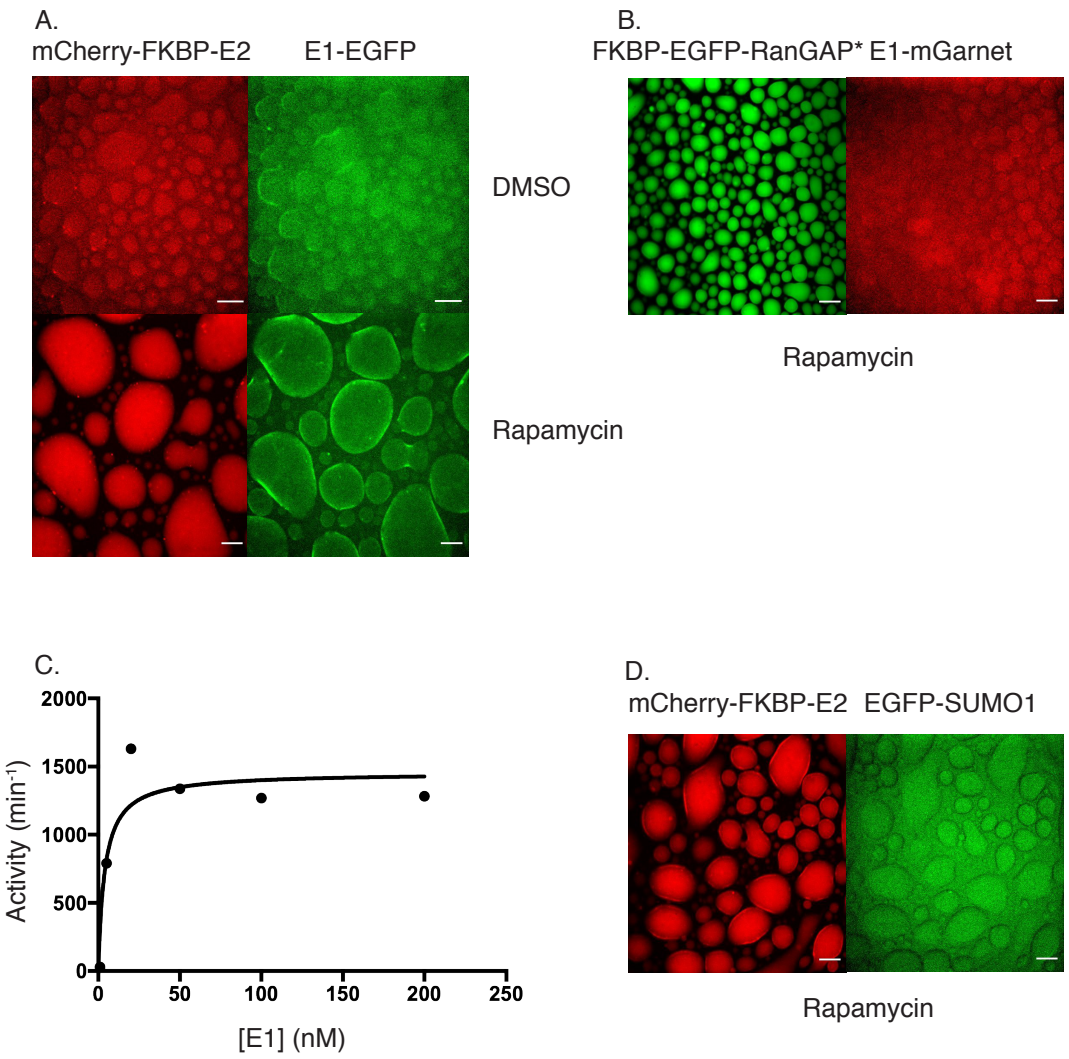


Figure S1

Supplementary Figure 1. E2-Mediated recruitment of E1, and no recruitment of SUMO.

(A) Confocal fluorescence microscopy images of mCherry-FKBP-E2 (red) and E1-EGFP (green) in the presence of FRB-polySH3$_3$-polyPRM$_5$ condensates with DMSO (top row) and rapamycin (bottom row). These images are representative of 3 independent experiments. Scale bar is 50um.

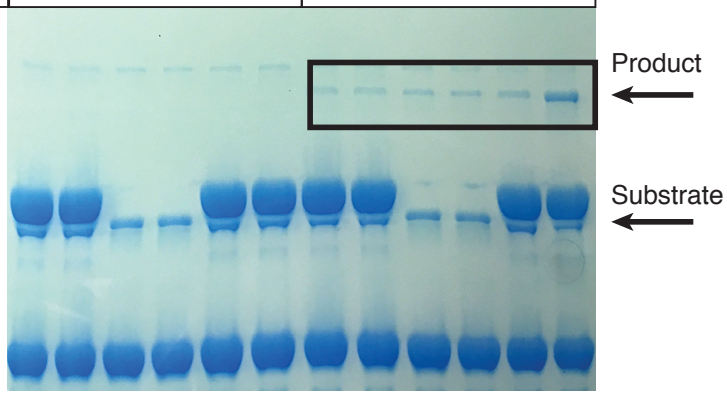
(B) Confocal fluorescence microscopy images of FKBP-EGFP-substrate (green) and E1-mGarnet (red) in the presence of FRB-polySH3$_3$-polyPRM$_5$ condensates and rapamycin. These images are representative of 4 independent experiments. Scale bar is 50um.

(C) Rate curve depicting SUMOylation of RanGAP* as a function of E1 concentration. Apparent E1 K_M is approximately 5 nM with no significant increase in rate above 50 nM.

(D) Confocal fluorescence microscopy images of mCherry-FKBP-E2 (red) and EGFP-SUMO1 (green) in the presence of FRB-polySH3$_3$-polyPRM$_5$ condensates and rapamycin. These images are representative of 3 independent experiments. Scale bar is 50um. All figure panels have associated raw data.

