Supplementary Information:

Prenylation of Ras facilitates hSos1-promoted nucleotide exchange, upon Ras binding to the regulatory site

Markos Pechlivanis, Rieke Ringel, Boriana Popkirova and Juergen Kuhlmann

Cloning, expression and purification of hSOS1-fragments:

All hSOS1-fragments were amplified by PCR using Pfu-Polymerase (Stratagene®) and cloned into pProExHTb-expression vectors (Invitrogen®) utilizing the BamHI and XhoI-site (SOSCDC25h: aa 743-1049; SOSCat: aa 564-1049 ; SOS568/1044: aa 568-1044; SOSPH: aa 422-551; SOSHLCat: aa 548-1049; SOSPHCat: aa 422-1049). Introduction of the W729E-mutation into SOSCat-cDNA-sequence was achieved with the Stratagene® Quikchange-Mutagenesis-Kit. CE-sequencing was done by MWG-Biotech® and confirmed correct DNA-sequences and fragment-boundaries. The SOSDHPHCat-fragment was a kind gift from Dr. Holger Sondermann (cloned into the pProExHTb-vector using NcoI and HindIII-restriction sites, respectively). Expression and purification of the hSOS1-fragments was performed essentially as described by Boriack-Sjodin et al., 1998; Margarit et al. 2003, Sondermann et al. 2004 and Chen et al. 1997(*1-4*).

Briefly E.coli BL21DE3 “Rosetta” were grown in LB (+100 mg/l Ampicillin; 30 mg/l Chloramphenicol) at 37°C to an OD600 of ~ 0.5-0.6. Protein expression was initiated by induction with 300 µM IPTG (AppliChem®) at 20-25°C; proteins were expressed at 20°C for 16-18 hours. Bacteria were harvested by centrifugation at 4000x g for 15 minutes, 4°C, washed once with ice-cold PBS-buffer, resuspended in NiNTA-buffer A[1](+ 200 µM PMSF (Serva®) + 5 mg DNase I (Roche®) and lysed with a microfluidizer (Microfluidics®). Cell debris was removed by ultracentrifugation at 100,000x g for 60 minutes at 4°C.

The following protein-purification was performed on an Äkta Prime (Amersham®) at 6-8°C. The supernatant was loaded onto a 20 ml Ni-NTA-column (QIAgen®) preequilibrated with NiNTA-buffer A. After loading the column was washed with 50 column volumes of NiNTA-buffer A, prior to elution of his6-tagged hSOS1-fragments with a linear gradient over 5 column volumes extending from NiNTA-buffer A to

[1] For compositions of buffers, see table S1.

NiNTA-buffer B. hSOS1-fragments were concentrated with Amicon Ultra (Mwco: 10/30K) ultrafiltration-devices (Millipore®), his6-tags were cleaved by incubation with TEV-protease while dialyzing against TEV–buffer. Passing the solution over a second Ni-NTA-column, preequilibrated with NiNTA-buffer C removed TEV-protease and non-cleaved his6-tagged hSOS1-fragments. The flow-through was collected, concentrated by ultrafiltration and passed over a Sephadex G75/G200-Gelfiltration column (Amersham®), preequilibrated with GF-buffer. For the SOSDHPHCat-fragment an additional Mono-Q-Sepharose-column (Amersham®) was used prior to gelfiltration. After loading of the MonoQ-Sepharose, the column was washed with 20 column-volumes of MonoQ-buffer A. A linear gradient over 20 column-volumes extending from MonoQ-buffer A to Mono Q-buffer B eluted the protein. Detailed purification conditions are listed in Table S1.

