Supporting Information

Phase-Separated Multienzyme Biosynthesis

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Abbreviations

IPTG: isopropyl β-D-1-thiogalactopyranoside

Tris: Tris(hydroxymethyl)aminomethane

EDTA: ethylenediaminetetraacetic acid

DTT: dithiothreitol

Trx: thioredoxin

NTA: nitrilotriacetic acid

SDS: sodium dodecyl sulfate

ThDP: thiamine diphosphate

TFA: trifluoroacetic acid

Cy5: cyanine5

DMF: N, N-dimethylformamide

DSC: N, N'-disuccinimidyl carbonate

DIPEA: N, N-diisopropylethylamine

DCM: dichloromethane

NHS: N-hydroxysuccinimide

DMSO: dimethyl sulfoxide

TEA: triethylamine

Materials

Unless otherwise noted, all reagents were purchased from commercial sources and used without further purification. Reagents were purchased from commercial suppliers, including Sigma-Aldrich Co. (USA), J&K Scientific Ltd. (Beijing, China), and BBI Life Sciences Co. (Shanghai, China). DNA oligonucleotides were synthesized by BGI (Shenzhen, China). Restriction endonucleases were purchased from Thermo Scientific (USA). Plasmids, PCR products and products of restriction digests were purified by commercial kit from Takara Biotech Co., Ltd (Dalian, China). Sequencing reactions were performed and analyzed at BGI (Shenzhen, China). Sulfo-Cy5 was kindly provided by Professor Jianhao Wang (Changzhou University, China).

Plasmids construction

All plasmids were constructed using traditional cloning methods. Plasmids encoding GKAP, Shank and Homer sequences were kindly provided by Prof. Mingjie Zhang (Hong Kong University of Science and Technology, China). Plasmids encoding CFP and YFP sequences were kindly provided by Prof. Adrian Keatinge-Clay (The University of Texas at Austin, USA). Plasmids encoding MenF, MenD and MenH sequences were kindly provided by Prof. Zhigong Guo (Hong Kong University of Science and Technology, China). Plasmids encoding Idi and IspA sequences were kindly provided by Prof. Tiangang Liu (Wuhan University, China). RI and RIAD sequences were synthesized and inserted into respective plasmids. Sequence information was shown in supporting tables.

Recombinant proteins expression and purification

Protein expression. All constructed plasmids were transformed into *E. coli* BL21 (DE3) cells and colonies were grown overnight at 37ºC in LB media supplemented with 100 μg/mL ampicillin or 50 μg/mL kanamycin. The start culture was grown overnight, and then was used to inoculate LB media supplemented with antibiotics at 1:100 ratio. The cell culture was grown at 37ºC to reach OD_{600} ~0.6 before 0.25 mM IPTG was added to induce protein expression. After grown at 16ºC overnight, cells were harvested for protein purification.

Purification of GKAP. Trx-His-GKAP was purified using a nickel-NTA agarose affinity column followed by size-exclusion chromatography (Superdex 200 or Superdex 75, GE Healthcare). Briefly, harvested cells were re-suspended in lysis buffer containing 50 mM Tris, pH 8.0, 500 mM NaCl, 5 mM imidazole. The cell suspension was lysed by homogenizer and centrifuged. The supernatant was collected, filtered, and loaded on the column. After washes with washing buffer containing 50mM Tris, pH 8.0, 500mM NaCl, 60mM imidazole, the bound protein was eluted by elution buffer containing 50mM Tris, pH8.0, 500mM NaCl, 1M imidazole. Eluted protein was further purified by Superdex 200 column with a column buffer containing 50 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 2 mM DTT. Then Trx-His tag was removed by HRV 3C protease incubating at 4 ℃ overnight. GKAP without tag was purified by Superdex 75 column with same column buffer.

Purification of Homer and RIAD-Homer. Homer and RIAD-Homer were purified using HisTrap™ HP column followed by Superdex 200 column (GE Healthcare). Briefly, harvested cells were re-suspended in lysis buffer containing 50 mM Tris, pH 8.0, 500 mM NaCl, 2 mM β-mercaptoethonol, 10 mM imidazole. The cell suspension was lysed by sonication and centrifuged. The supernatant was collected, filtered, and loaded on the column. The bound protein was eluted by imidazole gradient from 10 mM to 500 mM. Eluted protein was further

purified by Superdex 200 column with a column buffer containing 50 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA.

Purification of other proteins. Other proteins were purified using HisTrap™ HP column only. Pure protein fractions eluted with imidazole gradient were collected and exchanged with buffer containing 50 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA.

After purification, all proteins were flash frozen under liquid nitrogen and stored at -80 ℃.

Fluorescence recovery after photobleaching (FRAP) assay

FRAP assay was performed on a Leica SP8 confocal microscope at room temperature. GFP signal was bleached using a 488-nm laser beam. The scaffold concentration was set as 5 μM and GFP was 0.2 μM. The fluorescence intensity at pre-bleaching was normalized to 100%.