A.

FRB-polySH3$_3$	+		-		+		+		-		+	
polyPRM$_5$	-		+		+		-		+		+	
Rapamycin	-	+	-	+	-	+	-	+	-	+	-	+
Time (min)	0						5					



B.

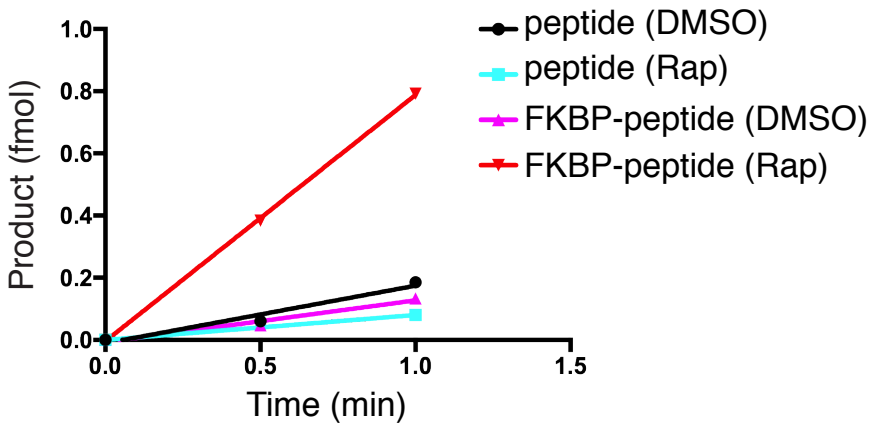


Figure S2

Supplementary Figure 2. Rate enhancement requires both scaffolds to be present and both E2 and substrate to be tethered to FRB-polySH3$_3$.

(A) SDS-PAGE gel showing the production of SUMOylated substrate over time in the presence of FRB-polySH3$_3$, polyPRM$_5$ or both, with and without rapamycin. B) Plot of Figure 2F depicting the simultaneous SUMOylation rates of peptide and FKBP-peptide when neither are recruited (DMSO) or both are recruited (Rap). All figure panels have associated raw data.

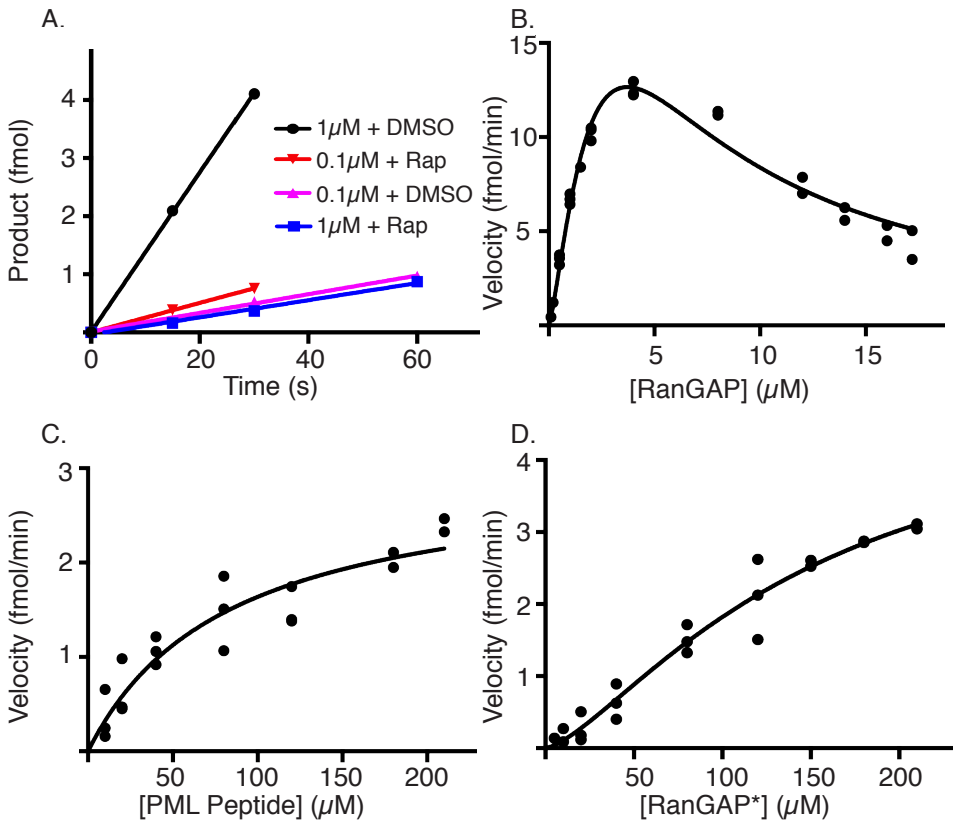


Figure S3

Supplementary Figure 3. SUMOylation of RanGAP, PML peptide and RanGAP* substrates.

(A) SUMOylation rate over time of RanGAP in the presence of FRB-polySH3$_3$-polyPRM$_5$ condensates at two different concentrations (1 and 0.1 μM) with and without rapamycin. 1 μM + DMSO (black circles), 1 μM + Rap (blue squares), 0.1 μM + DMSO (magenta triangles), and 0.1 μM + Rap (red inverted triangles). (B)-(D) SUMOylation velocity as a function of substrate concentration for RanGAP (B), PML peptide substrate (C), and RanGAP* mutant (D). RanGAP fit to substrate inhibition, while PML peptide and RanGAP* mutant fit to standard Michaelis-Menten. Each symbol represents the mean and standard deviation from n=3 (<150uM) and n=2 (≥150uM) independent experiments. Points without errors bars have standard deviations too small to show.

All figure panels have associated raw data.

A.