Cloning, expression and purification of N-Ras-proteins:

N-Ras wt fl. and N-Ras wt Δ181 cDNA-sequences were kindly provided by Dr. Melanie Wagner. N-Ras-cDNA was cloned into a ptac-expression vector using the SmaI and EcoRI restriction sites. Introduction of the Y64A-mutation into N-Ras wt-cDNA-sequence was achieved by using the Stratagene® Quikchange-Mutagenesis-Kit and confirmed by CE-DNA-sequencing (MWG-Biotech®). Expression and purification of N-Ras wt- and N-RasY64A-proteins was done essentially as described by Tucker et al., 1986 (*5*).

Briefly E.coli C600K were grown in LB (+ 100 mg/l Ampicillin; 30 mg/l Kanamycine) at 37°C to an OD600 of 0.5-0.6. Expression was induced by addition of 300 µM IPTG (AppliChem®) at 30°C. Proteins were expressed at 30°C for 16-18 hours. Bacteria were harvested by centrifugation at 4000x g for 15 minutes at 4°C, washed once with ice-cold PBS-buffer, resuspended in DEAE-buffer A (20 mM Tris pH 7.4, 5 mM $MgCl_2$, 2 mM DTE) + 200 µM PMSF (Serva®) + 5 mg DNase I (Roche®) and lysed with a microfluidizer (Microfluidics®). Cell debris was removed by ultracentrifugation at 100,000x g for 60 minutes, 4°C.

Protein-purification was performed on an Äkta Prime (Amersham®) at 6-8°C. The supernatant was loaded onto a 500 ml DEAE-Sepharose-column (Amersham®), preequilibrated with 2 column volumes of DEAE-buffer A (20 mM Tris pH 7.4, 5 mM $MgCl_2$, 2 mM DTE). The column was washed with at least 3 column volumes of DEAE-buffer A and protein elution was achieved by using a linear gradient over 5

column volumes extending from DEAE-buffer A to DEAE-buffer B (20 mM Tris pH 7.4, 5 mM $MgCl_2$, 1 M NaCl, 2 mM DTE). Fractions containing Ras were pooled and concentrated by ammonium-sulphate precipitation; the protein-solution was adjusted stepwise over a period of 1 hour to 3 M $(NH_4)_2SO_4$. The protein-precipitant was collected by centrifugation at 25,000x g for 35 minutes, 4°C and resolved in small volume of gelfiltration-buffer (20 mM Hepes pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT), prior to gelfiltration on a Sephadex G75-column (Amersham®). Fractions containing Ras were combined and concentrated by ultrafiltration using Amicon Ultra ultrafiltration-devices (Millipore®) (Mwco: 10K).

In vitro farnesylation and purification of farnesylated N-Ras-proteins

In vitro-farnesylation of N-Ras wt fl. and N-RasY64A fl.-proteins was performed by incubation of 500 nmol N-Ras, 2.5 mg rat FTase and 1 µmol farnesylpyrophosphate (FPP) in 30 mM Tris pH 7.8, 20 mM KCl, 1 mM MgCl2, 20 µM ZnCl2, 5 mM DTE and 50 µM GDP for 3 h at 30°C (volume: 5 ml). Addition of another 1 µmol FPP to the reaction mixture was repeated three times every 30 minutes for the first 90 minutes. Separation of farnesylated N-Ras from non-farnesylated N-Ras and rat FTase was achieved by TritonX114-extraction, essentially as described by Bordier (*6*). Briefly the reaction-mixture was filled up to a volume of 5 ml with DEAE-buffer A (20 mM Tris pH 7.4, 5 mM $MgCl_2$, 2 mM DTE), incubated on ice for 10 minutes, heated to 37°C for 3 minutes and phases were separated by centrifugation at 4,500x g for 5 minutes and 30°C. Residuals of the farnesylated Ras in the aqueous-phase were extracted from the aqueous-phase by adding 1 ml of 11% Triton X114, 30 mM Tris pH 7.4, 100 mM NaCl, incubating the mixture on ice for 10 minutes, heating it to 37°C for 3 minutes and separating the phases by centrifugation as described above. This step was repeated once. The combined Triton X114-phases were re-extracted twice with 10 ml DEAE-buffer A to remove non-modified N-Ras.