Synthesis of Sulfo-Cy5 NHS ester

Sulfo-Cy5 NHS ester was synthesized using a method modified from Jung. et al. and Kvach et al. [1,2] Briefly, Sulfo-Cy5 acid (0.02 mmol) was dissolved in 1 mL dry DMF. Then DSC (0.03 mmol) and DIPEA (0.1 mmol) were added to the stirred solution, and the mixture was stirred for 2 h at room temperature. TLC was used to monitor the reaction (20% methanol in DCM, v/v). After reaction, product was precipitated by cold diethyl ether, and stored at -20 ℃ for further use.

Analysis of chorismate and SHCHC by HPLC

The reactant chorismate and final product SHCHC of menaquinone biosynthesis enzymes were analyzed by Shimadzu HPLC system using Raptor™ C18 column (Restek Corporation, USA). Samples were eluted with the following gradient: Solvent A: water with 0.1%TFA; Solvent B: acetonitrile with 0.1% TFA; 5% B to 30% B in 11 min. Chorismate and SHCHC were monitored by absorbance at 274nm and 293nm respectively.

Standard curves were generated by injecting a series of chorismate and SHCHC using same column and HPLC condition and integrating the areas of specific peaks at 274nm or 293nm. The concentration of chorismate and SHCHC was determined by absorbance at 274nm and 293nm respectively. [3]

Estimation of protein concentration inside condensates

The concentrations of Idi-RIDD and RIDD-IspA in the terpene biosynthesis enzyme condensates were estimated by confocal microscopy using Cy5 labeled proteins through a

method modified from Zeng. et al. [4] In each measurement, only one enzyme was Cy5-labeled and labeled protein was adjusted to 2% by diluting a labeled protein into the unlabeled one. The 2% Cy5-labeled protein was further mixed with other unlabeled proteins at the concentration same in the enzymatic assay (5 μM scaffolds, 0.5 μM Idi-RIDD and 1 μM RIDD-IspA) to form condensates. The mixture was injected into a flow chamber and imaged by Leica SP8 confocal microscope with a 63x objective lens in a z stack mode with the step interval as 0.5 μm. To get the average Cy5 intensity in one droplet, first the fluorescence intensity was analyzed within the selected droplet area using ImageJ software for each z layer. Then the fluorescence intensities of the layers that the intensity <10% deviation from the maximal intensity layer were averaged to get the average Cy5 intensity in that droplet. Average Cy5 intensities from 10 different droplets in each group were analyzed for estimating the protein concentration.

Calibration curve of Cy5 fluorescence intensity was generated by injecting a series of Cy5 into the flow chamber and measuring the fluorescence intensity using the same imaging parameters and confocal settings as for the droplet quantifications. The concentration of Cy5 was determined by absorbance at 646 nm.

Table S1. List of protein sequences

GKAP (aa 328Q-421S + 916Q-992L, UniProt: Q9D415-1)

MSDKIIHLTDDSFDTDVLKADGAILVDFWAEWCGPCKMIAPILDEIADEYQGKLTVAKLNIDQNPGTAPKY
GIRGIPTLLLFKNGEVAATKVGALSKGQLKEFLDANLAGSGSGHMHHHHHHSSGLEVLFQ/GPGSEFQDE
WSGYTPRGKDDEIPCRRMRSGSYIKAMGDEDSGDSDTSPKPSPKVAARRESYLKATQPSLTELTTLKISNE
HSPKLQIRSHSYLRAVSEVSQMDPLDKKERRAPPPVPKKPAKGPAPLIRERSLESSQRQEARKRLMAAKRA
ASVRQNSATESAESIEIYIPEAQTRL*

HRV3C cut site shown in slash mark. GK binding repeats 328Q-421S were shown in blue; PDZ binding motif 916Q-992L was shown in green.

Homer (aa 1M-361P, Ser342 is an Arg, UniProt: Q9NSC5-1)

MHHHHHHSSGLEVLFQGPGSEFMSTAREQPIFSTRAHVFQIDPATKRNWIPAGKHALTVSYFYDATRNVY
RIISIGGAKAIINSTVTPNMTFTKTSQKFGQWADSRANTVYGLGFASEQHLTQFAEKFQEVKEAARLAREK
SQDGGELTSPALGLASHQVPPSPLVSANGPGEEKLFRSQSADAPGPTERERLKKMLSEGSVGEVQWEAEF
FALQDSNNKLAGALREANAAAAQWRQQLEAQRAEAERLRQRVAELEAQAASEVTPTGEKEGLGQGQSL
EQLEALVQTKDQEIQTLKSQTGGPREALEAAEREETQQKVQDLETRNAELEHQLRAMERSLEEARAERER
ARAEVGRAAQLLDVRLFELSELREGLARLAEAAP*

Homer3 1M-361P was shown in blue.

RIAD-Homer

MGSSHHHHHHSSGLVPRGSHMRSLEQYANQLADQIIKEATEGGSGGSEGGSGGSEFMSTAREQPIFSTR
AHVFQIDPATKRNWIPAGKHALTVSYFYDATRNVYRIISIGGAKAIINSTVTPNMTFTKTSQKFGQWADSR
ANTVYGLGFASEQHLTQFAEKFQEVKEAARLAREKSQDGGELTSPALGLASHQVPPSPLVSANGPGEEKLF
RSQSADAPGPTERERLKKMLSEGSVGEVQWEAEFFALQDSNNKLAGALREANAAAAQWRQQLEAQRA
EAERLRQRVAELEAQAASEVTPTGEKEGLGQGQSLEQLEALVQTKDQEIQTLKSQTGGPREALEAAEREET
QQKVQDLETRNAELEHQLRAMERSLEEARAERERARAEVGRAAQLLDVRLFELSELREGLARLAEAAP*

RIAD sequence was shown in red, Homer3 1M-361P was shown in blue.

