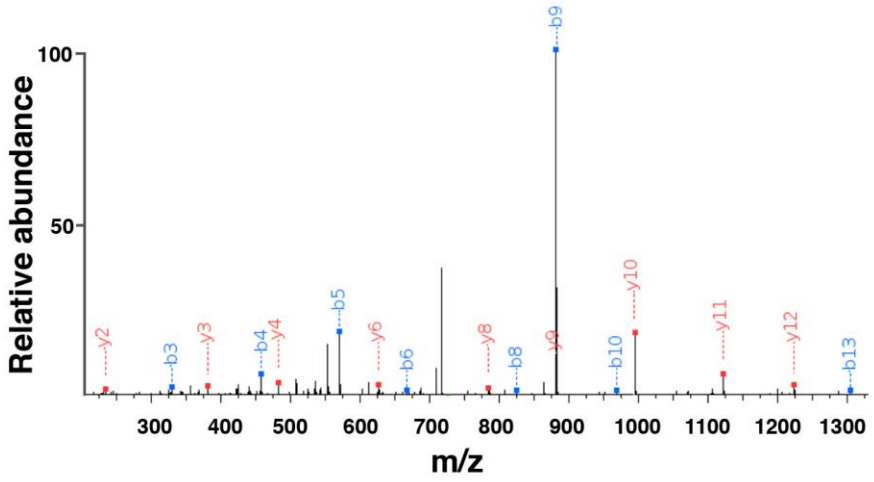
NW9 (amino acid sequence after TEV cleavage)

GSTFSKLREQLGPVTQEFWDNLEKETEGLRQEMSKDLEEVKAKVQPYLDDFQKKWQEEMELYRQKVEPLGEEMRDRARAHVDALR
THLAPYSDELRQRLAARLEALKENGGARLAEYHAKATEHLSTLSEKAKPALEDLRQGLLPVLESFKVSFLSALEEYTKK**LNTQLPGT**G
AAALEHHHHHH

Peptide sequence

LNTQLPGTGSTFSK



seq	#	b	y	#
L	1	114.091	---	14
N	2	228.134	1337.670	13
T	3	329.182	1223.627	12
Q	4	457.241	1122.579	11
L	5	570.325	994.520	10
P	6	667.377	881.436	9
G	7	724.399	784.384	8
T	8	825.446	727.362	7
G	9	882.468	626.314	6
S	10	969.500	569.293	5
T	11	1070.548	482.261	4
F	12	1217.616	381.213	3
S	13	1304.648	234.145	2
K	14	----	147.113	1

Supplementary Figure 1

Characterization of cNW9 by MS/MS confirms the ligation of the C terminus to the N terminus.

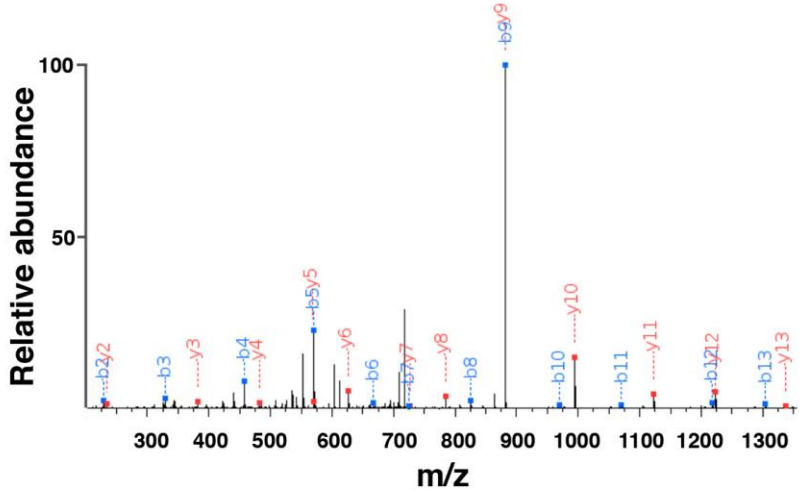
MS/MS spectrum of a tryptic fragment of cNW9 showing the ligation of the C-terminal motif (**LNTQLPGT**G-His_6) to the N-terminal residues (**GSTFSK**). Expected masses for b and y ions along with the peptide sequence are listed in the table. The b and y ions that were identified in the MS/MS spectrum are highlighted in blue and red. The full amino acid sequence of linear NW9 is shown at the top.

NW30 (amino acid sequence after TEV cleavage)

GSTFSKLREQLGPVTQEFWDNLEKETEGLRQEMSKDLEEVKAKVQPYLDDFQKKWQEEMELYRQKVEPLRAELQEGARQKLHELQ
EKLSPLGEEMRDRARAHVDALRTHLAPYSDELRQRLAARLEALKENGGARLAEYHAKATEHLSTLSEKAKPALEDLRQGLLPVLESFK
VSFLSALEEYTKKLNTQGTPVTQEFWDNLEKETEGLRQEMSKDLEEVKAKVQPYLDDFQKKWQEEMELYRQKVEPLRAELQEGAR
QKLHELQEKLSPLGEEMRDRARAHVDALRTHLAPYSDELRQRLAARLEALKENGGARLAEYHAKATEHLSTLSEKAKPALEDLRQGL
LPVLESFKVSFLSALEEYTKKLNTQGTPVTQEFWDNLEKETEGLRQEMSKDLEEVKAKVQPYLDDFQKKWQEEMELYRQKVEPLRA
ELQEGARQKLHELQEKLSPLGEEMRDRARAHVDALRTHLAPYSDELRQRLAARLEALKENGGARLAEYHAKATEHLSTLSEKAKPAL
EDLRQGLLPVLESFKVSFLSALEEYTKK**LNTQLPGT**GAAALEHHHHHH

Peptide sequence

LNTQLPGTGSTFSK



seq	#	b	y	#
L	1	114.091	---	14
N	2	228.134	1337.670	13
T	3	329.182	1223.627	12
Q	4	457.241	1122.579	11
L	5	570.325	994.520	10
P	6	667.377	881.436	9
G	7	724.399	784.384	8
T	8	825.446	727.362	7
G	9	882.468	626.314	6
S	10	969.500	569.293	5
T	11	1070.548	482.261	4
F	12	1217.616	381.213	3
S	13	1304.648	234.145	2
K	14	----	147.113	1

Supplementary Figure 2

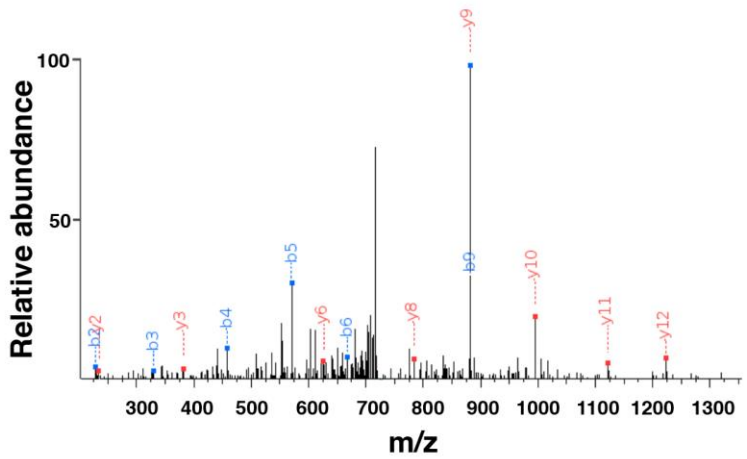
Characterization of cNW30 by MS/MS confirms the ligation of the C terminus to the N terminus.

MS/MS spectrum of a tryptic fragment of cNW30 showing the ligation of the C-terminal motif (**LNTQLPGT**G-His_6) to the N-terminal residues (**GSTFSK**). Expected masses for b and y ions along with the peptide sequence are listed in the table. The b and y ions that were identified in the MS/MS spectrum are highlighted in blue and red. The full amino acid sequence of linear NW30 is shown at the top.