The re-extracted Triton X114-phases were diluted 1:10 with DEAE-buffer A and loaded on a DEAE-Sepharose-column (Amersham), preequilibrated with DEAE-buffer A to remove Triton X114. After washing the column with 10 column volumes DEAE-buffer A, the farnesylated protein was eluted using a linear gradient over 5 column-volumes extending from DEAE-buffer A to DEAE-buffer B (20 mM Tris pH 7.4, 5 mM $MgCl_2$, 1 M NaCl, 2 mM DTE). Combined fractions containing the Ras-lipoprotein were concentrated by ultrafiltration using Amicon Ultra (Mwco: 10K) ultrafiltration-devices (Millipore®).

Preparation of small unilamelar vesicles (SUV)

Small unilamelar vesicles (SUV) were prepared by solving the required amounts of lipids (Avanti Polar Lipids®) in $CHCl_3$, in case of lipid mixtures by mixing the desired stock solutions, and subsequent evaporation of the solvent in a table top concentrator (Speed Vac concentrator, Eppendorf®). Lipids were afterwards solved in HBS-Mg-buffer (20 mM Hepes pH 7.4, 5 mM $MgCl_2$, 150 mM NaCl), vortexed, and sonicated in a Branson Sonifier 450-ultrasonicator using the microtip [Output 6 / Duty cycle 70%] until the solution becomes clear. Sonicated lipid solutions were frozen in liquid nitrogen for 1 minute then heated to 50°C for 3 minutes. This procedure was repeated 40 times. Prior to use SUV solutions were centrifuged at room temperature and 17,000x g for 5 minutes.

$SOS^{DHPHCat}$-promoted nucleotide exchange in presence of PIP_3-containing vesicles

Experiments were conducted at 20°C in HBS-Mg-buffer (20 mM Hepes pH 7.4, 5 mM $MgCl_2$, 150 mM NaCl) plus the indicated amounts of small unilamelar vesicles composed of POPC (1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-Phosphocholine) or POPC and PIP_3 (1,2-Dioleoyl-*sn*-Glycero-3-Phosphoinositol-3,4,5-trisphosphate). The concentration of vesicles used in the experiments was 100 µM POPC or 100 µM POPC doped with 1 mol% of PIP_3, respectively. Protein-concentrations are indicated in the figure legend.

Measurements were performed in Jobin Yvon® Fluoromax I or II fluorescence-spectrometers in 1 ml quartz cuvettes. Mant-fluorophore was excited at 366 nm and emission was collected at 450 nm. Nucleotide exchange reactions were started by addition of 240 µM GDP (final concentration).

Nucleotide exchange reactions in presence of 10 µM of a water soluble form of PIP_3 (1,2-Dioctanoyl-*sn*-Glycero-3-[Phosphoinositol-3,4,5-Trisphosphate]) (*7*) were done in the absence of lipid-vesicles, instrumental parameters as described above. Rates were determined by monoexponential curve fitting ($y = y_o + ae^{-kt}$) using the program Sigma-Plot, Systat-Software®.

Table S1: Purification Strategy for SOS-constructs

Figure S1: Purity of constructs used in the study was analyzed by SDS-PAGE and ESI mass spectrometry.

Figure S2: $SOS^{DHPHCat}$-promoted nucleotide-exchange-assay of N-Ras wt fl*mGDP (1 µM) in presence of PIP_3-doped lipid-vesicles. Final concentration of $SOS^{DHPHCat}$ 500 nM, N-RasY64A±Far*GppNHp 1 µM, POPC/PIP3 100 µM (1 µM PIP3). Presence of PIP_3 stimulates allosteric regulated $SOS^{DHPHCat}$ promoted nucleotide exchange on N-Ras wt fl.