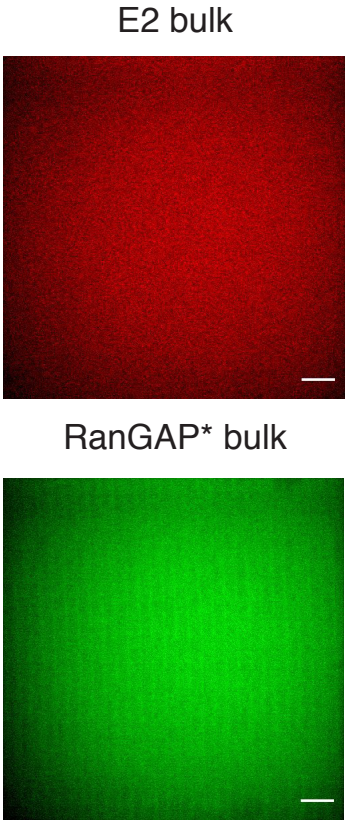


Figure S4

Supplementary Figure 4. Quantitative images of bulk solution after centrifugation.

A) Representative confocal fluorescence microscopy images of the bulk solution after clarification by centrifugation. Top row shows mCherry-FKBP-E2, bottom row shows FKBP-EGFP-RanGAP*. These images are representative of 4 independent experiments. Scale bar is 50um.

Figure has associated raw data.

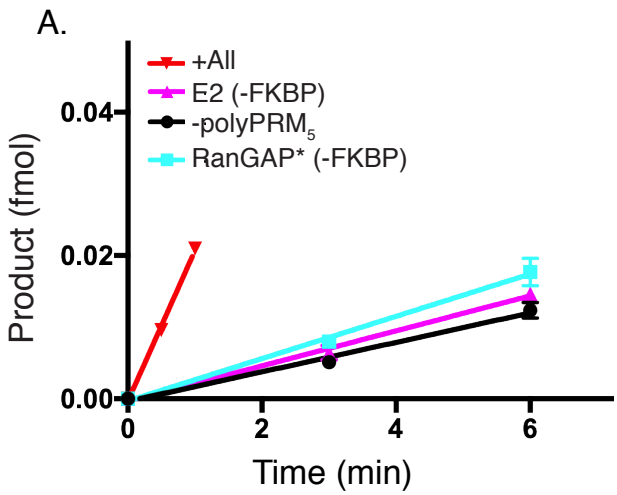


Figure S5

Supplementary Figure 5. Bulk scaffold enhancement requires both scaffolds to be present and both E2 and substrate to be tethered.

(A) RanGAP* SUMOylation over time at sub-critical concentrations of FRB-polySH3$_3$:polyPRM$_5$ without polyPRM$_5$ (-polyPRM$_5$, black circles), with substrate not tethered (Substrate (-FKBP), cyan squares), with E2 not tethered (E2 (-FKBP), magenta triangles), and with all components present and tethered (+All, red inverted triangles). Error bars represent the SEM of 3 independent experiments. For points with no error bars, the errors are too small to depict.

Figure has associated raw data.

A.

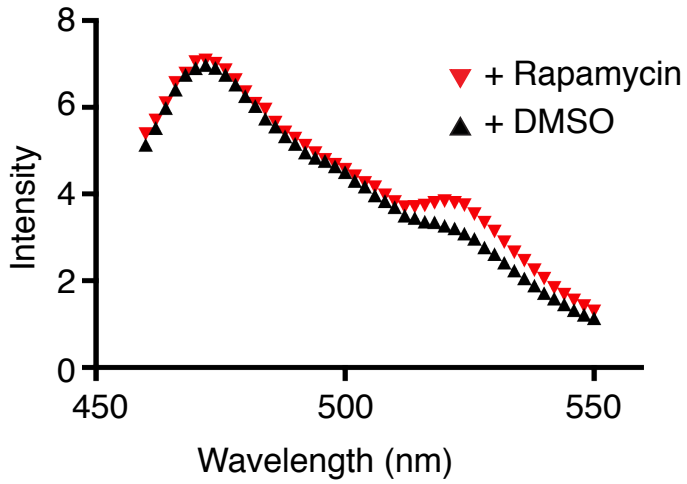


Figure S6

Supplementary Figure 6. FRET increase requires rapamycin.

(A) Fluorescence emission spectrum of FKBP-YPet-RanGAP* upon 445 nm excitation of CyPet-FKBP-E2. Spectra recorded in the presence of FRB-polySH3$_3$ + polyPRM$_5$ with either Rapamycin (inverted red triangles) or DMSO (black triangles).

Figure has associated raw data.

A.

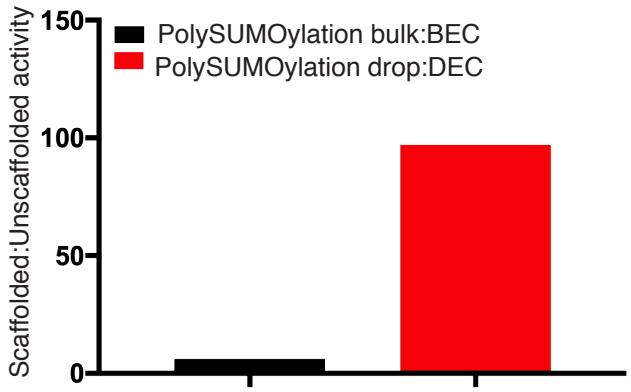


Figure S7

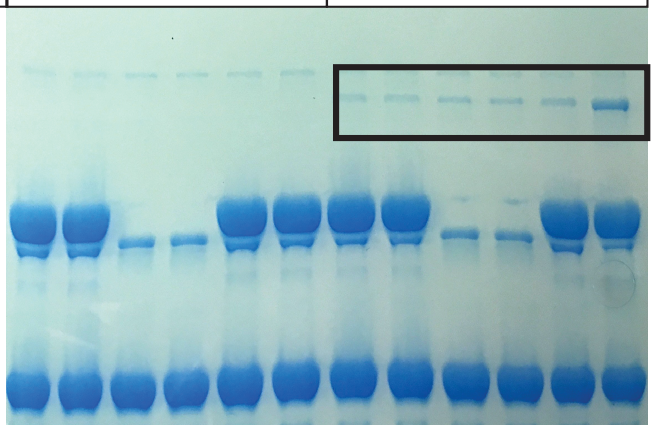
Supplementary Figure 7. PolySUMOylation acceleration is higher in droplets than in the bulk, suggesting effects beyond K_M and concentration

