition to MRI contrast, the iron biomineralization inside the encapsulins also allowed us to magnetically sort cells co-expressing the shell AFLAG with BCD P2A or with BM7 at significantly higher percentages than when human H-chain ferritin (HHF) was expressed or just yellow fluorescent protein (EYFP) (p = 0.0007, Kruskal–Wallis, with significant differences at α = 0.05 from Dunn’s multiple comparisons test, n = 6; Fig. 7c).

Encapsulins as markers for electron microscopy. Given that the iron loading of the encapsulins was very efficient and observable at the population level, we next assessed how well individual nanocompartments could be detected by electron microscopy in cells such that they could be used as genetically encoded markers. We thus grew HEK293T cells stably expressing the shell protein AFLAG and BCD P2A using a dual promoter vector on a transmission electron microscopy (TEM) grid, vitrified them by plunge-freezing and produced lamellae by cryo-focused ion beam (cryo-FIB) milling for in situ cellular cryo-electron tomography (cryo-ET). The heterologously expressed encapsulins were readily detected as clearly discernible nanospheres (Fig. 8a, Supplementary Fig. 6a, b) that exhibited electron-dense cores when we supplemented the growth media with ferrous iron (Fig. 8b) and were distributed as monodisperse spheres throughout the cytosol (Supplementary Fig. 6c, d). The electron density maps showed a high similarity to the structure published from encapsulin shells from M. xanthus EncA expressed in E. coli (pdb 4PT2; EMDataBank EMD-5917) that was obtained from M. xanthus EncA expressed in E. coli[1]. The cutaway views of the encapsulins show electron densities indicating the presence of cargo proteins (beige) and additional iron deposition (cyan and purple) as compared to published data from the EncA shell[1] that were obtained in the absence of cargo proteins.

Discussion

In summary, we genetically controlled multifunctional orthogonal compartments in mammalian cells via expressing N- or
C-terminally modified encapsulins, which we found to auto-assemble into abundant nanocompartments which readily encapsulated sets of natural and engineered cargo proteins and enabled size-constrained metal biomineralization.

The efficiency of self-targeting and auto-packaging of the various cargo proteins in mammalian cells was remarkable given that the number of possible protein interactions is even a few-fold higher than in the original prokaryotic host organism. We found that about 60 cargo proteins of a canonical fluorophores protein could be bound to the inner encapsulin surface via the minimal encapsulation signal. Higher loading factors could be achieved by providing cargo proteins with multidentate adapters such that the entire encapsulin volume could be filled. We furthermore observed that without co-expression of endogenous or engineered cargo, the abundance of the 60-mer encapsulin shell with $T=1$ symmetry was increased, which is in line with a previous observation made from M. xanthus encapsulin expressed in E. coli and suggests that encapsulation of cargo leads to the preferred assembly of the 180-mer in $T=3$ symmetry.

Also, iron storage inside the capsule via the ferritin-like enzymes B or C targeted to the encapsulins was very efficient, indicating that there was sufficient access to ferrous iron. Whereas encapsulins heterologously expressed in E. coli were shown to load iron, this could so far not been shown in mammalian cells. We also found that just co-expression of B (or C) with A is sufficient for robust iron storage such that a single-piece reporter construct of just ~2.1 kb in size can be used.

In the context of optimizing $T_2$ contrast in MRI, it would certainly be valuable to explore modifications of the outer surface that may control the agglomeration state and thus could modulate the apparent relaxivity of encapsulin ensembles. In this context, it would also be desirable to explore capsid architectures with more storage capacity such as ones with $T=1$ symmetry. Furthermore, modifications of the inner surface of the shell may be engineered and/or additional cargo could be designed that could facilitate the nucleation process to support higher iron packing densities or alter environmental parameters (e.g., pH and redox potential) to potentially even generate superparamagnetic iron-oxides which possess a substantially larger magnetization. Iterative optimization schemes such as directed evolution could also be employed based on rescue assays from excess iron or magnetic microfluidic sorting and could also be complemented by parallel screens in prokaryotes if enough iron-influx can be achieved there.