NW50 (amino acid sequence after TEV cleavage)

QGSTFSKLREQLGPVTQEFWDNLEKETEGLRQEMSKDLEEVKAKVQPYLDDFQKKWQEEMELYRQKVEPLRAELQEGARQKLHEL
QEKLSPLGEEMRDRARAHVDALRTHLAPYSDELRQRLAARLEALKENGGARLAEYHAKATEHLSTLSEKAKPALEDLRQGLLPVLES
FKVSFLSALEEYTKKLNTQGTPVTQEFWDNLEKETEGLRQEMSKDLEEVKAKVQPYLDDFQKKWQEEMELYRQKVEPLRAELQEGA
RQKLHELQEKLSPLGEEMRDRARAHVDALRTHLAPYSDELRQRLAARLEALKENGGARLAEYHAKATEHLSTLSEKAKPALEDLRQG
LLPVLESFKVSFLSALEEYTKKLNTQGTPVTQEFWDNLEKETEGLRQEMSKDLEEVKAKVQPYLDDFQKKWQEEMELYRQKVEPLR
AELQEGARQKLHELQEKLSPLGEEMRDRARAHVDALRTHLAPYSDELRQRLAARLEALKENGGARLAEYHAKATEHLSTLSEKAKPA
LEDLRQGLLPVLESFKVSFLSALEEYTKKLNTQGTPVTQEFWDNLEKETEGLRQEMSKDLEEVKAKVQPYLDDFQKKWQEEMELYR
QKVEPLRAELQEGARQKLHELQEKLSPLGEEMRDRARAHVDALRTHLAPYSDELRQRLAARLEALKENGGARLAEYHAKATEHLSTL
SEKAKPALEDLRQGLLPVLESFKVSFLSALEEYTKKLNTQGTPVTQEFWDNLEKETEGLRQEMSKDLEEVKAKVQPYLDDFQKKWQE
EMELYRQKVEPLRAELQEGARQKLHELQEKLSPLGEEMRDRARAHVDALRTHLAPYSDELRQRLAARLEALKENGGARLAEYHAKA
TEHLSTLSEKAKPALEDLRQGLLPVLESFKVSFLSALEEYTKK**LNTQLPGT**GAAALEHHHHHH

Peptide sequence
LNTQLPGTGSTFSK

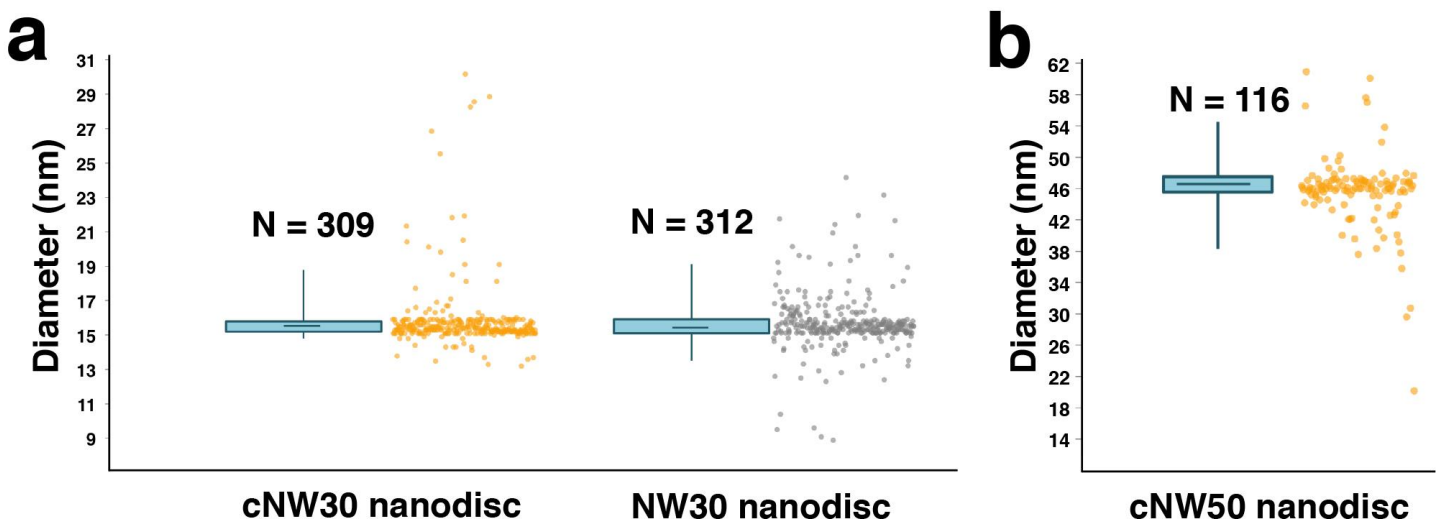


seq	#	b	y	#
L	1	114.091	---	14
N	2	228.134	1337.670	13
T	3	329.182	1223.627	12
Q	4	457.241	1122.579	11
L	5	570.325	994.520	10
P	6	667.377	881.436	9
G	7	724.399	784.384	8
T	8	825.446	727.362	7
G	9	882.468	626.314	6
S	10	969.500	569.293	5
T	11	1070.548	482.261	4
F	12	1217.616	381.213	3
S	13	1304.648	234.145	2
K	14	----	147.113	1

Supplementary Figure 3

Characterization of cNW50 by MS/MS confirms the ligation of the C terminus to the N terminus.

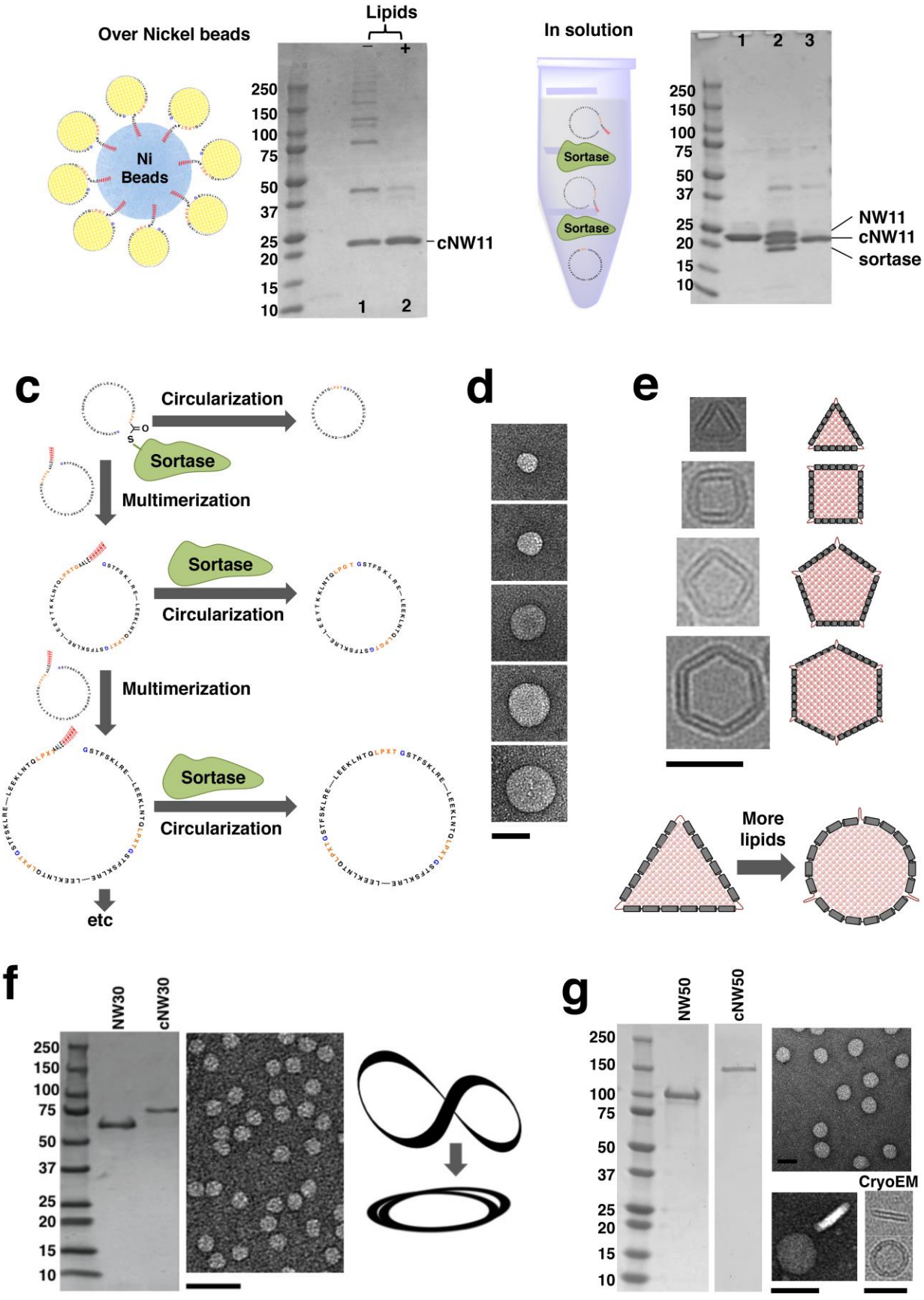
Spectrum of a tryptic fragment of cNW50 showing the ligation of the C-terminal motif (**LNTQLPGT**G-His_6) to the N-terminal residues (**GSTFSK**). Expected masses for b and y ions along with the peptide sequence are listed in the table. The b and y ions that were positively identified in the MS/MS spectrum are highlighted in blue and red. The amino acid sequence of linear NW50 (after TEV cleavage) is shown at the top.



Supplementary Figure 4

Diameter distribution for nanodiscs assembled using cNW30, NW30 and cNW50 proteins.

(**a**) Diameter distribution for nanodiscs assembled using cNW30 (left) and NW30 (right). There is less variance in the lengths of cNW30 nanodiscs compared to NW30 nanodiscs (p=0.002). (**b**) Diameter distribution for nanodiscs made using cNW50. In the box-and-whisker plots, center lines show the means; box limits indicate the 25th and 75th percentiles; whiskers extend to the 5th and 95th percentiles. Raw data (jittered along x for clarity) are shown next to its representative plot. There is less variance in the lengths of cNW30 compared to NW30 nanodiscs (p = 0.002). Measurements were performed with the ImageJ software[30].