Figure S3: SOS^{PH}-competition assay. SOS^{PH} was used in 30fold molar excess over SOS^{PHCat} (1 µM); RasY64A (0.5 µM). The isolated PH-domain of hSOS1 could not specifically compete for farnesylated N-RasY64A in a SOS^{PHCat}-promoted nucleotide-exchange-assay or significantly reduce SOS^{PHCat}-promoted nucleotide exchange to levels without allosteric regulation.

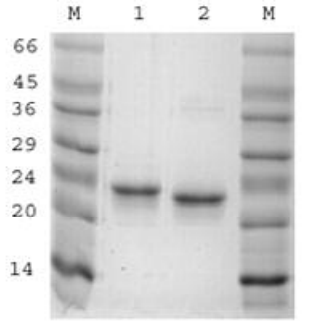
Figure S4: CD-Spectrum of $hSOS1^{PH}$-domain (45 µM) in 12.5 mM Na_2HPO_4-buffer pH 7.5 (10fold accumulated). Measurements were conducted with a Jasco J710-CD-spectrometer (Japan Spectroscopic Co. LTD, Tokio, Japan) in 0.01 mm-quartz-cuvettes (0.5 nm steps, 20 nm / min^{-1}, 1 sec. integration).
CD-spectroscopy revealed folding of SOS^{PH}.

Table S1: Purification Strategy for SOS-constructs

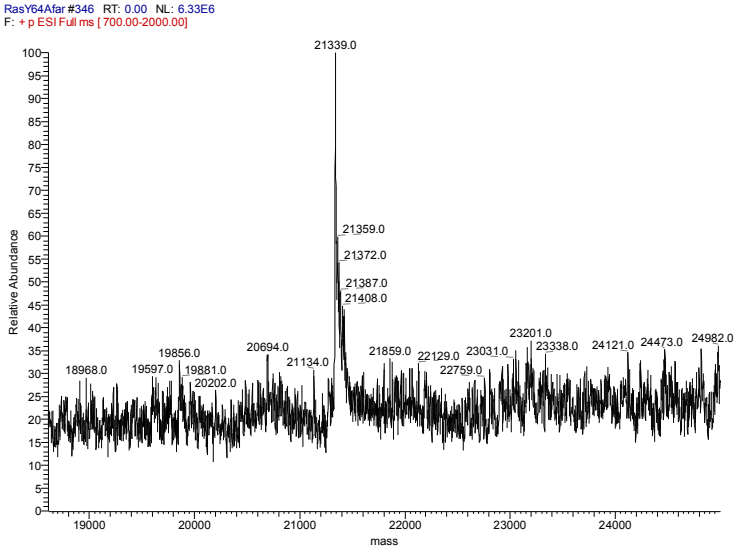
	SOSCDC25 / SOSPH	**SOSCat**	**SOS568/1044**	**SOSPHCat**	**SOSDHPHCat**	**SOSHLCat**
NiNTA-buffer A	20 mM Tris pH 8.0 / 300 mM NaCl / 1 mM β-ME / 25 mM Imidazol	20 mM Tris pH 8.0 / 250 mM NaC l/ 2 mM β-ME / 30 mM Imidazol	20 mM Tris pH 8.0 / 250 mM NaC l/ 2 mM β-ME / 30 mM Imidazol	20 mM Tris pH 8.0 / 300 mM NaCl / 1 mM β-ME / 25 mM Imidazol	20 mM Tris pH 8.0 / 500 mM NaCl / 2 mM β-ME / 20 mM Imidazol	20 mM Tris pH 8.0 / 250 mM NaCl / 2 mM β-ME / 30 mM Imidazol
NiNTA -buffer B	20 mM Tris pH 8.0/ 500 mM NaCl / 1 mM b-ME / 500 mM Imidazol	20 mM Tris pH 8.0 / 250 mM NaCl / 2 mM β-ME / 500 mM Imidazol	20 mM Tris pH 8.0 / 250 mM NaCl / 2 mM β-ME / 500 mM Imidazol	20 mM Tris pH 8.0/ 500 mM NaCl/ 1 mM β-ME/ 500 mM Imidazol	20 mM Tris pH 8.0/ 500 mM NaCl/ 2 mM β-ME/ 500 mM Imidazol	20 mM Tris pH 8.0 / 250 mM NaCl / 2 mM β-ME / 500 mM Imidazol
NiNTA -buffer C	20 mM Tris pH 8.0 / 300 mM NaCl	20 mM Tris pH 8.0 / 150 mM NaCl	20 mM Tris pH 8.0 / 150 mM NaCl	20 mM Tris pH 8.0 / 300 mM NaCl	20 mM Tris pH 8.3 / 50 mM NaCl	20 mM Tris pH 8.0 / 200 mM NaCl
TEV-buffer	20 mM Tris pH 8.0 / 300 mM NaCl / 1 mM DTT / 0.5 mM EDTA	20 mM Tris pH 8.0 / 100 mM NaCl / 1 mM DTT / 0.5 mM EDTA	20 mM Tris pH 8.0 / 100 mM NaCl / 1 mM DTT / 0.5 mM EDTA	20 mM Tris pH 8.0 / 300 mM NaCl / 1 mM DTT / 0.5 mM EDTA	20 mM Tris pH 8.3 / 50 mM NaCl / 1 mM DTT / 0.5 mM EDTA	20 mM Tris pH 8.0 / 100 mM NaCl / 1 mM DTT / 0.5 mM EDTA
MonoQ-buffer A					20 mM Tris pH 8.3 / 50 mM NaCl / 1 mM DTT	
MonoQ-buffer B					20 mM Tris pH 8.3 / 500 mM NaCl / 1 mM DTT	
GF-buffer	20 mM Hepes 8.0 / 300 mM NaCl / 5 mM $MgCl_2$ / 1 mM DTT	20 mM Hepes 7.4 / 150 mM NaCl / 5 mM $MgCl_2$ / 1 mM DTT	20 mM Tris pH 8.0 / 200 mM NaCl / 1 mM DTT	20 mM Hepes 7.4 / 300 mM NaCl / 5 mM $MgCl_2$ / 1 mM DTT	20 mM Hepes 7.4 / 300 mM NaCl / 5 mM $MgCl_2$ / 1 mM DTT	20 mM Hepes 7.4 / 150 mM NaCl / 5 mM $MgCl_2$ / 1 mM DTT