Their dense monodisperse distribution, spherical shape, and sufficient size, also render encapsulins excellent genetically expressed EM markers in mammalian cells (Supplementary Movie 1) that are much more readily detectable than ferritins, which have been visualized by EM in E. coli and yeast. In addition, the iron-based contrast in encapsulins has the advantage over semi-genetic methods such as metallothionein (MT), miniSOG, erHRP, or APEX/APEX2 that no fixation and delivery of fluorescent proteins via photoactivated localization microscopy (PALM) and combine this with cryo-ET as was demonstrated for photoactivatable GFP (cryo-PALM). Besides allowing the inflow of metals for size-constrained biominerlization for the type of applications discussed above, the pore size of ~5 Å inside the encapsulin shell also affords selective passage of small substrates, whereas reaction products may be trapped inside the nanoshell. We have exploited this feature by encapsulating tyrosinase for confined enzymatic production of the toxic polymer melanin and utilized the engineered “nanomelanosomes” as genetically encoded reporters for optoacoustic imaging.

In future applications, encapsulins could thus be used as versatile reaction chambers for, e.g., metabolic engineering of orthogonal reactions in eukaryotic cells. The toolbox for genetically controlled compartmentalization in mammalian cells which we introduce here could, for instance, enable multi-step enzymatic production involving labile or toxic intermediates but yielding end-products that may have beneficial intracellular effects or serve as molecular signals upon “quantal” release from the nanocompartment. The approach could for instance also endow genetically modified mammalian cells used for cell therapies with metabolic pathways that may augment their therapeutic efficacy. Complementarily, endogenously produced toxic products could be contained and detoxified in engineered compartments for causal studies or potentially for cell or gene therapies.

In addition to the encapsulins presented here, heterologous expression of compartments with different sizes and shapes seem possible, which could offer different sets of endogenous and engineered cargo molecules with different subcellular targeting. These alternative systems would ideally also be orthogonal to each other such that multiplexing (maybe even nesting) of several engineered compartments and multicomponent processes could be achieved.

More generally, genetically controlled compartmentalization of multi-component processes in eukaryotic cells—as demonstrated for encapsulins here—is a fundamental biotechnological capability that has profound implications for mammalian cell engineering and emerging cell therapies.