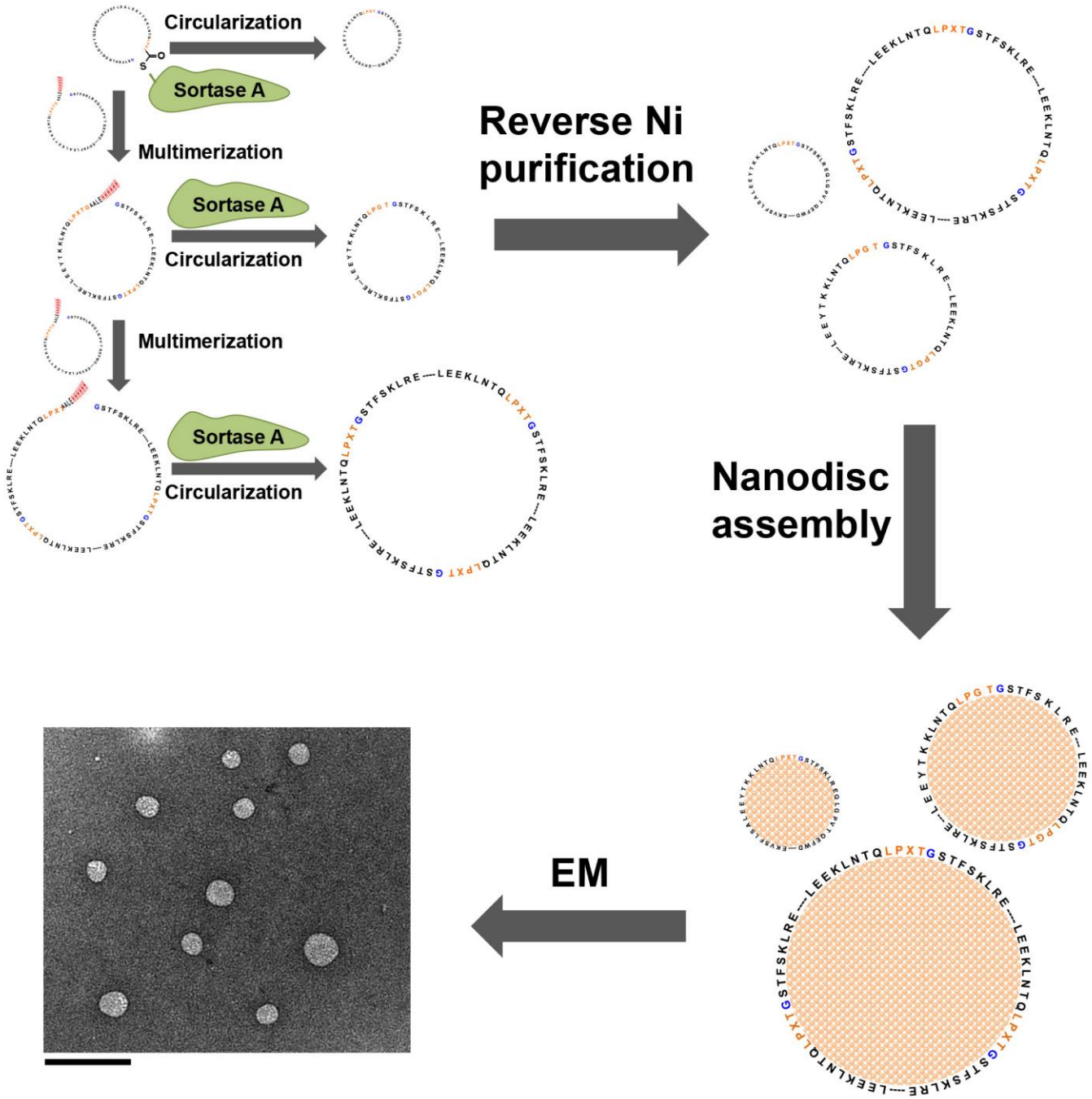
Nature Methods: doi:10.1038/nmeth.4079

Supplementary Figure 5

Producing covalently circularized nanodiscs with defined sizes and shapes.

(**a**) SDS-PAGE gel showing the final products after circularization over Ni beads with or without lipids. (**b**) Adding evolved sortase to diluted NW11 solution results mainly in NW11 circularization. Sortase was added to a dilute NW11 solution ([NW11] < 15 μM) to suppress linking two or more copies of NW11. (**c**) Adding evolved sortase to a concentrated NW11 solution leads to multimerization followed by circularization. (**d**) Negative-stain EM images showing large nanodiscs made using oligomeric circularized NW11. (**e**) Cryo-EM images of individual nanodiscs with different shapes. A triangle-shaped nanodisc is made using 3 copies of NW11 covalently linked together and circularized. Adding more lipids can change the shape back to a circular disc. Right: potential molecular arrangements of NW11 molecules around the differently shaped nanodiscs. (**f**) SDS-PAGE analysis of NW30 before and after circularization. Circularized NW30 migrates slower than its linear form. Right: negative-stain EM analysis of the nanodiscs made using circularized NW30 (cNW30) shows the formation of ~15 nm nanodiscs. (**g**) SDS-PAGE analysis of NW50 before and after circularization. Uncropped images are shown in **Supplementary note 2**. Right: negative-stain EM analysis of the nanodiscs made using circularized NW50 (cNW50) shows the formation of ~50 nm nanodiscs. Bottom: Negative-stain (left) and cryo-EM images (right) for individual nanodiscs showing top and side views. Scale bar, 50 nm.

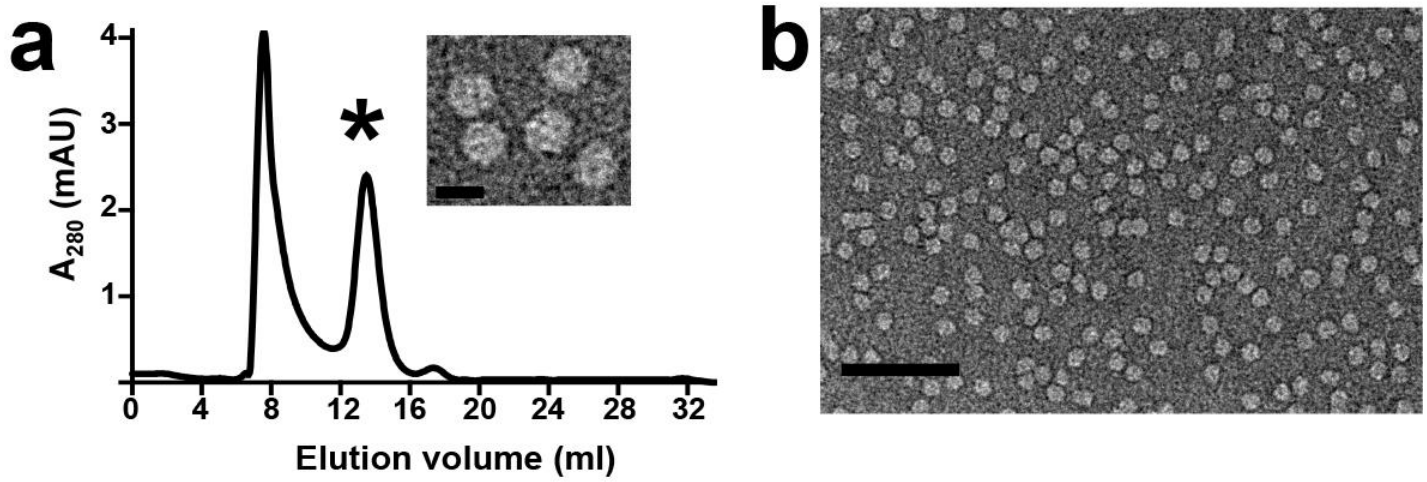
NW30 and NW50 are sortaggable variants that we designed to assemble ~ 30- and 50-nm nanodiscs, respectively. Surprisingly, the cNW30 forms very homogenous ~15 nm instead of 30 nm nanodiscs On the other hand, as predicted, circularized NW50 assembled mainly ~ 50 nm nanodiscs.



Supplementary Figure 6

The oligomeric, circularized species containing variable numbers of NW11 assemble into nanodiscs of various sizes.