Shank (aa 533D-665R + 1294D-1323P + 1400A-1426P + 1654G-1730S, UniProt: Q4ACU6-1)

MQYKLALNGKTLKGETTTEAVDAATAEKVFKQYANDNGVDGEWTYDDATKTFTVTEHMHHHHHHSSG
LEVLFQGPGSEFDTRHETREDRTKRLFRHYTVGSYDSLTSHSDYVIDDKVAILQKRDHEGFGFVLRGAKAE
TPIEEFTPTPAFPALQYLESVDVEGVAWRAGLRTGDFLIEVNGVNVVKVGHKQVVGLIRQGGNRLVMKVV
SVTRDEETREELARIGLVPPPEEFANGILLTTPPADGHTFLLEKPPVPPKPKLKSPLGKGPGPRRPFQQKPLQ
LWSKFDVGDWLESIHLGEHRDRFEDHEIEGAHLPALTKEDFVELGVTRVGHRMNIERALRQLDGS*

GB1 tag was shown in red. N-terminal extended PDZ domain 533D-665R was show in green; SAM domain 1654G-1730S was shown in blue.

RIAD-Shank

MQYKLALNGKTLKGETTTEAVDAATAEKVFKQYANDNGVDGEWTYDDATKTFTVTEHMHHHHHHSSG
LEVLFQGPGSLEQYANQLADQIIKEATEGGSGGSEGGSGGSEFDTRHETREDRTKRLFRHYTVGSYDSLTS
HSDYVIDDKVAILQKRDHEGFGFVLRGAKAETPIEEFTPTPAFPALQYLESVDVEGVAWRAGLRTGDFLIEV
NGVNVVKVGHKQVVGLIRQGGNRLVMKVVSVTRDEETREELARIGLVPPPEEFANGILLTTPPADGHTFLL
EKPPVPPKPKLKSPLGKGPGPRRPFQQKPLQLWSKFDVGDWLESIHLGEHRDRFEDHEIEGAHLPALTKE
DFVELGVTRVGHRMNIERALRQLDGS*

GB1 tag was shown in red. RIAD sequences were shown in green. Shank3 sequences were show in blue.

EGFP-RIDD

MHHHHHHMASMTGGQQMGRGSMVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTL
KFICTTGKLPVPWPTLVTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKF
EGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQ
NTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAAGITLGMDELYKELRRQASGGGGSGGG
GSGGGGCGSLRECELYVQKHNIQALLKDSIVQLCTARPERPMAFLREYFERLEKEEAK*

EGFP sequences were show in green. RIDD sequences were shown in red.

CFP-RIDD (CFP is mTurquoise variant [5])

MGSSHHHHHHSSGLVPRGSHMVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICT
TGKLPVPWPTLVTTLSWGVQCFARYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGD
TLVNRIELKGIDFKEDGNILGHKLEYNYFSDNVYITADKQKNGIKANFKIRHNIEDGGVQLADHYQQNTPIG
DGPVLLPDNHYLSTQSKLSKDPNEKRDHMVLLEFVTAAGITLGMDELYKGSEFELRRQASGGGGSGGGG
SGGGGCGSLRECELYVQKHNIQALLKDSIVQLCTARPERPMAFLREYFERLEKEEAK*

CFP sequences were show in blue. RIDD sequences were shown in red.

YFP-RIDD (YFP is mVenus variant [6])

MGSSHHHHHHSSGLVPRGSHMSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKLICTT
GKLPVPWPTLVTTLGYGLQCFARYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTL
VNRIELKGIDFKEGGNILGHKLEYNYNSHNVYITADKQKNGIKANFKIRHNIEDGGVQLADHYQQNTPIGD

GPVLLPDNHYLSYQSALSKDPNEKRDHMVLLEFVTAAGITHGMDELYKGSEFELRRQASGGGGSGGGGS
GGGGCGSLRECELYVQKHNIQALLKDSIVQLCTARPERPMAFLREYFERLEKEEAK*

YFP sequences were show in gold. RIDD sequences were shown in red.

MenF (aa 1M-431E, UniProt: P38051-1)

MHHHHHHSSGLVPRGSMQSLTTALENLLRHLSQEIPATPGIRVIDIPFPLKDAFDALSWLASQQTYPQFY
WQQRNGDEEAVVLGAITRFTSLDQAQRFLRQHPEHADLRIWGLNAFDPSQGNLLLPRLEWRRCGGKAT
LRLTLFSESSLQHDAIQAKEFIATLVSIKPLPGLHLTTTREQHWPDKTGWTQLIELATKTIAEGELDKVVLARA
TDLHFASPVNAAAMMAASRRLNLNCYHFYMAFDGENAFLGSSPERLWRRRDKALRTEALAGTVANNP
DDKQAQQLGEWLMADDKNQRENMLVVEDICQRLQADTQTLDVLPPQVLRLRKVQHLRRCIWTSLNKA
DDVICLHQLQPTAAVAGLPRDLARQFIARHEPFTREWYAGSAGYLSLQQSEFCVSLRSAKISGNVVRLYAG
AGIVRGSDPEQEWQEIDNKAAGLRTLLQME*

MenF 1M-431E was shown in blue.