**Methods**

**Genetic constructs.** Mammalian codon-optimized MxEncA (UniProt: MXAN_3556) MxEncB, MxEncC, and MxEncD (UniProt: MXAN_3557, MXAN_4464, MXAN_2410) were custom synthesized by Integrated DNA Technologies and cloned into pcDNA 3.1 (+) Zeocin (Invitrogen) using restriction cloning or Gibson assembly. The MxEncA surface tag (FLAG or StrepTagII) were C-terminally appended using Q5 Site-Directed Mutagenesis (New England Biolabs). N-terminal Myc epitopes were added accordingly to the cargo proteins. Multigene expression of B, C, and D was achieved by generating a single reading frame containing all three genes separated by P2A peptides yielding BCDP2A.A was designed for photoactivatable GFP (cryo-PALM) and cloned into the Cytomegalovirus promoter (CMV) driven expression cassette of the vector via restriction cloning. To generate AAV enabling multigene expression of MxEncAFLAG and MxB-Mms7ct, two strategies were employed: MxEncAFLAG was cloned upstream of an ECMV internal ribosome entry site (IRES) whereas MxB-Mms7ct was auto-cleaved followed by a P2A peptide in between the genes as previously described. For generating stable clones expressing MxEncABCD, MxEncAFLAG was cloned into the Cytomegalovirus promoter (CMV) driven expression cassette of pBudCE4.1 (Invitrogen) and BCDP2A was cloned into the elongation factor 1 alpha promoter (EF1a) driven expression cassette of the vector via restriction cloning. To generate AAV enabling multigene expression of MxEncAFLAG and MxB-Mms7ct, two strategies were employed: MxEncAFLAG was cloned upstream of an ECMV internal ribosome entry site (IRES) whereas MxB-Mms7ct was inserted downstream. The second approach employs MxB-Mms7ct followed by a P2A peptide and MxEncAFLAG. The two cassettes were subcloned into pAAV-CamKIIa (https://www.addgene.org/26969/) with BamHI and EcoRI. AAVs were custom prepared by the UNC Vector Core of the University of North Carolina at Chapel Hill. To test the bistrictic expression constructs used for the AAVs in HEK293T cells, the cassettes were also sub-cloned into the pcDNA 3.1 (+) Zeocin with EcoRI and NotI. To target PAMCherry1 and mEos4b as cargo to the encapsulin nanocompartments, the fluorescent proteins were C-terminally fused to 2 × GGGS linker following the minimal encapsulation signal LTVGSLRR.
buffer were loaded onto pre-cast NativePAGE® Novex® 3–12% Bis-Tris gels. NativeMark® Unstained Protein Standard (Life Technologies) covering a size range between 100 and 200 kDa was used as a marker. The total cell lysate samples loaded per well was adjusted to ~1–3 µg. Blue native (BN) gels were run for 90–180 min at 150 V according to the protocol of the manufacturer. Gels loaded with samples from Co-IP/purification were silver-stained using SilverStain™ Silver Staining Kit (Novex) or Coomassie-stained using Bio-Safe™ Coomassie Stain (Bio-Rad Laboratories). For protein detection, gels loaded with whole cell lysate samples were Coomassie-stained accordingly. For detection of iron-containing proteins, gels loaded with samples containing iron loaded encapsulins were Prussian Blue (PB) stained. Briefly, gels were incubated in 2% potassium hexacyanoferrate(II) in 0.15 M NaCl for 20 min. For 3.3% Co-IP samples, the gels were washed three times with ddH2O and incubated in 0.1 M phosphate buffer (pH 7.4) containing 0.025% DAB and 0.005% H2O2 until dark-brown bands appeared. To stop DAB polymerization, gels were washed three times with ddH2O. For detection of fluorescent signals from native encapsulins bands (fluorescent cargos: mEos4b, MxEncR1, split FAM) or mineralized C55S, unstained BN gels were imaged on a Fusion FX7 SL advance imaging system (PQlab Biotechnologie GmbH) using the UV fluorescence mode. For on-gel detection of luminescence signal generated by encapsulated split NanoLuciferase, unstained BN gels were soaked in 1 ml of Nano-Glo® Luciferase substrate (Nano-Glo® Luciferase Assay, Promega) and imaged on a Fusion FX7 SL advance imaging system (PQlab Biotechnologie GmbH) in chemiluminescence mode. For cell lysates luminescence detection, cell lysates were mixed with the substrate at a 1:1 ratio and luminescence readings were taken on a Centro LB 960 (Berthold Technologies) at 0.1 s acquisition