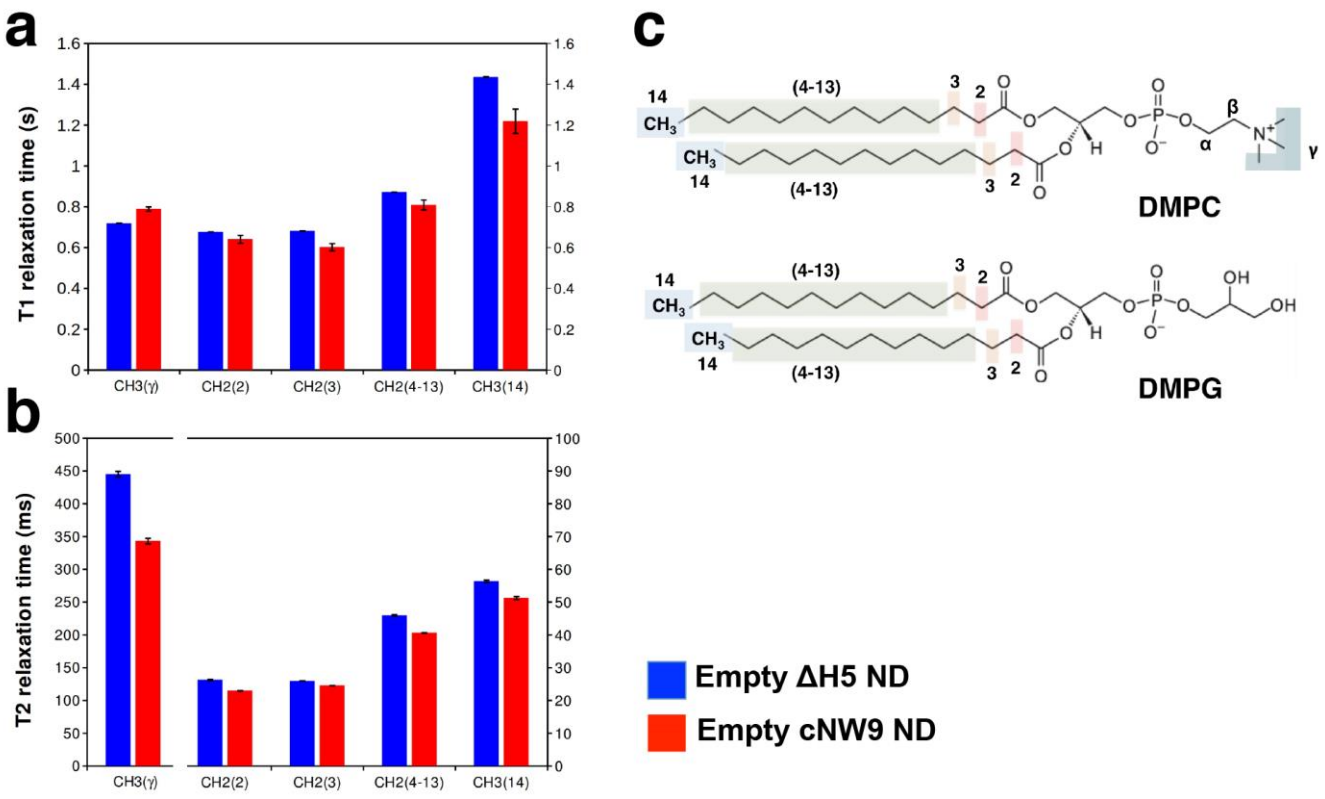
Adding evolved sortase to concentrated NW11 solution leads to multimerization of NW11 followed by circularization. **Bottom**: Negative-stain EM image showing large nanodiscs made using oligomeric, circularized NW11. Scale bar, 100 nm.



Supplementary Figure 7

Analysis of nanodiscs assembled using a cNW30:lipid ratio of 1:1000.

(**a**) Size-exclusion chromatography (SEC) analysis of the assembled nanodiscs. The SEC column (Superose 6 10/300) was equilibrated in 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.5 mM EDTA. The peak (labeled with *) was collected and analyzed by negative-stain EM (**b**). Scale bars, 15 nm (**a**) and 100 nm (**b**)

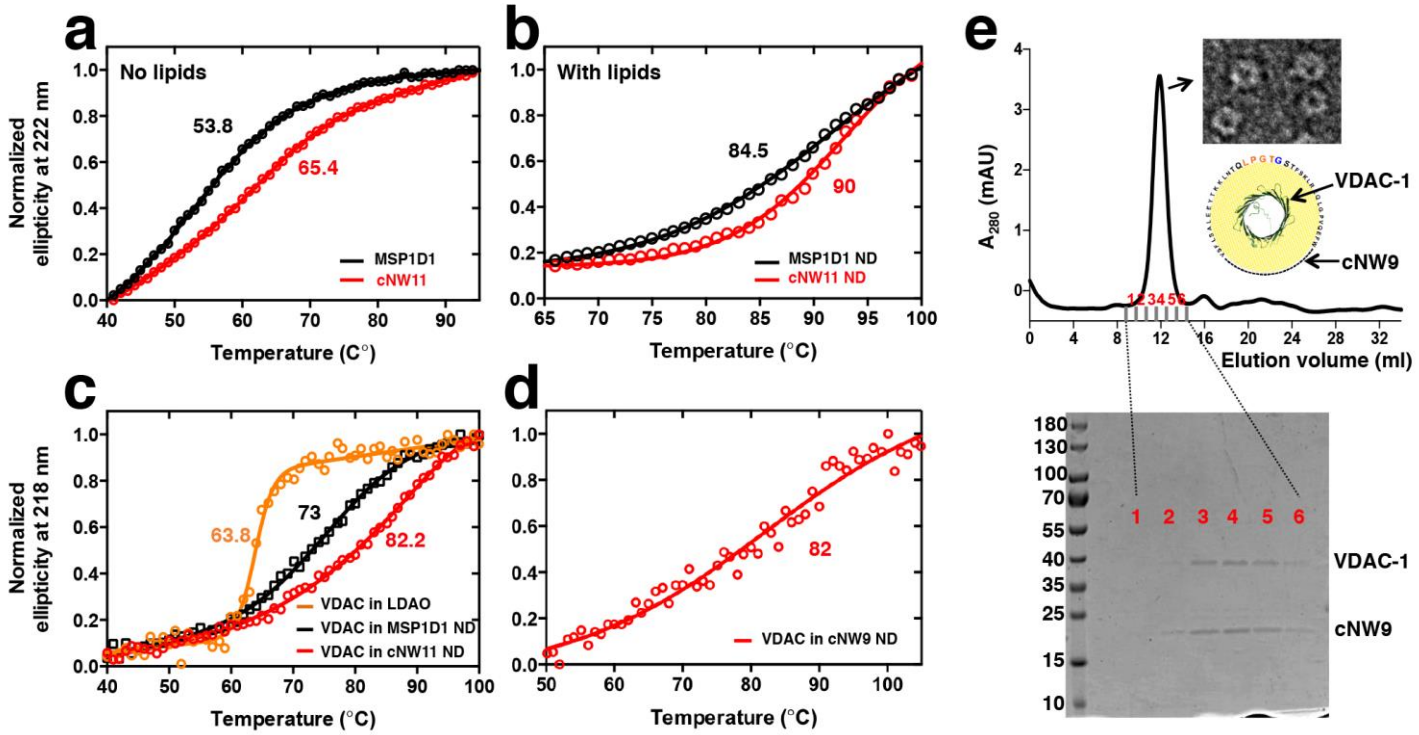


Supplementary Figure 8

Relaxation analysis of DMPC/DMPG (3:1) phospholipids in cNW and ΔH5 nanodiscs.

(**a**) ^{1}H-NMR T1 relaxation times of DMPC/DMPG lipid signals in empty ΔH5 (blue) and cNW9 (red) nanodiscs. (**b**) ^{1}H-NMR T2 relaxation times of DMPC/DMPG lipids signals in empty ΔH5 (blue) and cNW9 (red) nanodiscs (**c**) Chemical structure of DMPC and DMPG.

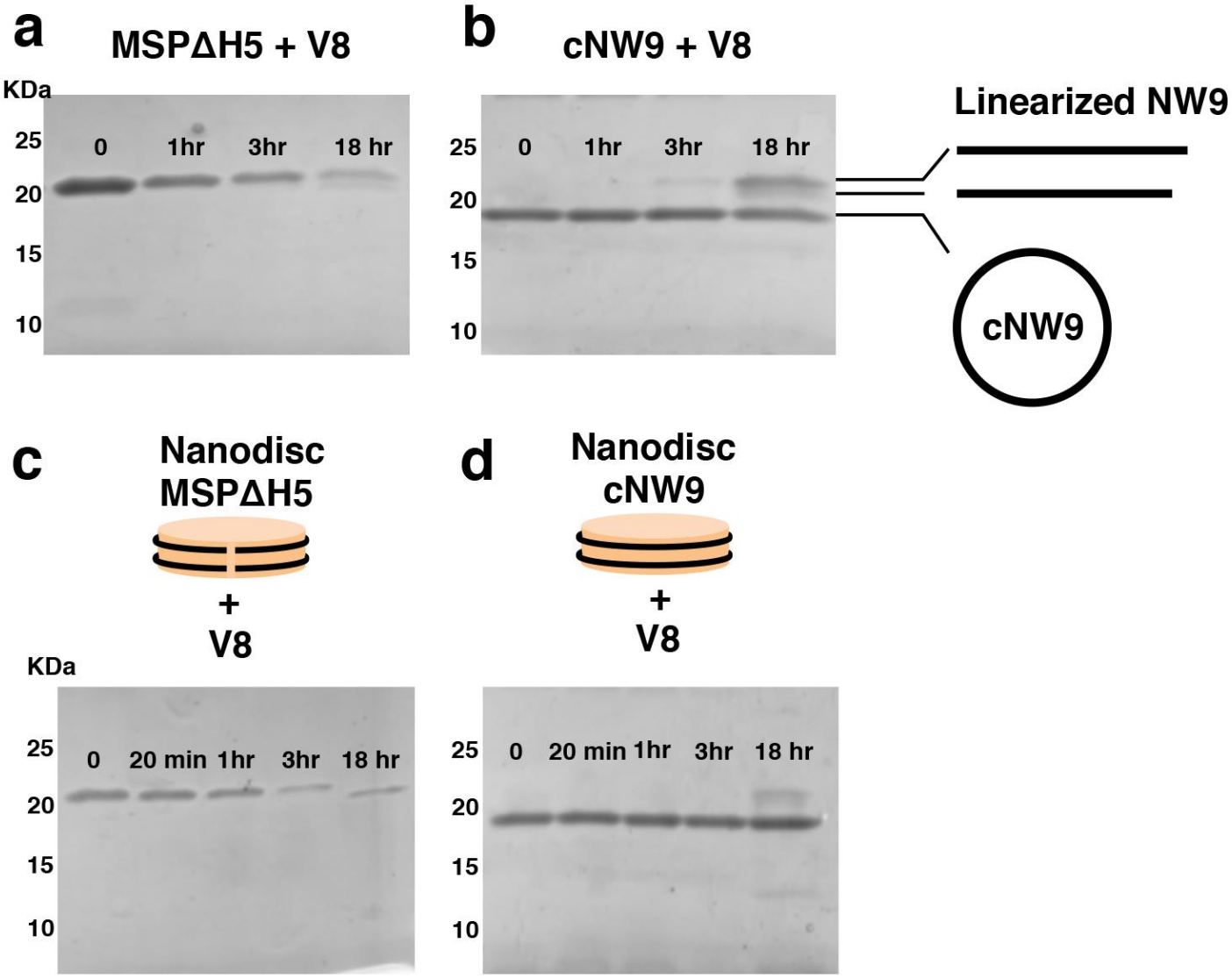
Except the DMPC γ methyl group located outside the bilayer region, the lipids in cNW9 show shorter T1 and T2 times consistent with smaller nanodisc size and dynamics indicative of the more restrictive circularized cNW9 belt. Measurements were acquired at 45ºC on a Bruker 500-MHz spectrometer using inversion recovery for T1 and CPMG refocusing train for T2.