MenD (aa 2S-556L, UniProt: P17109-1)

MHHHHHHHSSLVPRGSGSSVSAFNRRWAAVILEALTRHGVRHICIAPGSRSTPLTLAAAENSAFIHHTHF
DERGLGHLALGLAKVSKQPVAVIVTSGTAVANLYPALIEAGLTGEKLILLTADRPPELIDCGANQAIRQPGMF
ASHPTHSISLPRPTQDIPARWLVSTIDHALGTLHAGGVHINCPFAEPLYGEMDDTGLSWQQRLGDWWQ
DDKPWLREAPRLESEKQRDWFFWRQKRGVVVAGRMSAEEGKKVALWAQTLGWPLIGDVLSQTGQPLP
CADLWLGNAKATSELQQAQIVVQLGSSLTGKRLLQWQASCEPEEYWIVDDIEGRLDPAHHRGRRLIANIA
DWLELHPAEKRQPWCVEIPRLAEQAMQAVIARRDAFGEAQLAHRICDYLPEQGQLFVGNSLVVRLIDALS
QLPAGYPVYSNRGASGIDGLLSTAAGVQRASGKPTLAIVGDLSALYDLNALALLRQVSAPLVLIVVNNNGG
QIFSLLPTPQSERERFYLMPQNVHFEHAAAMFELKYHRPQNWQELETAFADAWRTPTTTVIEMVVNDT
DGAQTLQQLLAQVSHL*

MenD 2S-556L was shown in blue.

MenH-RIDD (MenH: aa 2I-252F, UniProt: P37355-1)

MGSSHHHHHHSSGLVPRGSHMASMTGGQQMGRGSILHAQAKHGKPGLPWLVFLHGFSGDCHEWQE
VGEAFADYSRLYVDLPGHGGSAAISVDGFDDVTDLLRKTLVSYNILDFWLVGYSLGGRVAMMAACQGLA
GLCGVIVEGGHPGLQNAEQRAERQRSDRQWVQRFLTEPLTAVFADWYQQPVFASLNDDQRRELVALRS
NNNGATLAAMLEATSLAVQPDLRANLSARTFAFYYLCGERDSKFRALAAELAADCHVIPRAGHNAHREN
PAGVIASLAQILRFQASGGGGSGGGGSGGGGCGSLRECELYVQKHNIQALLKDSIVQLCTARPERPMAFL
REYFERLEKEEAK*

MenH 2I-252F was shown in blue. RIDD sequences were shown in red.

Idi-RIDD (Idi: aa 1M-182K, UniProt: Q46822-1)

MGSSHHHHHHSSGLVPRGSHMQTEHVILLNAQGVPTGTLEKYAAHTADTRLHLAFSSWLFNAKGQLLV
TRRALSKKAWPGVWTNSVCGHPQLGESNEDAVIRRCRYELGVEITPPESIYPDFRYRATDPSGIVENEVC
PVFAARTTSALQINDDEVMDYQWCDLADVLHGIDATPWAFSPWMVMQATNREARKRLSAFTQLKQA
SGGGGSGGGGSGGGGCGSLRECELYVQKHNIQALLKDSIVQLCTARPERPMAFLREYFERLEKEEAK*

Idi 1M-182K was shown in blue. RIDD sequences were shown in red.

RIDD-IspA (IspA: aa 1M-299K, UniProt: P22939-1)

MGSSHHHHHHSSGLVPRGSHMASSLRECELYVQKHNIQALLKDSIVQLCTARPERPMAFLREYFERLEKE
EAKGGSGGSGGSEGGSGGSMDFPQQLEACVKQANQALSRFIAPLPFQNTPVVETMQYGALLGGKRLR
PFLVYATGHMFGVSTNTLDAPAAAVECIHAYSLIHDDLPAMDDDDLRRGLPTCHVKFGEANAILAGDAL
QTLAFSILSDADMPEVSDRDRISMISELASASGIAGMCGGQALDLDAEGKHVPLDALERIHRHKTGALIR
AAVRLGALSAGDKGRRALPVLDKYAESIGLAFQVQDDILDVVGDTATLGKRQGADQQLGKSTYPALLGL
EQARKKARDLIDDARQSLKQLAEQSLDTSALEALADYIIQRNKQAS*

IspA 1M-299K was shown in blue. RIDD sequences were shown in red.