Scaffolded:unscaffolded reaction rate ratio of the polySUMOylation reaction for both bulk and droplet. Scaffolded reactions contain FRB-polySH3$_3$:polyPRM$_5$. Reactions are carried out at identical total enzyme and substrate concentrations of 90nM E1, 200nM E2, and 1uM RanGAP*. PolySUMOylation rates represent the mean of two independent experiments and only consider the di- and tri-SUMOylated species.

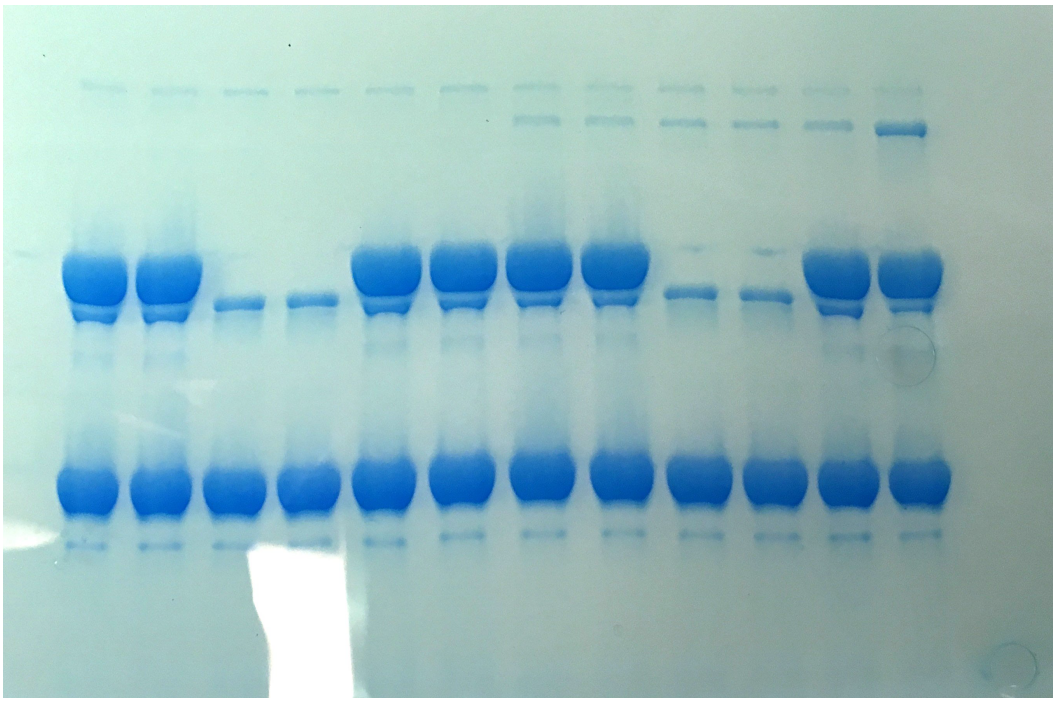
Figure has associated raw data.

A.

FRB-polySH3$_3$	+		-		+		+		-		+	
polyPRM$_5$	-		+		+		-		+		+	
Rapamycin	-	+	-	+	-	+	-	+	-	+	-	+
Time (min)	0						5					



Full gel



Supplementary Figure 8. Source Data for Supplementary Figure 2.

A) Supplementary Figure 2 alongside the uncropped gel.

Supplementary Table 1. Kinetic parameters of SUMOylation reactions.

Substrate	SH3 Scaffold	K_M (μM)	Vmax (fmol/min/μl)	Hill Coefficient	K_i (μM)
PML peptide	-	83 ± 26	3 ± 0.4	-	-
RanGAP	-	2.5 ± 2	14 ± 10	-	10 ± 18
RanGAP*	-	150 ± 70	5 ± 1.5	1.4 ± 0.3	-
RanGAP*	polySH3$_3$	50 ± 15	3.7 ± 2	0.7 ± 0.1	-
RanGAP*	polySH3$_5$	93 ± 45	4.7 ± 1.7	1.8 ± 0.7	-

Supplementary Table 1.

Kinetic parameters obtained for the PML peptide, wildtype RanGAP and mutant RanGAP* substrates in the absence of scaffolds and for RanGAP* with sub-critical concentrations of FRB-polySH3$_3$:polyPRM$_5$ and FRB-polySH3$_5$:polyPRM$_5$.

Supplementary Table 2. Protein concentrations in droplets and bulk solution.

Protein	Total Concentration (μM)	Droplet Concentration (μM)	Bulk Concentration (μM)	Partition Coefficient
E1	0.09	0.17 ± 0.01	0.09 ± 0.01	1.9 ± 0.2
E2	0.1	1.4 ± 0.1	0.09 ± 0.01	14 ± 2
Substrate	1.0	31 ± 2	0.65 ± 0.03	48 ± 4
SUMO1	1.0	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1
FRB-polySH3$_5$	15.0	660 ± 16	6.3 ± 0.3	105 ± 6

Supplementary Table 2.

Protein concentration values measured in FRB-polySH3$_3$:polyPRM$_5$ droplets and bulk as well the total input concentrations and the partition coefficient for each component.

Supplementary Table 3. Constructs used in this study. Protein sequences after proteolytic removal of affinity tags are shown.