Supplementary Figure 9

Covalent circularization enhances thermal stability of MSPs and embedded VDAC-1.

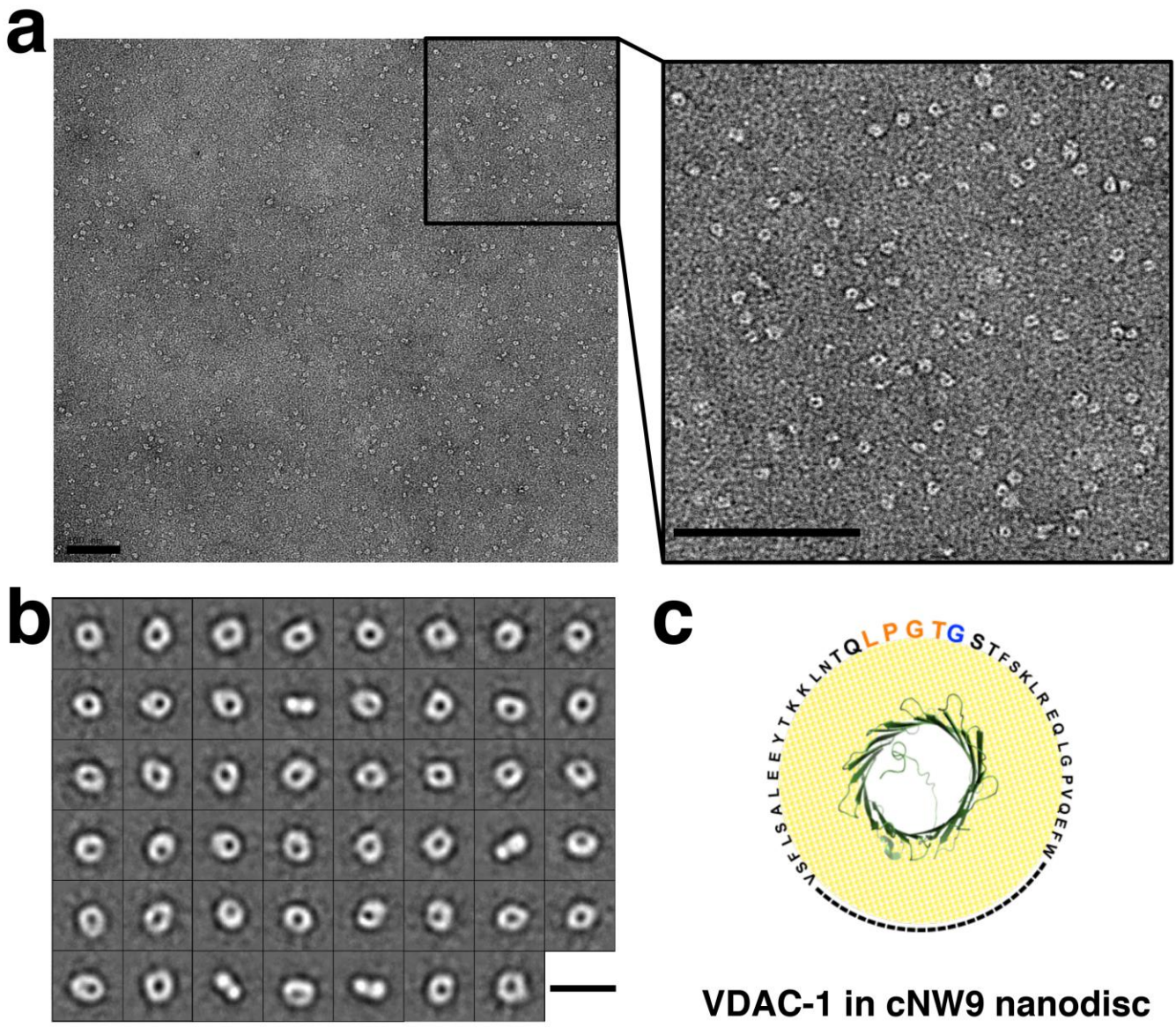
Thermal unfolding of MSP1D1 (black) and cNW11 (red) without **(a)** and with lipids (**b**) followed by circular dichroism (CD) spectroscopy at 222 nm, the wavelength most characteristic of helical secondary structure. A lipid mixture of POPC:POPG at a molar ratio of 3:2 was used to generate nanodiscs. (**c**) Reconstitution into MSP1D1 nanodiscs increases the melting temperature, T_m, of VDAC1 by 9.2°C over that of VDAC1 in an LDAO micelle environment, and covalent circularization of the scaffold protein (cNW11) raises the T_m by an additional 9.2°C. Thermal unfolding of human VDAC-1 was followed by CD spectroscopy at 218 nm, the wavelength most characteristic of β-sheet secondary structure. Orange: VDAC1 in 0.1% LDAO, black: VDAC1 reconstituted into conventional nanodiscs (assembled using MSP1D1), and red: VDAC1 reconstituted into circularized nanodiscs (assembled using cNW11). Nanodiscs were made with a 3:2 POPC:POPG mixture. (**d**) Thermal unfolding of VDAC1 reconstituted into circularized nanodiscs (assembled using cNW9) followed by CD spectroscopy at 218 nm. Nanodiscs were made with POPC/POPG lipids at a molar ratio of 3:2. All samples were in 20 mM Tris-HCl, pH 7.5, 100 mM NaCl. **(e)** Analysis of the VDAC1 nanodisc assembly reaction. Top: size-exclusion chromatography and negative-stain EM of VDAC1 in cNW9 nanodiscs. The negative-stain EM shows nanodiscs containing a single channel. The stain-filled channels appear as dark spots inside the nanodiscs. Bottom: SDS-PAGE analysis of the nanodisc assembly. Fractions 1-6 were collected and analyzed.



Supplementary Figure 10

Covalent circularization stabilizes MSPs against digestion by V8 protease without and with lipids.