Figure S1:

Purity of *in vitro* farnesylated N-Ras-proteins / ESI-MS:



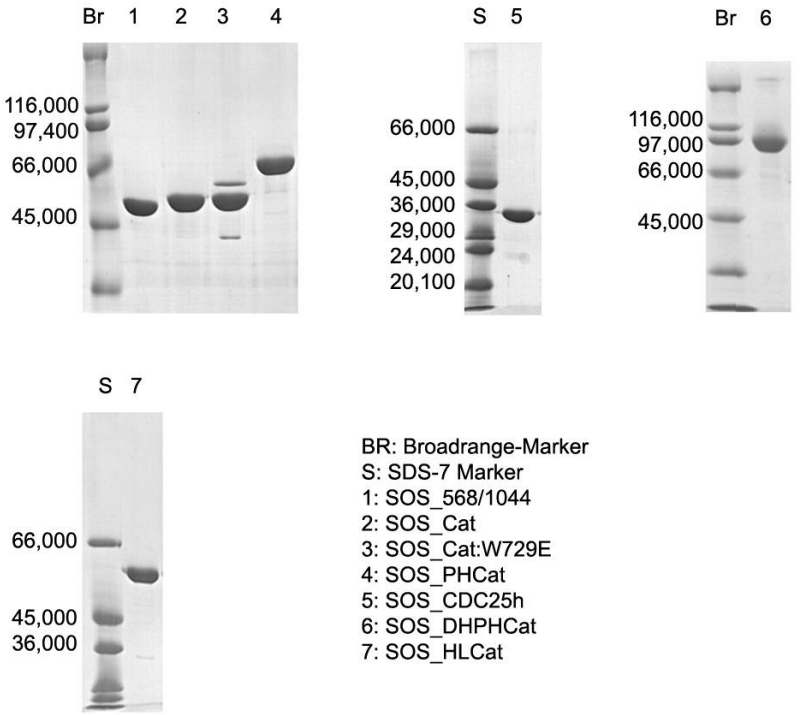
M: SDS7
1: N-RasY64A f.l.
2: N-RasY64A_far_GppNHp