Construct	Protein sequence	Tag(s)
polyPRM$_5$	GHMKGGSWGGSKKKKTAPTPPKRSGGSGGSGGSGGSKKKK TAPTPPKRSGGSGGSGGSGGSKKKKTAPTPPKRSGGSGGS GGSGGSKKKKTAPTPPKRSGGSGGSGGSGGSKKKKTAPTP PKRSGGSGSENLYFQ	N-terminal MBP (maltose binding protein) C-terminal His$_6$
FRB-polySH3$_3$	GEFMLEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMME RGPQTLKETSFNQAYGRDLMEAQEWCRKYMKSGNVKDLTQ AWDLYYHVFRRISKQVDGGSGGSGGSGGSHMDLNMPAYVK FNYMAEREDELSLIKGTKVIVMEKSSDGWWRGSYNGQVGW FPSNYVTEEGDSPLASGAGGSEGGGSEGGTSGATDLNMPA YVKFNYMAEREDELSLIKGTKVIVMEKSSDGWWRGSYNGQ VGWFPSNYVTEEGDSPLASGAGGSEGGGSEGGTSGATDLN MPAYVKFNYMAEREDELSLIKGTKVIVMEKSSDGWWRGSY NGQVGWFPSNYVTEEGDSPLGGGSENLYFQ	N-terminal MBP C-terminal His$_6$
FRB-polySH3$_5$	GEFMLEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMME RGPQTLKETSFNQAYGRDLMEAQEWCRKYMKSGNVKDLTQ AWDLYYHVFRRISKQVDGGSGGSGGSGGSKDAQTNSSSNN NNNNNNNNLGIEGRISHMDLNMPAYVKFNYMAEREDELSL IKGTKVIVMEKSSDGWWRGSYNGQVGWFPSNYVTEEGDSP LASGAGGSEGGGSEGGTSGATDLNMPAYVKFNYMAEREDE LSLIKGTKVIVMEKSSDGWWRGSYNGQVGWFPSNYVTEEG DSPLASGAGGSEGGGSEGGTSGATHMDLNMPAYVKFNYMA EREDELSLIKGTKVIVMEKSSDGWWRGSYNGQVGWFPSNY VTEEGDSPLASGAGGSEGGGSEGGTSGATDLNMPAYVKFN YMAEREDELSLIKGTKVIVMEKSSDGWWRGSYNGQVGWFP SNYVTEEGDSPLASGAGGSEGGGSEGGTSGATDLNMPAYV KFNYMAEREDELSLIKGTKVIVMEKSSDGWWRGSYNGQVG WFPSNYVTEEGDSPLGGGSENLYFQ	N-terminal MBP C-terminal His$_6$
ShadowG-SUMO1	GEFVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDA TYGKLTLKLICTTGKLPVPWPTLVTTFGYGLMCFARYPDH MKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGD TLVNRIELKGIDFKEDGNILGHKLEYNWNSHNVYIMADKQ KNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPD NHYLSTQSKLSKDPNEKRDHMVLLEFVTAAGITLGMDELY KSGLRSRAQASNSAVDGTMSDQEAKPSTEDLGDKKEGEYI KLKVIGQDSSEIHFKVKMTTHLKKLKESYCQRQGVPMNSL RFLFEGQRIADNHTPKELGMEEEDVIEVYQEQTGG	N-terminal His$_8$
SAE1	GEFGGSGGSGGSGGSMVEKEEAGGGISEEEAAQYDRQIRL WGLEAQKRLRASRVLLVGLKGLGAEIAKNLILAGVKGLTM LDHEQVTPEDPGAQFLIRTGSVGRNRAEASLERAQNLNPM VDVKVDTEDIEKKPESFFTQFDAVCLTCCSRDVIVKVDQI CHKNSIKFFTGDVFGYHGYTFANLGEHEFVEEKTKVAKVS QGVEDGPGPDTKRAKLDSSETTMVKKKVVFCPVKEALEVD WSSEKAKAALKRTTSDYFLLQVLLKFRTDKGRDPSSDTYE EDSELLLQIRNDVLDSLGISPDLLPEDFVRYCFSEMAPVC AVVGGILAQEIVKALSQRDPPHNNFFFFDGMKGNGIVECL GPK	N-terminal His$_6$
SAE2-mGarnet	GEFSGGSGGSGGSMALSRGLPRELAEAVAGGRVLVVGAGG IGCELLKNLVLTGFSHIDLIDLDTIDVSNLNRQFLFQKKH	N-terminal-MBP