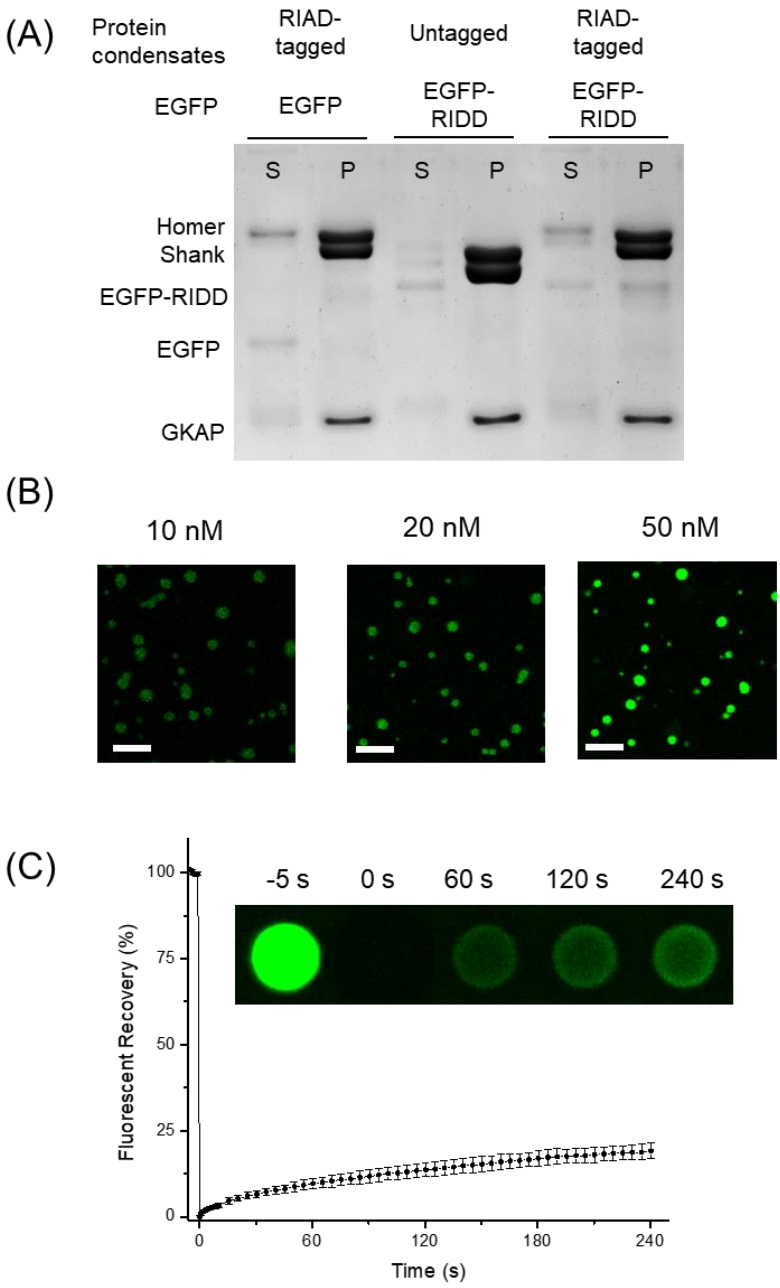


Figure S1. Assembly of EGFP in the protein condensate. **(A)** SDS-PAGE analysis showing the distribution of phase scaffolds and EGFP variants between diluted phase/supernatant (S) and condensed phase/pellet (P). More than 50% of EGFP-RIDD added to the mixture was found in P phase when mixed with RIAD-tagged phase scaffolds. Protein scaffolds 5 μM, EGFP or EGFP-RIDD 0.5 μM. **(B)** Confocal images showing EGFP-RIDD can assembled in the RIAD-tagged condensates at indicated concentration. Scale bar 10 μm, Protein scaffold 5 μM. **(C)** FRAP analysis showing EGFP-RIDD was dynamically exchanged between condensed protein condensates and solution. Protein scaffolds 5 μM, EGFP-RIDD 0.2 μM. Quantification curve used the results of 10 condensate droplets with diameter of ~5 μm.