Expected Mass of in vitro-farnesylated N-RasY64A: 21341 Da

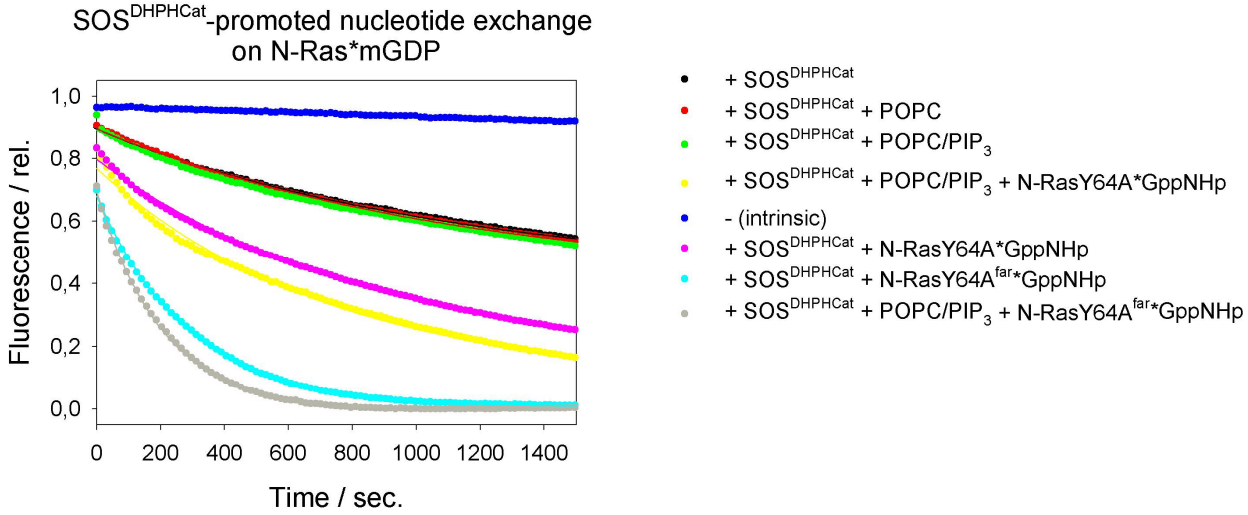
Actual mass of in vitro-farnesylated N-RasY64A: 21339 Da

Purity of hSOS1-constructs:



BR: Broadrange-Marker
S: SDS-7 Marker
1: SOS_568/1044
2: SOS_Cat
3: SOS_Cat:W729E
4: SOS_PHCat
5: SOS_CDC25h
6: SOS_DHPHCat
7: SOS_HLCat

Figure S2:


SOS^DHPHCat-promoted nucleotide exchange on N-Ras*mGDP
Fluorescence / rel.
1,0
0,8
0,6
0,4
0,2
0,0
0
200
400
600
800
1000
1200
1400
Time / sec.
+ SOS^DHPHCat
+ SOS^DHPHCat + POPC
+ SOS^DHPHCat + POPC/PIP3
+ SOS^DHPHCat + POPC/PIP3 + N-RasY64A*GppNHp
- (intrinsic)
+ SOS^DHPHCat + N-RasY64A*GppNHp
+ SOS^DHPHCat + N-RasY64A^far*GppNHp
+ SOS^DHPHCat + POPC/PIP3 + N-RasY64A^far*GppNHp