	VGRSKAQVAKESVLQFYPKANIVAYHDSIMNPDYNVEFFR QFILVMNALDNRAARNHVNRMCLAADVPLIESGTAGYLGQ VTTIKKGVTECYECHPKPTQRTFPGCTIRNTPSEPIHCIV WAKYLFNQLFGEEDADQEVSPDRADPEAAWEPTEAEARAR ASNEDGDIKRISTKEWAKSTGYDPVKLFTKLFKDDIRYLL TMDKLWRKRKPPVPLDWAEVQSQGEETNASDQQNEPQLGL KDQQVLDVKSYARLFSKSIETLRVHLAEKGDGAELIWDKD DPSAMDFVTSAANLRMHIFSMNMKSRFDIKSMAGNIIPAI ATTNAVIAGLIVLEGLKILSGKIDQCRTIFLNKQPNPRKK LLVPCALDPPNPNCYVCASKPEVTVRLNVHKVTVLTLQDK IVKEKFAMVAPDVQIEDGKGTILISSEEGETEANNHKKLS EFGIRNGSRLQADDFLQDYTLLINILHSEDLGKDVEFEVV GDAPEKVGPKQAEDAAKSITNGSDDGAQPSTSTAQEQDDV LIVDSDEEDSSNNADVSEEERSRKRKLDEKENLSAKRSRI EQKEELDDVIALDLDLRSRAQASNSAVDGTNSLIKENMRM KVVLEGSVNGHQFKCTGEGEGNPYMGTQTMRIKVIEGGPL PFAFDILATSFMYGSKTFIKYPKGIPDFFKQSFPEGFTWE RVTRYEDGGVITVMQDTSLEDGCLVYHAQVRGVNFPSNGA VMQKKTKGWEPNTEMMYPADGGLRGYNHMALKVDGGGHLS CSLVTTYRSKKTVGNIKMPGIHAVDRRLERLEESDNEMFV VQREHAVAKFAGLGGG	
mCherry-E2	GEFVSKGEEDNMAIIKEFMRFKVHMEGSVNGHEFEIEGEG EGRPYEGTQTAKLKVTKGGPLPFAWDILSPQFMYGSKAYV KHPADIPDYLKLSFPEGFKWERVMNFEDGGVVTVTQDSSL QDGEFIYKVKLRGTNFPSDGPVMQKKTMGWEASSERMYPE DGALKGEIKQRLKLKDGGHYDAEVKTTYKAKKPVQLPGAY NVNIKLDITSHNEDCTIVEQYERAEGRHSTGGMDELYKSG GGSGGSGGSGGSMSGIALSRLAQERKAWRKDHPFGFVAVP TKNPDGTMNLMNWECAIPGKKGTPWEGGLFKIRMLFKDDY PSSPPKCKFEPPLFHPNVYPSGTVCLSILEEDKDWRPAIT IKQILLGIQELLNEPNIQDPAQAEAYTIYCQNRVEYEKRV RAQAKKFAPSLEENLYFQ	N-terminal His_{10} C-terminal RK5 (5 repeats of arginine and lysine)
mCherry-FKBP-E2	GEFVSKGEEDNMAIIKEFMRFKVHMEGSVNGHEFEIEGEG EGRPYEGTQTAKLKVTKGGPLPFAWDILSPQFMYGSKAYV KHPADIPDYLKLSFPEGFKWERVMNFEDGGVVTVTQDSSL QDGEFIYKVKLRGTNFPSDGPVMQKKTMGWEASSERMYPE DGALKGEIKQRLKLKDGGHYDAEVKTTYKAKKPVQLPGAY NVNIKLDITSHNEDCTIVEQYERAEGRHSTGGMDELYKQL MGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKFDSS RDRNKPFKFMLGKQEVIRGWEEGVAQMSVGQRAKLTISPD YAYGATGHPGIIPPHATLVFDVELLKLNEGGSGGSGGSGG SLRSRAQASNSAVDGTMSGIALSRLAQERKAWRKDHPFGF VAVPTKNPDGTMNLMNWECAIPGKKGTPWEGGLFKLRMLF KDDYPSSPPKCKFEPPLFHPNVYPSGTVCLSILEEDKDWR PAITIKQILLGIQELLNEPNIQDPAQAEAYTIYCQNRVEY EKRVRAQAKKFAPSLENLYFQ	N-terminal His_{10} C-terminal RK5
CyPet-FKBP-E2	GEFVSKGEELFGGIVPILVELEGDVNGHKFSVSGEGEGDA TYGKLTLKFICTTGKLPVPWPTLVTTLTWGVQCFSRYPDH MKQHDFFKSVMPEGYVQERTIFFKDDGNYKTRAEVKFEGD TLVNRIELKGIDFKEDGNILGHKLEYNYISHNVYITADKQ KNGIKANFKARHNITDGSVQLADHYQQNTPIGDGPVILPD NHYLSTQSALSKDPNEKRDHMVLLEFVTAAGITHGMDELY KQLMGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKF	N-terminal His_{10} C-terminal RK5

	DSSRDRNKPFKFMLGKQEVIRGWEEGVAQMSVGQRAKLTI SPDYAYGATGHPGIIPPHATLVFDVELLKLNEGGSGGSGG SGGSLRSRAQASNSAVDGTMSGIALSRLAQERKAWRKDHP FGFVAVPTKNPDGTMNLMNWECAIPGKKGTPWEGGLFKLR MLFKDDYPSSPPKCKFEPPLFHPNVYPSGTVCLSILEEDK DWRPAITIKQILLGIQELLNEPNIQDPAQAEAYTIYCQNR VEYEKRVRAQAKKFAPSLENLYFQ	
FKBP- EGFP- RanGAP	GEFMGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKF DSSRDRNKPFKFMLGKQEVIRGWEEGVAQMSVGQRAKLTI SPDYAYGATGHPGIIPPHATLVFDVELLKLNEGGSGGSGG SGGSVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGD ATYGKLTLKFICTTGKLPVPWPTLVTTLTYGVQCFSRYPD HMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEG DTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADK QKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLP DNHYLSTQSKLSKDPNEKRDHMVLLEFVTAAGITLGMDEL YKSGLRSPQQRGQGEKSATPSRKILDPNTGEPAPVLSSPP PADVSTFLAFPSPEKLLRLGPKSSVLIAQQTDTSDPEKVV SAFLKVSSVFKDEATVRMAVQDAVDALMQKAFNSSSFNSN TFLTRLLVHMGLLKSEDKVKAIANLYGPLMALNHMVQQDY FPKALAPLLLAFVTKPNSALESCSFARHSLLQTLSKVGSE NLYFQ	N-terminal MBP C-terminal His_6
FKBP- EGFP- RanGAP*	GEFMGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKF DSSRDRNKPFKFMLGKQEVIRGWEEGVAQMSVGQRAKLTI SPDYAYGATGHPGIIPPHATLVFDVELLKLNEGGSGGSGG SGGSVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGD ATYGKLTLKFICTTGKLPVPWPTLVTTLTYGVQCFSRYPD HMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEG DTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADK QKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLP DNHYLSTQSKLSKDPNEKRDHMVLLEFVTAAGITLGMDEL YKSGLRSPQQRGQGEKSATPSRKILDPNTGEPAPVLSSPP PADVSTFLAFPSPEKLLRLGPKSSVLIAQQTDTSDPEKVV SAFLKVSSVFKDEATVRMAVQDAVDALMQKAFNSSSFNSN TFLTRLLVHMGLLKSEDKVKAIANLYGPLMALNHMVQQDY FPKALAPLLLAAVTKPNSALESCSFARHSLLQTLSKVGSE NLYFQ	N-terminal MBP C-terminal His_6 Mutation site in red
FKBP- YPet- RanGAP*	GEFMGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKF DSSRDRNKPFKFMLGKQEVIRGWEEGVAQMSVGQRAKLTI SPDYAYGATGHPGIIPPHATLVFDVELLKLNEGGSGGSGG SGGSVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGD ATYGKLTLKLLCTTGKLPVPWPTLVTTLGYGVQCFARYPD HMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEG DTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYITADK QKNGIKANFKIRHNIEDGGVQLADHYQQNTPIGDGPVLLP DNHYLSYQSALFKDPNEKRDHMVLLEFLTAAGITEGMNEL YKSGLRSPQQRGQGEKSATPSRKILDPNTGEPAPVLSSPP PADVSTFLAFPSPEKLLRLGPKSSVLIAQQTDTSDPEKVV SAFLKVSSVFKDEATVRMAVQDAVDALMQKAFNSSSFNSN TFLTRLLVHMGLLKSEDKVKAIANLYGPLMALNHMVQQDY FPKALAPLLLAAVTKPNSALESCSFARHSLLQTLSKVGSE NLYFQ	N-terminal MBP C-terminal His_6 Mutation site in red
FKBP-	GEFMGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKF	N-terminal MBP