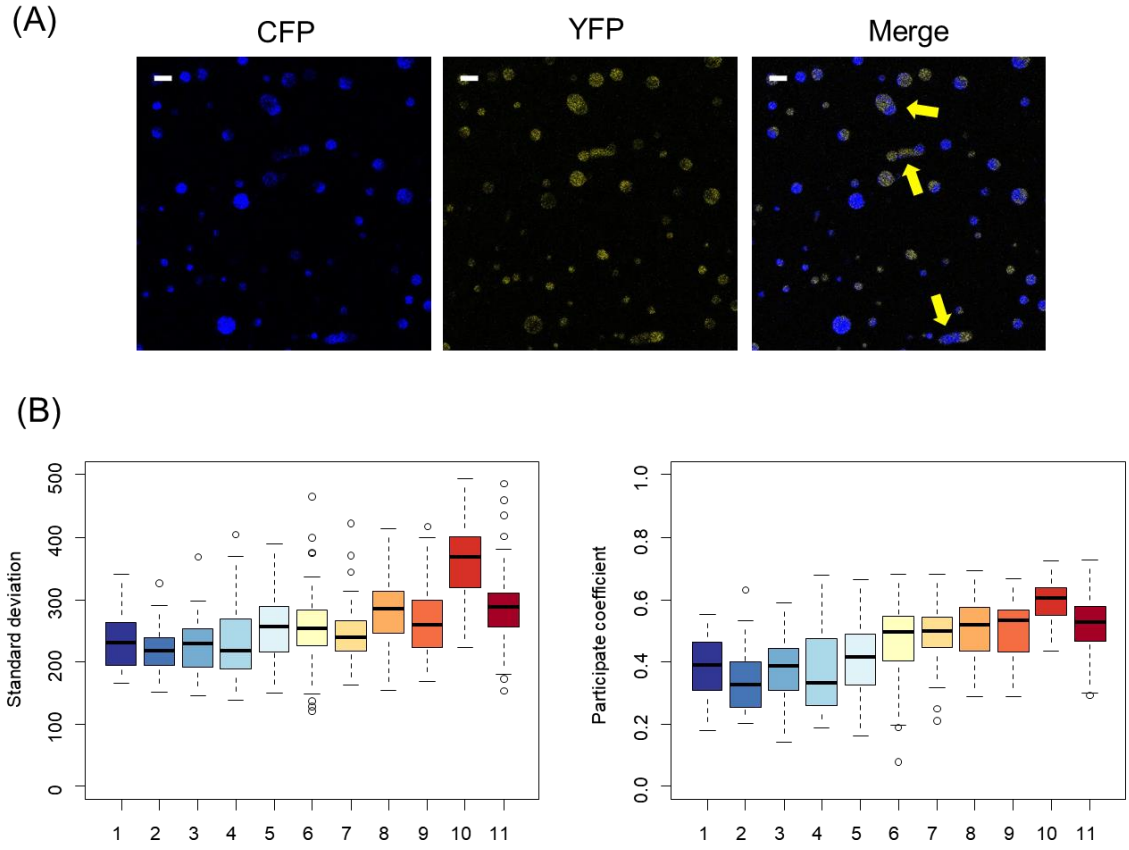


Figure S2. (A) Exchange of fluorescent proteins between CFP only and YFP only condensates. Two systems, RIAD-tagged condensates with CFP-RIDD only and RIAD-tagged condensates with YFP-RIDD only were formed separately. After 10 minutes two condensates were mixed and injected into a flow chamber. Confocal image of the mixture showing the merge and exchange of fluorescent proteins between condensates with different kind of fluorescent proteins. Scale bar, 5 μm. Protein scaffolds 5 μM, CFP-RIDD or YFP-RIDD 0.2 μM. Yellow arrows point to droplets that in the fusion process. (B) Difference of the fluorescent signal distribution in CFP only and CFP+YFP condensates. Boxplots of the standard deviation (left) and participate coefficient (right) of all the condensates in five CFP only images (numbered 1-5 in cold color) and CFP+YFP images (numbered 6-11 in warm color). The circles indicate outliers.