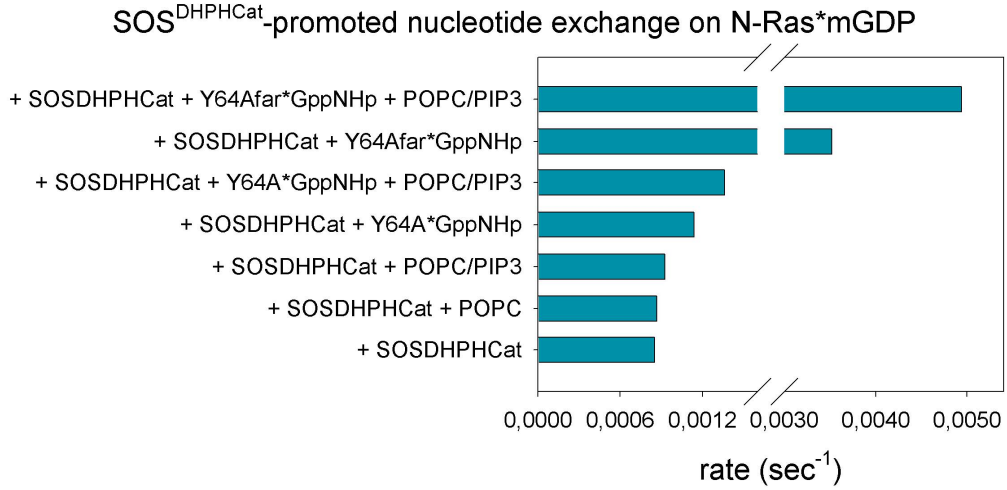

SOS^DHPHCat-promoted nucleotide exchange on N-Ras*mGDP
+ SOSDHPHCat + Y64Afar*GppNHp + POPC/PIP3
+ SOSDHPHCat + Y64Afar*GppNHp
+ SOSDHPHCat + Y64A*GppNHp + POPC/PIP3
+ SOSDHPHCat + Y64A*GppNHp
+ SOSDHPHCat + POPC/PIP3
+ SOSDHPHCat + POPC
+ SOSDHPHCat
0,0000
0,0006
0,0012
0,0030
0,0040
0,0050
rate (sec^-1)

Figure S3:

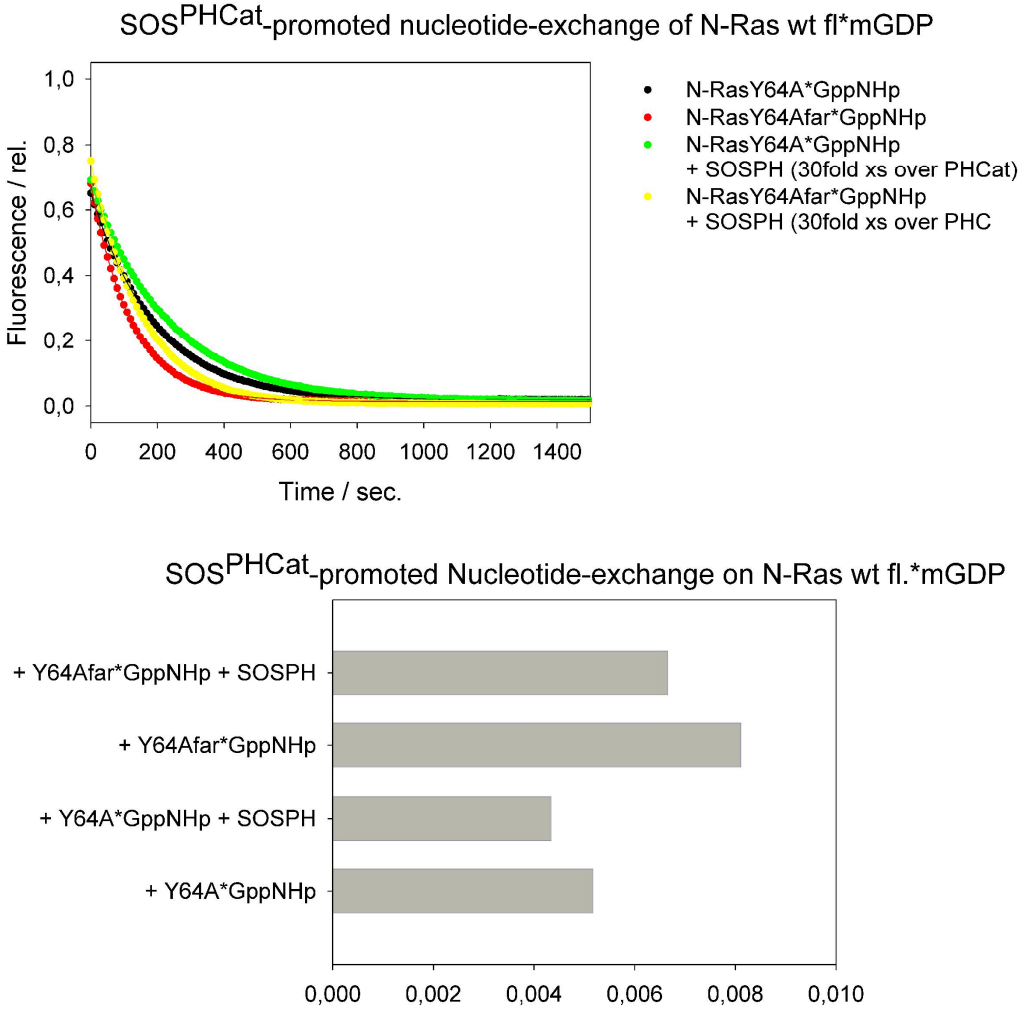
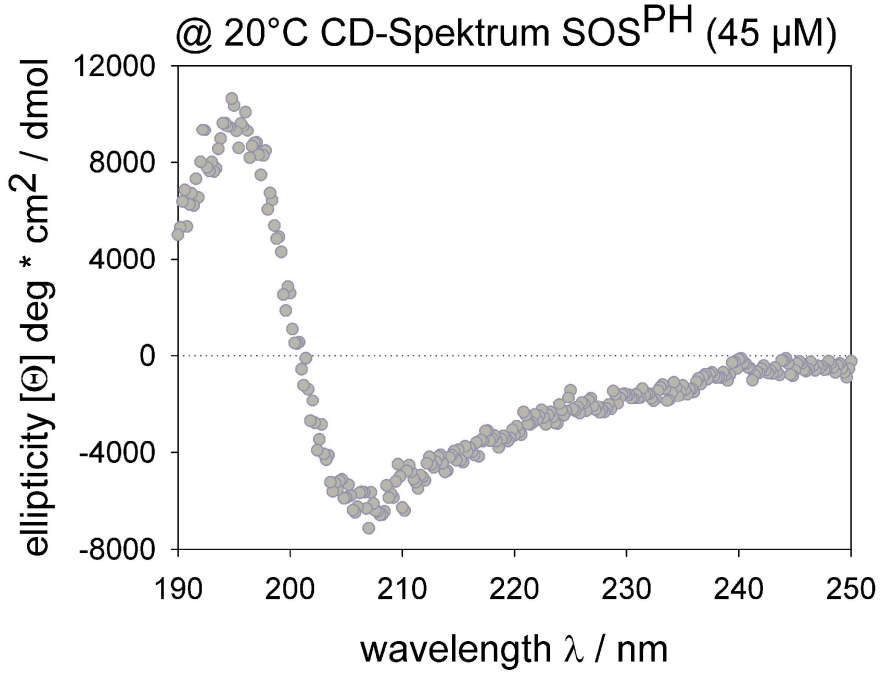


Figure S4:



References

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