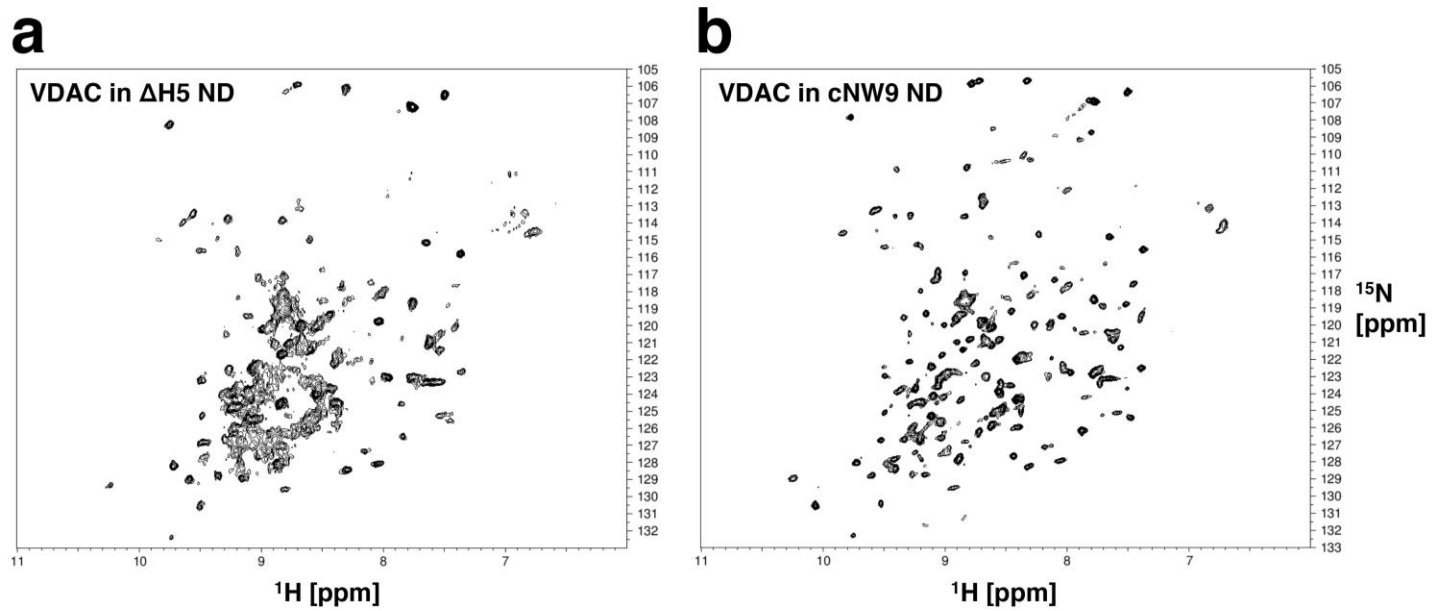
(**a, b**) SDS-PAGE analysis of the proteolysis of lipid-free MSPΔH5 and cNW9. Samples were treated with V8 protease for 0 min (before addition of V8), and for 1, 3 and 18 hours. Lanes are labeled with times of protease treatment. Proteolysis was performed in 20 mM Tris-HCl, pH 7.5, 100 mM NaCl at 37°C and using a protein:protease ratio (w/w) of 1000:1. Treatment of MSPΔH5 with V8 resulted in the appearance of a large peptide, which is close in size to MSPΔH5 and is not discernible until about 18 hours after addition of V8; this peptide may be generated earlier but is not visible due to the low amount or overlap with uncleaved MSPΔH5. The intensity of the MSPΔH5 band decreased by 75% after 3 hours and by 93% after 18 hours. On the other hand, the intensity of the cNW9 band decreased by only 20% after 18 hours. A band that corresponds to linearized NW9 was observed after 3 hours and increased in intensity after 18 hours. (**c, d**) SDS-PAGE analysis of the proteolysis of nanodiscs assembled with MSPΔH5 and cNW9. Samples were treated with V8 protease for 0 min (before addition of V8), and for 20 min, 1, 3 and 18 hours. Proteolysis was performed at 37°C at pH 7.5 in 20 mM Tris-HCl, 100 mM NaCl using a 100:1 protein:protease (w/w). The band intensity of MSPΔH5 decreased by 81% after 3 hours. There was no decrease in the cNW9 band intensity up to 3 hours. The ImageJ software was used for analyzing band intensities.



Supplementary Figure 11

Single-particle EM analysis of negatively stained VDAC-1 in cNW9 nanodiscs.

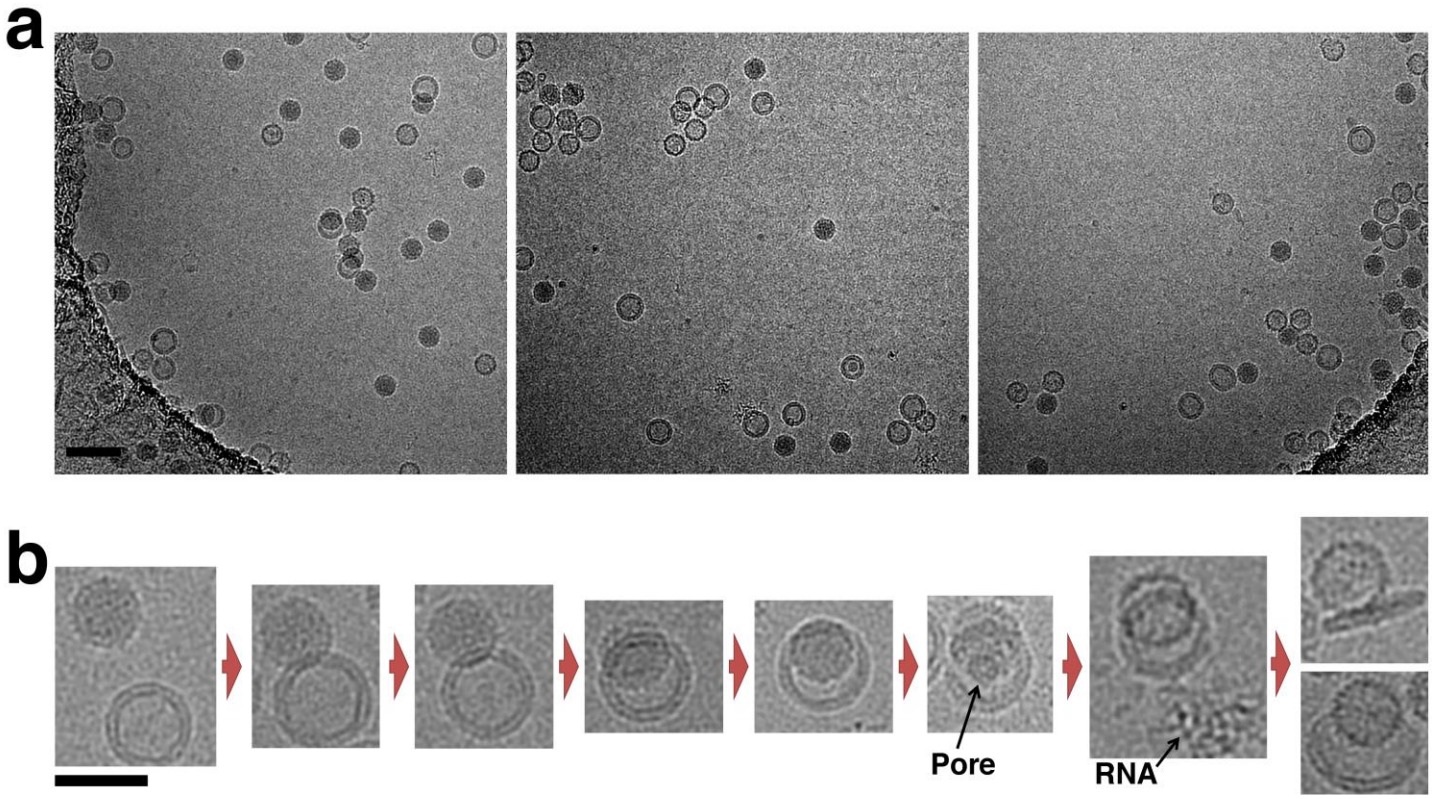
(**a**) Representative EM image of VDAC-1 in cNW9. Right. Enlarged image of the rectangular area in panel a, showing one VDAC-1 channel per nanodisc. Images were collected on a Tecnai T12 electron microscope (FEI) operated at 120 kV. (**b**) Representative two-dimensional (2D) class averages of VDAC-1 in cNW9 nanodisc, showing distinct views (top, tilted and side views). (**c**) Cartoon representation of VDAC-1 in a cNW9 nanodisc. Scale bars, 100 nm (**a**) and 20 nm (**b**)



Supplementary Figure 12

Comparison of the quality of NMR spectra of VDAC-1 in ΔH5 and cNW9 nanodiscs.

(**a**) ^{1}H-^{15}N TROSY HSQC recorded at 45°C of 100 uM ^{15}N-^{2}D-labeled VDAC-1 in ΔH5 3:1 DMPC:DMPG nanodiscs acquired overnight on a 600-MHz spectrometer. (**b**) ^{1}H-^{15}N TROSY HSQC recorded at 45°C of 100 μM ^{15}N-^{2}D-labeled VDAC1 in cNW9 3:1 DMPC:DMPG nanodiscs acquired overnight on a 800-MHz spectrometer. The spectral quality and sample stability are greatly improved by using cNW9 as compared to ΔH5 nanodiscs.