time. For detection of APEX2 peroxidase activity inside encapsulins, unstained BN gels were incubated in 0.1 M phosphate buffer (pH 7.4) containing 0.025% DAB and 0.005% H2O2 for 15 min. For microscopic gel observation, gels were fixed in 4% PFA in PBS for 15 min. Subsequently, gels were incubated in 0.1 M phosphate buffer (pH 7.4) containing 0.025% DAB and 0.005% H2O2 for 5 min. The reaction was stopped by washing three times with PBS. For the on-gel detection of melanin generation associated with encapsulins, gels loaded with whole cell lysates of HEK293T cells expressing encapsulins loaded with tyrosinase were incubated in PBS containing 2 mM l-tyrosinase and 100 µM CuCl2 for 1 h at 37 °C until a black encasement band became visible. Size exclusion chromatography. Size exclusion chromatography (SEC) of purified AFLAG*-tagged with or without DD-mEos4b-EncSig was performed on an Akta Purifier (GE Healthcare). Equilibration with 6 mM 100 mM NaCl, 1 mM EDTA, pH 7.4, at 4 °C was used. In total, 100 µl samples were run at a flow rate of 0.4 ml/min in 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 7.4, at a concentration of 0.3 mg/ml. Dynamic light scattering. Dynamic light scattering experiments were performed on a DynaPro NanoStar instrument and analyzed with DYNAMICS 7.1.9 software (Wyatt Technology). Measurements were performed in doubly distilled water at 4°C. Recombinant samples were analyzed at a concentration of 0.1–0.45 µg/ml corresponding to an estimated monomer concentration ranging from 0.1 to 14 µM. Gold-coated nanoelectrodes needles were made in-house from borosilicate capillaries (Kwik-Fil, World Precision Instruments, Sarasota, FL) and pulled (Sutter, Novato, CA) and being coated by using an Edwards Scanout six piranha 501 sputter coater (Edwards Laboratories, Milpitas, USA). Measurements were carried out in positive ion mode on a modified Q-ToF 2 (Waters, UK) instrument15, operated at elevated pressure in the source region (~10 mbar), using Xenon as collision gas at 210−2 mbar in the collision cell. Capillary and sample cone voltage was set to 1400 V and 150 V, respectively. The voltage before the collision cell was either set to 2*104 V and 150 V, respectively. Spectra were calibrated using an aqueous solution of cesium iodide (25 mg/ml) and exported from MassLynx. All further data analysis was performed with in-house developed python scripts (Python 3.6). When applicable, charges were assigned to charge state resolved peak series by extracting the top position for consecutive charge states and minimizing the standard deviation (SD) of the average mass by trying different charge states. Centroids for empty and cargo filled encapsulins (1−3) were calculated using all data points above 40% of the base peaks intensity in the appropriate region (m/z 30,000−40,000 for empty and m/z 35,000−45,000 for cargo filled encapsulins). The average was taken over three technical replicates and the error represents the
standard deviation (SD). To estimate the mass from the m/z position, we fitted 77 empirical determined masses and their corresponding m/z positions to the equation $m/z = 12 + 41.4 \times z$. These 77 proteins consist of encapsulin T = 1, which was determined in this study as well as 76 other assemblies, which were already measured and reported in previous publications (3,4). The resulting formula $\text{Mass} \times 10^{-4} = 1.63 \times 10^{-14} m/z^{1.2}$ was used to calculate the mass from the average m/z positions of empty and cargo filled encapsulins (T = 3). Since mass and m/z positions do not have a real relation to each other, the upper dimensional projection from the m/z dimension projected in the mass domain. Cargo load was estimated based on the difference between the predicted masses of empty and cargo loaded encapsulins (T = 3). The error of the mass difference was calculated using the equation $\text{Mass}_{\text{Diff}} = m/z^2 - \text{mass}_{\text{empty}}^2/2$. The difference in mass was then divided by 41.4 kDa, the mass of the DD-mEos4b-EncSig monomer protein. The $A^{\text{FLAG}}$ monomer mass was calculated as the weighted average for the different proteofoms, using the summed intensities over the charge states for each species.