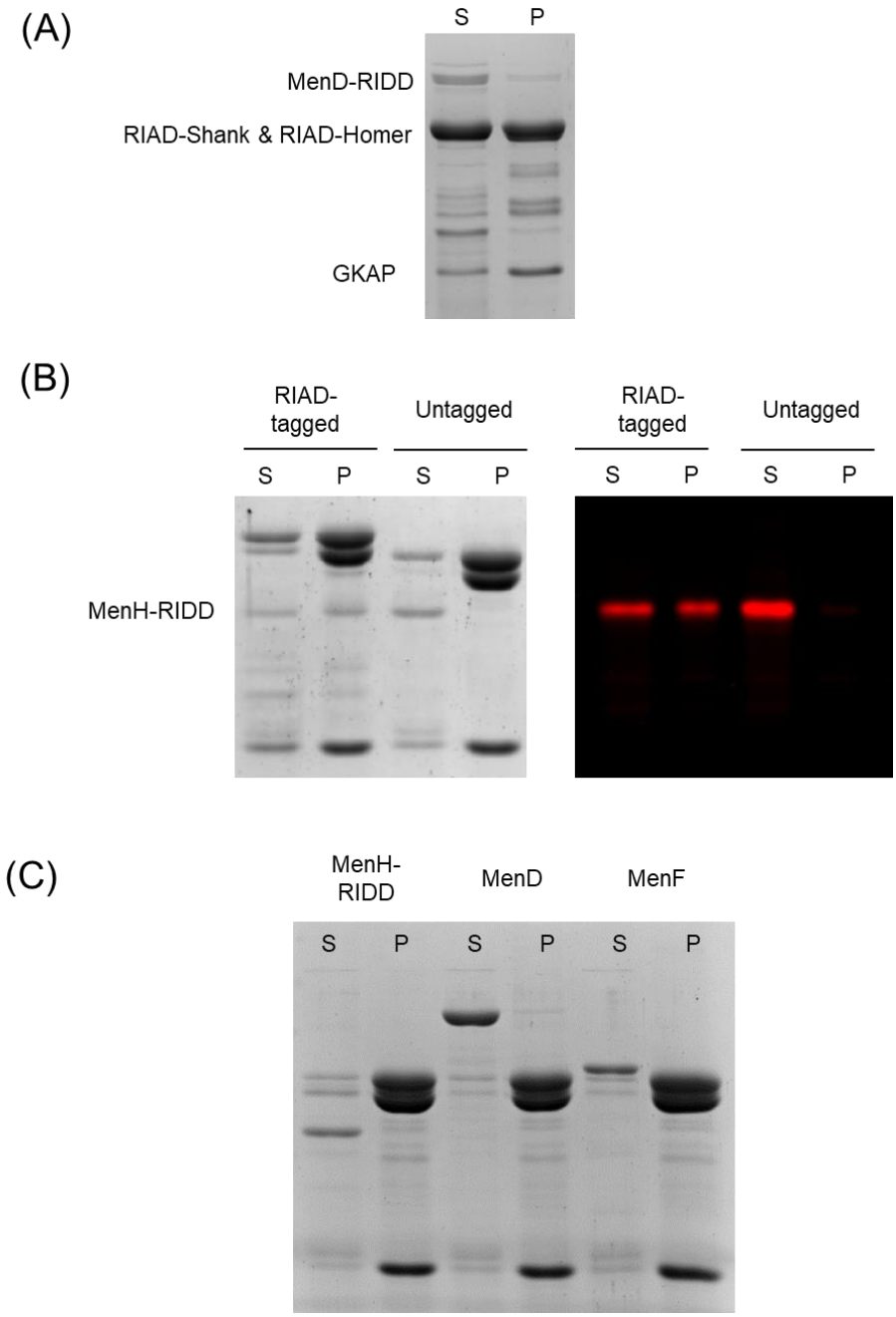


Figure S3. Centrifugation/SDS-PAGE analysis showing the protein distributions of the menaquinone biosynthetic enzymes in the protein condensate. **(A)** Assembly of MenD-RIDD disrupts phase formation. When MenD-RIDD was mixed with RIAD-tagged phase scaffolds, most of the MenD-RIDD was found in S phase and about 50% of the scaffold proteins were found in S phase. Protein scaffolds, 5 μM; MenD-RIDD, 0.5 μM. **(B)** Assembly of MenH-RIDD in RIAD-tagged condensates shown by Coomassie blue stain and Cy5 fluorescent of SDS-PAGE. About 50% of MenH-RIDD was found in P phase. Protein scaffold, 5 μM; MenH-RIDD 1 μM with 5% Cy5 labelled. **(C)** Spontaneous enrichment of the three enzymes in the protein condensates without RIAD tags in the absence of peptide-peptide interactions. Most of the enzyme were found in S phase when incubated with untagged scaffold proteins. Protein scaffolds, 5 μM; enzymes, 1 μM.

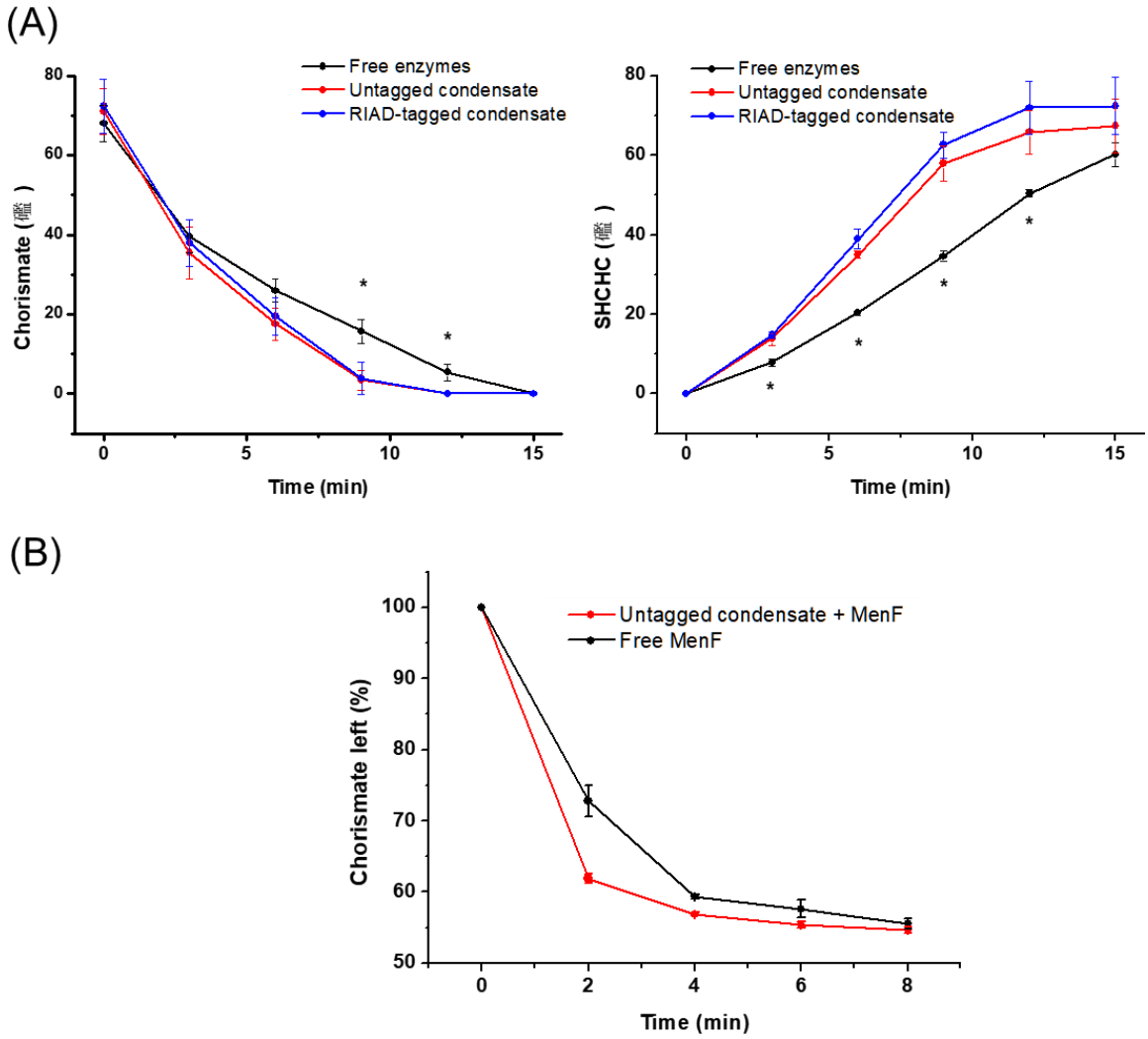
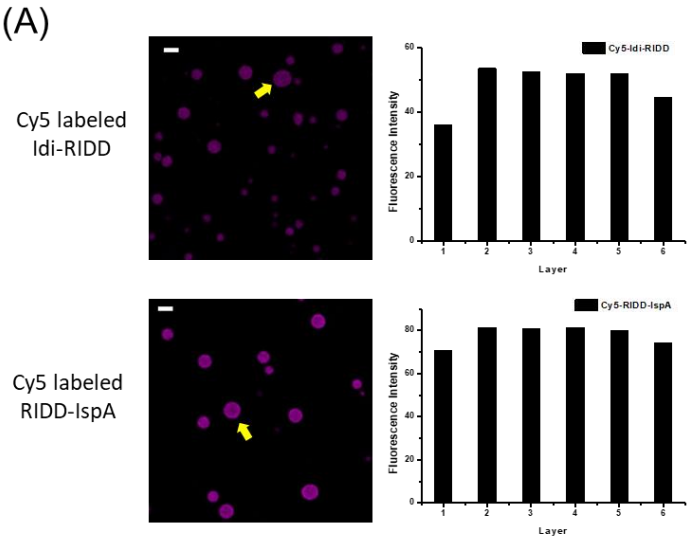
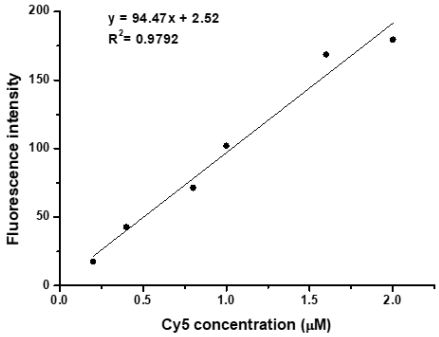