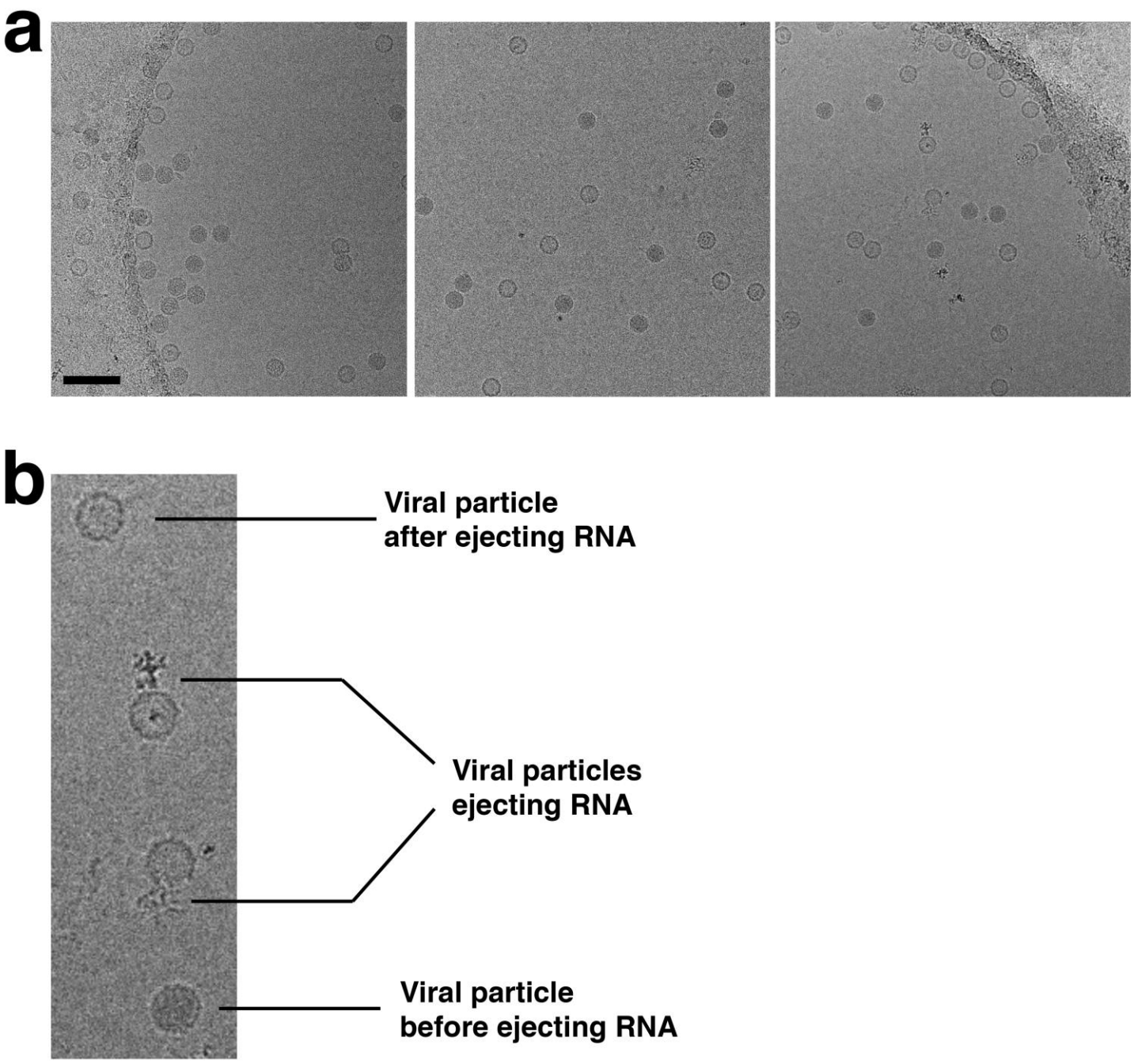


Supplementary Figure 13

Cryo-EM images of poliovirus plus CD155-decorated 50-nm nanodiscs.

(**a**) Representative cryo-EM images of poliovirus plus CD155-decorated 50-nm nanodiscs in vitreous ice. Scale bar represents 100 nm. (**b**) Representative cryo-EM images of poliovirus-nanodisc complexes showing different intermediate states. Scale bars, 100 nm (**a**) and 50 nm (**b**).

We find it unlikely that the putative pore in the nanodisc is a hexametric arrangement of CD155 since we used only the ectodomains of the receptor linked to lipid molecules via His tags. Scale bars, 100 nm (**a**) and 50 nm (**b**)



Supplementary Figure 14

Cryo-EM images of poliovirus plus CD155-decorated 15-nm nanodiscs.

(a) Representative cryo-EM images of poliovirus plus CD155-decorated 15-nm nanodiscs in vitreous ice. Scale bar represents 100 nm. **(b)** Cryo-EM image showing individual viruses ejecting RNA after incubation with CD155-decorated 15-nm nanodiscs. Scale bar, 100 nm.

Supplementary Note 1: Amino acid sequences of NWs

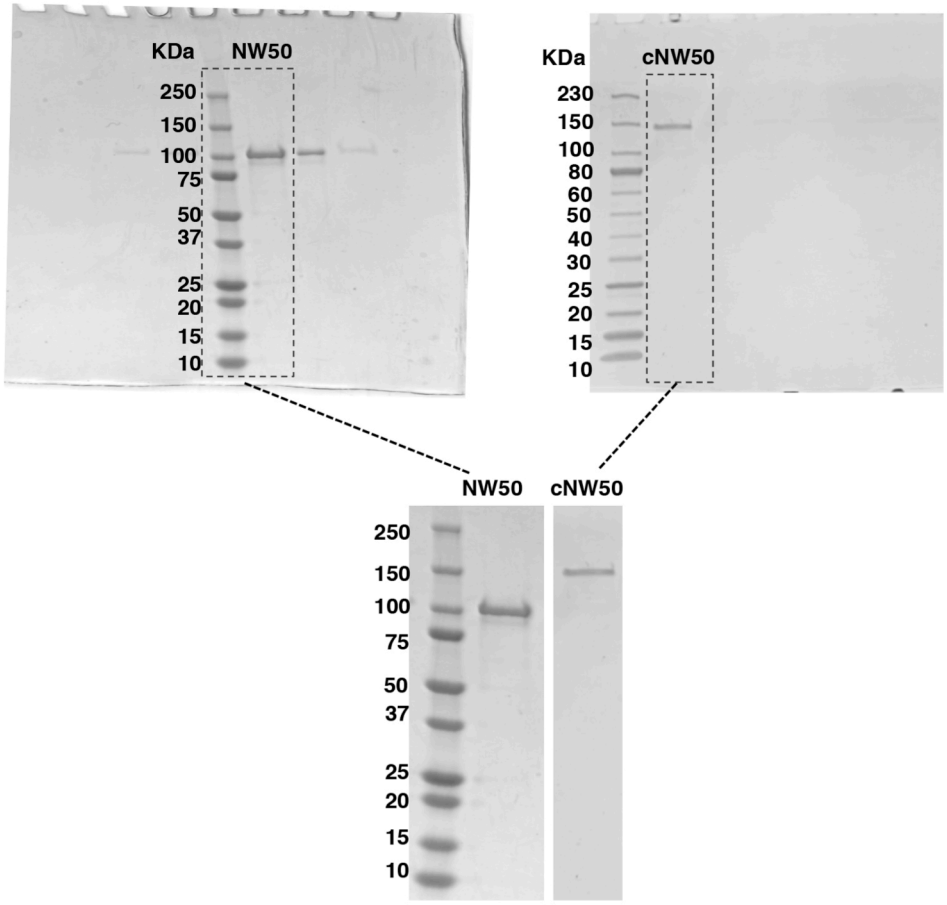
NW9 (assembles ~ 8.5 nm nanodisc)
MGSSHHHHHHENLYFQGSTFSKLREQLGPVTQEFWDNLEKETEGLRQEMSKDLEEVKAKV
QPYLDDFQKKWQEEMELYRQKVEPLGEEMRDRARAHVDALRTHLAPYSDELRQRLAARLE
ALKENGGARLAEYHAKATEHLSTLSEKAKPALEDLRQGLLPVLESFKVSFLSALEEYTKKLNT
QLPGTGAAALEHHHHHH

NW11 (assembles ~ 11 nm nanodiscs)
MGSSHHHHHHENLYFQGSTFSKLREQLGPVTQEFWDNLEKETEGLRQEMSKDLEEVKAKV
QPYLDDFQKKWQEEMELYRQKVEPLRAELQEGARQKLHELQEKLSPLGEEMRDRARAHVD
ALRTHLAPYSDELRQRLAARLEALKENGGARLAEYHAKATEHLSTLSEKAKPALEDLRQGLL
PVLESFKVSFLSALEEYTKKLNTQLPGTGAAALEHHHHHH

NW30 (assembles ~15 nm nanodisc)
MGSSHHHHHHENLYFQGSTFSKLREQLGPVTQEFWDNLEKETEGLRQEMSKDLEEVKAKV
QPYLDDFQKKWQEEMELYRQKVEPLRAELQEGARQKLHELQEKLSPLGEEMRDRARAHVD
ALRTHLAPYSDELRQRLAARLEALKENGGARLAEYHAKATEHLSTLSEKAKPALEDLRQGLL
PVLESFKVSFLSALEEYTKKLNTQGTPVTQEFWDNLEKETEGLRQEMSKDLEEVKAKVQPY
LDDFQKKWQEEMELYRQKVEPLRAELQEGARQKLHELQEKLSPLGEEMRDRARAHVDALR
THLAPYSDELRQRLAARLEALKENGGARLAEYHAKATEHLSTLSEKAKPALEDLRQGLLPVL
ESFKVSFLSALEEYTKKLNTQGTPVTQEFWDNLEKETEGLRQEMSKDLEEVKAKVQPYLDD
FQKKWQEEMELYRQKVEPLRAELQEGARQKLHELQEKLSPLGEEMRDRARAHVDALRTHL
APYSDELRQRLAARLEALKENGGARLAEYHAKATEHLSTLSEKAKPALEDLRQGLLPVLESF
KVSFLSALEEYTKKLNTQLPGTGAAALEHHHHHH