EGFP-PML peptide	DSSRDRNKPFKFMLGKQEVIRGWEEGVAQMSVGQRAKLTI SPDYAYGATGHPGIIPPHATLVFDVELLKLNEGGSGGSGG SGGSVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGD ATYGKLTLKFICTTGKLPVPWPTLVTTLTYGVQCFSRYPD HMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEG DTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADK QKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLP DNHYLSTQSKLSKDPNEKRDHMVLLEFVTAAGITLGMDEL YKGGSGGSGGSKVDVIDLTIESSSDEEEDPPAKRGSAGSA GSAGSAGSAGSAGSAGSAGSAGSAGSASQTQSPRKVI KMESEEGSENLYFQ	C-terminal His_6
EGFP-PML peptide	GEFVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDA TYGKLTLKFICTTGKLPVPWPTLVTTLTYGVQCFSRYPDH MKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGD TLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQ KNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPD NHYLSTQSKLSKDPNEKRDHMVLLEFVTAAGITLGMDELY KGGSGGSGGSKVDVIDLTIESSSDEEEDPPAKRGSAGSAG SAGSAGSAGSAGSAGSAGSAGSAGSASQTQSPRKVIK MESEEGSENLYFQ	N-terminal MBP C-terminal His_6
EGFP-RanGAP*	GEFVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDA TYGKLTLKFICTTGKLPVPWPTLVTTLTYGVQCFSRYPDH MKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGD TLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQ KNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPD NHYLSTQSKLSKDPNEKRDHMVLLEFVTAAGITLGMDELY KSGLRSPQQRGQGEKSATPSRKILDPNTGEPAPVLSSPPP ADVSTFLAFPSPEKLLRLGPKSSVLIAQQTDTSDPEKVVS AFLKVSSVFKDEATVRMAVQDAVDALMQKAFNSSSFNSNT FLTRLLVHMGLLKSEDKVKAIANLYGPLMALNHMVQQDYF PKALAPLLLA**A**VTKPNSALESCSFARHSLLQTLSKVGSEN LYFQ	N-terminal MBP C-terminal His_6 Mutation site in red

Supplementary Methods: Modeling

All modeling was performed in MATLAB (Mathworks). Models were generated using the Michaelis-Menten (MM) equation to describe reaction rates in the droplet and bulk compartments, $R = k_{cat}[E]*[S]/K_M+[S]$, where [E] and [S] are the concentrations of enzyme and substrate in the respective compartment and K_M can either be the scaffolded ($K_{M,S}$) or unscaffolded ($K_{M,US}$) value. The model assumed identical k_{cat} in both compartments. The droplet and bulk concentrations are related to the total concentration according to: $V_T*C_T= V_D*C_D+V_B*C_B$, where C is concentration, V is volume, T is total, D is droplet, and B is bulk. By setting $V_T = 1$, defining the partition coefficient (PC) = C_D/C_B (assumed, for simplicity, to be identical for E and S), and assigning a value to the compartment (droplet or bulk) volume fraction, this equation can be rearranged to express C_D or C_B as a function of C_T and PC, which can be substituted into the MM equation to yield the enzymatic rate in a given compartment.

When E and S are recruited into droplets, the droplet and bulk rates can be expressed as:

$$\mathrm{R_D} = \frac{k_{cat}[E]_D[S]_D}{K_{M,S} + [S]_D} \quad (1)$$

and:

$$\mathrm{R_B} = \frac{k_{cat}[E]_B[S]_B}{K_{M,S} + [S]_B} \quad (2)$$

Rearranging $C_T*V_T=V_D*C_D+V_B*C_B$, and assuming a 1% droplet volume gives:

$$\mathrm{C_D} = \frac{100C_T * PC}{PC + 99} \quad (3)$$

and

$$\mathrm{C_B} = \frac{100[E]t}{99 + PC} \tag{4}$$

Substituting equations (3) and (4) into equations (1) and (2), respectively, gives droplet and bulk rates of:

$$R_D = k_{cat}[E]_D \frac{100[S]_T * PC}{K_{M,S}(PC + 99) + 100[S]_T * PC} \tag{5}$$

and:

$$R_B = \frac{k_{cat}[E]_D}{PC} * \frac{100[S]_T}{K_{M,S}(PC + 99) + 100[S]_T} \tag{6}$$