**Complementation of split PAmCherry1 inside encapsulins.** Cells transfected with C-PAS1 and B-PAS2 with or without $A^{\text{FLAG}}$ were seeded onto 8-well Poly-l-lysine-coated microscopy chips (Ibidi). Thirty-six hours post transfection, live cell confocal microscopy was conducted on a Leica SP5 system (Leica Microsystems). For photoactivation of split PAmCherry1, samples were illuminated with a 405 nm laser for 60 s at 40% laser power. The signal of complemented split PAmCherry1 was excited using the 561 nm laser. To quantify the complementation of split PAmCherry1 with or without the encapsulin shell, the ratio of the total mean fluorescence after photoactivation divided by the signal before was calculated. ImageJ was used to quantify mean fluorescence values from randomly chosen areas on the well.

**Multispectral optoacoustic tomography.** Optoacoustic images of cells co-expressing $A^{\text{FLAG}}$ and $D^{\text{DD}}$-BnTyr were acquired on an inVision 256-TF system (iThera Medical GmbH). Briefly, $10^5$ HEK293T cells in 12-well plates were exposed to the genes treated with 10 µM CuCl2 and 1 mM l-tyrosine 24 h prior to the measurement were detached using trypsin, washed with PBS, embedded into 1% low melting agar yielding a tabular phantom of ~300 µl volume. The cell phantom and an ink column from the magnetic separator, cells were eluted with 1 ml DPBS. The total volume of 1 µl of viral particles was injected at 0.1 µl/min. Injection cannulae (the side of injection for $A^{\text{FLAG}}$) were then lowered into bilateral guide cannulae (22 Gauge, Plastics One, Roanoke, VA, USA) connected via polyethylene tubing and centrifuged at 500 × g and resuspended in growth media. Cell suspensions were backfilled into two injection cannulae (28 Gauge, Plastics One, Roanoke, VA, USA) connected via plastic tubing to 25 µl Hamilton glass syringes clamped in a remote dual syringe pump (PHD 22/2,000; Harvard Apparatus, Holliston, MA, USA). Injection cannulae (the side of the capsules for $A^{\text{FLAG}}$ or control were switched between experiments) were then lowered into bilateral guide cannulae (22 Gauge, Plastics One, Roanoke, VA) that were previously implanted in Sprague–Dawley rats (1). Rats were then centered in the bore of a 7 T 20 cm inner diameter, horizontal bore magnet (Bruker BioSpin MRI GmbH, Ettlingen, Germany) and gradient echo scans (FOV = 2.5 cm × 2.5 cm, matrix size = 256 × 256; seven slices with 1 mm slice thickness) were taken at a TR = 800 ms and different echo times (5, 10, 15, 20, 25 ms) to compute relaxation rate maps and perform ROI analysis (circular ROIs of 1 mm diameter placed on injection sites) using custom routines in Matlab (Mathworks, Natick, MA, USA). All procedures on rats were conducted in accordance with the National Institutes of Health guidelines and with the approval of the MIT Committee on Animal Care.

**Cell viability assays.** Iron-related cytotoxicity was monitored via the Roche Cytotoxicity Detection Kit (LDH) (Roche Diagnostics) according to the protocol of the manufacturer. Briefly, $7.5 \times 10^4$ HEK293T cells were seeded on poly-l-lysine-coated 24-well plates. Twenty four hours post seeding, cells were transfected with different combinations of genes using X-tremeGENE HP (Roche). The Zip14 DNA amount was kept constant in all samples expressing Zip14 (5% of total DNA). For expression of combinations of $A^{\text{FLAG}}$ with cargo proteins, 60% of the total DNA amount was encoding $A^{\text{FLAG}}$ and the remaining 35% were used for the respective cargo molecule. 24 h post transfection, cells were transfected with different combinations of genes using X-tremeGENE HP (Roche). Twenty four hours post addition of FAS, cells were assayed for LDH release. In order to evaluate gene-related toxicity in the absence of iron, the assay was performed accordingly but without iron supplementation and cells were assayed 48 h post transfection. The Luciferase-based viability assay (RealTime-Glo™ MT Cell Viability Assay, Promega) was performed according to the protocol of the manufacturer in 96-well plate format as an endpoint measurement. Luminescence readings were taken on a Centro LB 960 (Berthold Technologies) at 0.5 s acquisition time.

**Electron microscopy.** Please refer to Supplementary Methods in the Supplementary Information for a detailed description of the electron microscopy techniques used.

**Data availability.** Data are available upon request to the corresponding author. The cryo-EM maps of non-iron loaded and iron loaded encapsulins in HEK293T cells have been deposited under EMDB-4392 and 4393 respectively.

Received: 4 December 2017 Accepted: 16 April 2018
Published online: 18 May 2018

**References**

1. DeLoache, W. C. & Dueber, J. E. Compartimentalization metabolic pathways in organelles. *Nat. Biotechnol.* **31**, 320–321 (2013).
2. Küchler, A., Yoshimoto, M., Luginbühl, S., Mavelli, F. & Walde, P. Enzymatic relaxation properties of superparamagnetic particles. Biochim. Biophys. Acta 1802, 606–608 (2008).

28. Papaefthymiou, G. C. The Mössbauer and magnetic properties of ferritin proteins. Biochim. Biophys. Acta 1800, 886–897 (2010).

30. Gossuin, Y., Gillis, P., Hocq, A., Vuong, Q. L. & Roch, A. Magnetic resonance relaxation properties of superparamagnetic particles. Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol. 1, 299–310 (2009).