Figure S4. Catalytic property of the menaquinone biosynthetic enzyme condensates. **(A)** The decrease of substrate chorismate and increase of the final product SHCHC of the whole reaction processes showing the efficiencies of the MenF/MenD/MenH-RIDD cascades. There was significant difference between control group and phase separation group at the indicated time point marked with star. And there was no significant difference between untagged phase group and RIAD-tagged phase group at any time of the whole reaction process. Welch's t-test, * $p < 0.05$, $n = 3$. **(B)** HPLC data of chorismate showing that MenF turned chrosimate into equilibrium faster when protein phase separation formed.

(A)



(B)



(C)

2% Cy5 labeled protein	Added concentration (μM)	Average Cy5 intensity in droplet	Cy5-protein concentration in droplet (μM)	Total protein concentration in droplet (μM)
Idi-RIDD	0.5	54.1 ± 3.2	0.562 ± 0.034	28.1 ± 1.7
RIDD-IspA	1	83.0 ± 6.1	0.863 ± 0.064	43.1 ± 3.2

(D)

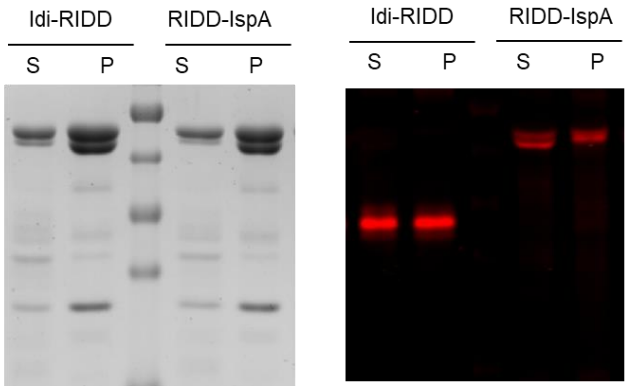


Figure S5. Estimating concentration of terpene biosynthesis enzymes inside the protein condensate. (A) Representative confocal images showing the Cy5 fluorescence of labeled enzymes in the condensate. The measured fluorescence intensities of each layers for the droplets marked with yellow arrow were plotted on the right. (B) Standard curve between Cy5 concentration and Cy5 fluorescence intensity measured by confocal microscope. (C) Summary of the average Cy5 intensities analyzed from confocal images and the calculated protein concentrations. Values are represented as mean ± SD of 10 different droplets. (D) Centrifugation/SDS-PAGE analysis showing the protein distributions of the Idi-RIDD and RIDD-IspA in the protein condensate. Protein scaffold, 5 μM; Idi-RIDD 1 μM with 10% Cy5 labelled; RIDD-IspA 1 μM with 5% Cy5 labelled.

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