For a droplet volume of 1%, the total scaffolded reaction rate, $R_{T,S} = 0.01R_D + 0.99\ R_B$:

$$R_{T,S} = k_{cat}[E]_D \left\{ \frac{[S]_T * PC}{K_{M,S}(PC + 99) + 100[S]_T * PC} + \frac{1}{PC} * \frac{99[S]_T}{K_{M,S}(PC + 99) + 100[S]_T} \right\} \tag{7}$$

The total unscaffolded reaction rate is:

$$\mathrm{R_{T,US}} = \frac{k_{cat}[E]_T[S]_T}{K_{M,US} + [S]_T} \tag{8}$$

Or

$$R_{T,US} = k_{cat}[E]_D * \frac{(PC + 99)}{100PC} * \frac{[S]_T}{K_{M,US} + [S]_T}$$

Thus, the ratio of total scaffolded to total unscaffolded rates, $R_{T,S}/R_{T,US}$ is:

$$\frac{R_{T,S}}{R_{T,US}} = \frac{100PC}{PC+99} * \left\{ \frac{PC * \left(K_{M,US} + [S]_T\right)}{K_{M,S}(PC+99) + 100[S]_T * PC} + \frac{1}{PC} * \frac{99\left(K_{M,US} + [S]_T\right)}{K_{M,S}(PC+99) + 100[S]_T} \right\} \quad (9)$$

The ratio of droplet to bulk rates is given by dividing equation (5) by equation (6):

$$\frac{R_D}{R_B} = \frac{PC^2\ (K_{M,S}(PC+99) + 100[S]_T)}{K_{M,S}(PC+99) + 100[S]_T * PC} \quad (10)$$

This is the ratio of rates per volume, which can be converted to the ratio of total activities by dividing by 99.

MATLAB code for the modeling figures are as follows:

Extended Data Figure 1A:

```
[X,Y] = meshgrid(0.1:10,1:100);
Z=((100.*Y)./(99+Y)).*(((Y.*(10+10.*X))./(((10/3).*(Y+99))+(1000.*Y.*X)))+((1./Y).*((99.*(10+10.*
X))./(((10/3).*(Y+99))+1000.*X))));
surf(X,Y,Z);
xlabel('[substrate]/Km')
ylabel('partition coefficient')
zlabel('total S/ total US rate)
```

Extended Data Figure 1A inset:

```
x = 0.1:10;
```

```
y=(5000./149).*(((50.*(10+10.*x))./(((10/3).*(50+99))+(1000.*50.*x)))+((1./50).*((99.*(10+10.*x))./
(((10/3).*(50+99))+1000.*x))));
plot(x,y)
```

Extended Data Figure 1B:

```
[X,Y] = meshgrid(1:100,1:100);
Z=(7700./(((1+(99./Y)).^2).*((70./X)+(700./((1+(99./Y)))))))+(762300./(((99+Y).^2).*((70./X)+(700.
/(99+Y)))));
surf(X,Y,Z);
xlabel('Kmus/Kms')
ylabel('partition coefficient')
zlabel('total S/total US rate)
```

Extended Data Figure 1C:

```
[X,Y] = meshgrid(1:100,1:100);
Z=(77000./(((1+(99./Y)).^2).*((70./X)+(70000./((1+(99./Y)))))))+(7623000./(((99+Y).^2).*((70./X)+
(70000./(99+Y)))));
surf(X,Y,Z);
xlabel('Kmus/Kms')
ylabel('partition coefficient')
zlabel('total S/total US rate)
```

Extended Data Figure 2A:

```
[X,Y] = meshgrid(1:100,1:100);
Z=((7000+(7000.*X))./(100.*(1+(99./Y)).*(70+((7000.*X)./(1+(99./Y))))))+((693000+(693000.*X)).
/(100.*(99+Y).*(70+((7000.*X)./(99+Y)))));
surf(X,Y,Z);
```

```
xlabel('[substrate]/Km')
ylabel('partitioning coefficient')
zlabel('total S/ total US rate)
```

Extended Data Figure 2B:

```
[X,Y] = meshgrid(1:100,1:100);
Z=((7000+(7000.*X))./(10.9.*(1+(99./Y)).*(70+((7000.*X)./(1+(99./Y))))))+((693000+(693000.*X))
./(109.*(99+Y).*(70+((7000.*X)./(99+Y)))));
surf(X,Y,Z);
xlabel('[substrate]/Km')
ylabel('partitioning coefficient')
zlabel('total S/ total US rate)
```

Extended Data Figure 2C:

```
[X,Y] = meshgrid(1:100,1:100);
Z=((7000+(7000.*X))./(1.99.*(1+(99./Y)).*(70+((7000.*X)./(1+(99./Y))))))+((693000+(693000.*X))
./(199.*(99+Y).*(70+((7000.*X)./(99+Y)))));
surf(X,Y,Z);
xlabel('[substrate]/Km')
ylabel('partition coefficient')
zlabel('total S/ total US rate)
```

Extended Data Figure 3A:

```
[X,Y] = meshgrid(0.1:10,1:100);
Z = (Y.^2).*(17+((7000.*X)./(99+Y)))./(17+((7000.*X)./(1+(99./Y))));
surf(X,Y,Z);
```

```
xlabel('[substrate]/Km')
ylabel('partitioning coefficient')
zlabel('droplet/bulk rate)
```

Extended Data Figure 3B:

```
[X,Y] = meshgrid(0.1:10,1:100);
Z=(1./(((1+(99./Y)).^2).*(17+((1700.*X)./(1+(99./Y)))))./((1./(((1+(99./Y)).^2).*(17+((1700.*X)./(1+
(99./Y))))))+(99./(((Y+99).^2).*(17+((1700.*X)./(99+Y))))));
surf(X,Y,Z);
xlabel('[substrate]/Km')
ylabel('partitioning coefficient')
zlabel('total fractional droplet rate)
```