31. Douglas, T. et al. Protein engineering of a viral cage for constrained nanomaterials synthesis. Adv. Mater. 14, 415 (2002).

36. Moon, H., Lee, J., Min, J. & Kang, S. Developing genetically engineered encapsulin protein cage nanoparticles as a targeted delivery nanoplatform. Biomacromolecules 13, 3794–3801 (2014).

38. Kanekiyo, M. et al. Rational design of an Epstein-Barr virus vaccine targeting the receptor-binding site. Cell 162, 1090–1100 (2015).

40. Kickhoefer, V. A. et al. Engineering of vault nanocapsules with enzymatic and mechanical properties. Nat. Commun. 7, 12186 (2016).

48. Park, J. et al. Engineering of protein nanocompartments for targeted enzyme localization. Cell 148, 1229–1243 (2012).

51. Totty, M. et al. Structural basis of enzyme encapsulation into a bacterial nanocompartment. Nat. Struct. Mol. Biol. 15, 939–947 (2008).

53. Moon, H., Lee, J., Min, J. & Kang, S. Developing genetically engineered encapsulin protein cage nanoparticles as a targeted delivery nanoplatform. Biomacromolecules 13, 3794–3801 (2014).

55. Dunleavy, J. et al. Widespread distribution of encapsulin protein cage nanoparticles in redox control of iron biomineralization in Magnetospirillum magneticum. Nat. Nanotechnol. 7, 5593–5598 (2010).

59. Giessen, T. W. & Silver, P. A. Widespread distribution of encapsulin protein cage nanoparticles in redox control of iron biomineralization in Magnetospirillum magneticum. Nat. Nanotechnol. 7, 5593–5598 (2010).

64. Strittmatter, J. M. et al. Proteome-wide analysis of chaperonin-dependent protein folding in Escherichia coli. Proc. Natl Acad. Sci. USA 112, 797–802 (2015).

69. Kerner, M. J. et al. Proteome-wide analysis of chaperonin-dependent protein folding in Escherichia coli. Proc. Natl Acad. Sci. USA 112, 797–802 (2015).

74. Lehto, M. et al. Structural characterization of encapsulated ferritin provides insight into iron storage in bacterial nanocompartments. Elife 5, e18972 (2016).

76. Pati, R. M., Fredy, J. W., Cornelissen, J. J. L. M., Koay, M. S. T. & Katsonis, N. Labelling bacterial nanocages with photo-switchable fluorophores. ChemPhysChem 17, 1815–1816 (2016).

79. Kanekiyo, M. et al. Rational design of an Epstein-Barr virus vaccine targeting the receptor-binding site. Cell 162, 1090–1100 (2015).

81. Giessen, T. W. & Silver, P. A. Converting a natural protein compartment into a nanofactory for the size-constrained synthesis of antimicrobial silver nanoparticles. ACS Synth. Biol. 5, 1497–1504 (2016).

82. Tamura, A. et al. Packaging guest proteins into the encapsulin nanocompartment from Rhodococcus erythropolis N771. Biotechnol. Bioeng. 112, 4517–4527 (2015).

83. Zhang, B. et al. Coordinated protein co-expression in plants by harnessing the synergy between an in vivo and a viral 2A peptide. Plant Biotechnol. J. 15, 718–728 (2017).

84. Paez-Segala, M. G. et al. Fixation-resistant photoactivatable fluorescent proteins for CLEM. Nat. Methods 12, 215–218 (2013). doi:10.1038/nmeth.2619.

85. Maynard-Smith, L. A., Chen, L.-C., Banaszynski, L. A., Ooi, A. G. L. & Wandless, T. J. A directed approach for engineering conditional protein stability using biologically silent small molecules. J. Biol. Chem. 282, 24866–24872 (2007).

86. Nickerson, A., Huang, T., Lin, L.-J. & Nan, X. Photoactivated localization microscopy with bimolecular fluorescence complementation (BiFC-PALM) for nanoscale imaging of protein-protein interactions in cells. PLoS ONE 9, e100589 (2014).

87. Dixon, A. S. et al. NanoLuc complementation reporter optimized for accurate measurement of protein interactions in cells. ACS Chem. Biol. 11, 400–408 (2016).

88. Strittmatter, J. et al. Vaccinia virus-mediated melanin production allows MR and optoacoustic deep tissue imaging and laser-induced theranostics of cancer. Proc. Natl Acad. Sci. USA 110, 3316–3320 (2013).

89. Jathoul, A. P. et al. Deep in vivo photoacoustic imaging of mammalian tissues using a tyrosinase-based genetic reporter. Nat. Photonics 9, 239–246 (2015).

90. Ntzichristos, V. Going deeper than microscopy: the optical imaging frontier in biology. Nature Methods 7, 603–614 (2010).

91. Ntzichristos, V. & Razansky, D. Molecular imaging by means of multispectral optoacoustic tomography (MSOT). Chem. Rev. 110, 2783–2797 (2010).

92. Raposo, G. & Marks, M. S. Melanosomes—dark organelles enlighten endosomal membrane transport. Nat. Rev. Mol. Cell Biol. 8, 786–797 (2007).

93. Haase, R. & T. Tyrasields expressing neuronal cell line as in vitro model of Parkinson’s disease. Int. J. Mol. Sci. 11, 1082–1098 (2010).

94. Sendovski, M., Kanteev, M., Ben-Yosef, V. S., Adir, N. & Fishman, A. First structures of an active bacterial tyrosinase reveal copper plasticity. J. Mol. Biol. 405, 227–237 (2017).

95. Lam, S. S. et al. Directed evolution of APEX2 for electron microscopy and proximity labeling. Nat. Methods 12, 51–54 (2015).

96. Wittig, I., Braun, H.-P. & Schägger, H. Blue native PAGE. Nat. Protoc. 1, 418–428 (2006).

97. Dunleavy, R., Lu, L., Kiel, C. J., McIntosh, S. & Berger, B. W. Single-molecule visualization of RNA in vivo and in vitro using optical nanoprobes. Biowater. Res. 21, 18 (2014).

98. Jathoul, A. et al. Structural characterization of encapsulated ferritin provides insight into iron storage in bacterial nanocompartments. Elife 5, e18972 (2016).

99. Pati, R. M., Fredy, J. W., Cornelissen, J. J. L. M., Koay, M. S. T. & Katsonis, N. Labelling bacterial nanocages with photo-switchable fluorophores. ChemPhysChem 17, 1815–1816 (2016).

100. Panemño, Y. & Jasanoff, A. T2 relaxation induced by clusters of superparamagnetic nanoparticles: Monte Carlo simulations. Magn. Reson. Imaging 26, 994–998 (2008).
Acknowledgements

We are grateful for support from the European Research Council under grant agreements ERC-StG: 311552 (F.S., A.S., H.R., G.G.W.). T.W., J.S. and A.J.R.H acknowledge support from the Netherlands Organization for Scientific Research (NWO) funding the large-scale proteomics facility Proteins@Work (project 184.032.201) embedded in the Netherlands Proteomics Centre. We acknowledge Susanne Pettinger for assistance with DLS measurements.

Author contributions

F.S. co-designed the study, generated all constructs, conducted all cell and biochemical experiments, analyzed data, generated figures and co-wrote the manuscript; C.M. made important contributions to the iron-loading experiments and performed in vitro MRI experiments; P.E. designed, conducted and analyzed cryo-EM experiments supervised by J.P.; A.S. supported cell experiments; H.R. supported animal experiments; M.D. and S.B. conducted in vivo MRI experiments supervised by A.J.; A.G. helped with HPLC purification and DLS analysis; T.P.W. and J.S. conducted and analyzed native mass spectrometry experiments supervised by A.J/R.H., H.F. and M.H. supervised in vitro MRI experiments; V.N. supervised MSOT experiments; G.G.W. conceptualized and co-designed the study, analyzed data and generated figures, supervised the project, and wrote the manuscript.

Additional information

Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-018-04227-3.

Competing interests: The authors declare no competing interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

© The Author(s) 2018