NW50 (assembles ~ 50 nm nanodisc)
MGSSHHHHHHENLYFQGSTFSKLREQLGPVTQEFWDNLEKETEGLRQEMSKDLEEVKAKV
QPYLDDFQKKWQEEMELYRQKVEPLRAELQEGARQKLHELQEKLSPLGEEMRDRARAHVD
ALRTHLAPYSDELRQRLAARLEALKENGGARLAEYHAKATEHLSTLSEKAKPALEDLRQGLL
PVLESFKVSFLSALEEYTKKLNTQGTPVTQEFWDNLEKETEGLRQEMSKDLEEVKAKVQPY
LDDFQKKWQEEMELYRQKVEPLRAELQEGARQKLHELQEKLSPLGEEMRDRARAHVDALR
THLAPYSDELRQRLAARLEALKENGGARLAEYHAKATEHLSTLSEKAKPALEDLRQGLLPVL
ESFKVSFLSALEEYTKKLNTQGTPVTQEFWDNLEKETEGLRQEMSKDLEEVKAKVQPYLDD
FQKKWQEEMELYRQKVEPLRAELQEGARQKLHELQEKLSPLGEEMRDRARAHVDALRTHL
APYSDELRQRLAARLEALKENGGARLAEYHAKATEHLSTLSEKAKPALEDLRQGLLPVLESF
KVSFLSALEEYTKKLNTQGTPVTQEFWDNLEKETEGLRQEMSKDLEEVKAKVQPYLDDFQK
KWQEEMELYRQKVEPLRAELQEGARQKLHELQEKLSPLGEEMRDRARAHVDALRTHLAPY
SDELRQRLAARLEALKENGGARLAEYHAKATEHLSTLSEKAKPALEDLRQGLLPVLESFKVS
FLSALEEYTKKLNTQGTPVTQEFWDNLEKETEGLRQEMSKDLEEVKAKVQPYLDDFQKKW
QEEMELYRQKVEPLRAELQEGARQKLHELQEKLSPLGEEMRDRARAHVDALRTHLAPYSD
ELRQRLAARLEALKENGGARLAEYHAKATEHLSTLSEKAKPALEDLRQGLLPVLESFKVSFLS
ALEEYTKKLNTQLPGTGAAALEHHHHHH

Supplementary Note 2: Full view of cropped figure 5g.



Miniature cropped SDS-PAGE (bottom) presented in supplementary figure 5g and corresponding uncropped SDS-PAGE (top).

Supplementary Table 1. Characterization of intact NW proteins by mass spectrometry.

		Mass, Da		
	Linear (calculated)	Linear (observed)	Circularized (Calculated)	Circularized (Observed)
NW9	21191.8	21192.8	19838.4	19838.5
NW11	23747.7	23752.4	22394.3	22398.1
NW30	65506.9	65520.6	64153.5	64162.4
NW50	107266.1	107304.7	105912.7	105940.7

Supplementary Protocols

A. Expression of cNWs proteins in *E. coli*

1) Transform 20 µl *E. coli* BL21-Gold (DE3) cells (or derivatives) with 20 ng of pET28a-NWs plasmid DNA.
2) Plate the cells on LB agar plate containing 50 µg/mL Kanamycin and grow colonies at 37°C overnight.
3) Inoculate a colony into a sterile flask containing 70 mL LB supplemented with 50 µg/mL Kanamycin.
4) Grow seed culture overnight at 37°C, shaking at 200 rpm.
5) Inoculate 6L LB medium supplemented with 50 µg/mL Kanamycin and shake at 200 rpm at 37°C until OD_{600} reaches 0.6.
6) Induce at OD_{600} of 0.6 with 1 mM IPTG (1 M stock in H_2O, filtered).
7) Grow cells for another 3 hours at 37°C (for NW9 and NW11) or another 16 hours at 18°C (for NW30 and NW50).
8) Harvest cells by centrifugation (7000x*g*, 15 minutes, 4°C).
9) Remove supernatant and store cell pellet at -80°C.

B. Purification of NW9 and NW11

1) Thaw cells at room temperature (RT) for 40 minutes then resuspend in 5 mL of lysis buffer (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 1 % Triton X-100) per gram of cell. Make sure you add one protease inhibitor cocktail tablet per 50 ml of cell suspension. Sometimes, we supplement lysis buffer with 1 mM EDTA In addition to the protease inhibitor tablet (optional).
2) Add ~1 mg lysozyme per mL of cell suspension
3) Stir in the cold room for 30-40 minutes.
4) Sonicate cells on ice (20 min net sonication, 30% duty cycle, 1 second on - 3 seconds off)
5) Add 5 mM $MgCl_2$ (if you supplemented the lysis buffer with EDTA) and 100U Benzonase Nuclease then stir on ice or at 4°C for 45 minutes.
6) Spin down cell debris and insoluble materials at 35000 g and 4°C for 50 min.

7) Apply (3 times) supernatant to Ni-NTA column equilibrated with 50 mM Tris-HCl, pH 8.0, 500 mM NaCl (= buffer A) +1 % Triton X-100.
8) Wash with 10 column volumes (CV) buffer A+ 1% TritonX-100.
9) Wash with 10 CV buffer A + 50 mM Cholate.
10) Wash with 10 CV buffer A
11) Wash with 10 CV buffer A + 20 mM imidazole.
12) Elute protein with 3-5 CV buffer A+ 500 mM imidazole
13) Check protein purity with SDS-PAGE then dialyze against 50 mM Tris-HCl, pH 8.0, 100 mM NaCl using a 12-14 kD MWCO membrane.
14) Add 2 mM DTT and TEV protease (His6-tagged; produced in-house), incubate at 4°C for ~ 8-16 hours. Add TEV at a protease to NW protein ratio of 1: 50 (w/w).
15) Run SDS-PAGE to make sure the N- terminal His tag cleavage is complete.
16) After TEV cleavage is complete, concentrate up to 500-600 μM, flash freeze and store at -80°C. Alternatively, move on directly to the circularization reaction (preferred).

Optional

As a lot of NW9 and NW11 proteins can be found in the insoluble fraction after cell lysis, we perform purification under denaturing conditions as follows;

1) Dissolve the pellet in 6 M guanidine hydrochloride (Gn-HCl), 50 mM Tris-HCl, pH 8.0, 500 mM NaCl.
2) Centrifuge (35000 g, 50 min, 4°C) to remove insoluble debris.
3) Apply (3 times) onto NiNTA equilibrated with 6 M Gn-HCl buffer
4) Wash with 10 CV 6 M Gn-HCl buffer
5) Wash with 10 CV buffer A to refold the NW9 and NW11 on the NiNTA column.
6) Wash with buffer A + Triton X-100, buffer A + cholate, buffer A, and buffer A + 20 mM Imidazole as described above in the standard protocol.
7) Elute with buffer A + 500 mM Imidazole.
8) Perform dialyses and TEV cleavage as mentioned above.

C. Purification of NW30 and NW50

NW30 and NW50 were purified under denaturing conditions and refolded as follows:

1) Dissolve the cell pellet in 6 M Gn-HCl, 50 mM Tris-HCl, pH 8.0, 500 mM NaCl.
2) Centrifuge (35000 g, 50 min, 4°C) to remove insoluble debris.
3) Apply (3 times) onto NiNTA equilibrated with 6 M Gn-HCl buffer
4) Wash with 10 CV 6 M Gn-HCl buffer.
5) Wash with 10 CV buffer A to refold the NW30 and NW50 on the NiNTA column.
6) Wash with buffer A + Triton X-100, buffer A + cholate, buffer A, and buffer A + 20 mM Imidazole as described above under section **B** (steps 8-11).
7) Elute with buffer A + 500 mM Imidazole.
8) Perform dialyses and TEV cleavage as described above under section **B** (steps 13-16).
9) NW30 and NW50 (still containing a C-terminal His_6 tag) were further purified by size exclusion chromatography (SEC; Superdex 200 16/60 [GE Healthcare] equilibrated in 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 50 mM sodium cholate, 0.5 mM EDTA).
10) SEC fractions containing NW30 and NW50 were further purified over Ni^{2+}-NTA resin to remove truncation products (which lack a C-terminal His_6 tag). (Follow steps 7-13 from section **B**)
11) Purified proteins were concentrated, flash frozen and stored at -80°C. Alternatively, move on directly to the circularization reaction (preferred).

General Comments about NWs productions.

- In order to minimize the truncation products (for NW30 and NW50), expression is optionally being done at lower temperatures (20°C or below) and an additional His purification step is added after SEC.
- Make sure the TEV reaction is complete before moving on to the next step.
- We do not remove TEV after N terminal His tag cleavage. Instead, we remove it together with the sortase enzyme at a later step.

D. MSP circularization

1) Dilute the purified proteins into sortase reaction buffer (300 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$) so that the final NWs concentration is less than 15 µM.

2) Add freshly made evolved sortase (Addgene plasmid # 75144) so that the final sortase: protein molar ratio is 1: 2. Continue incubation at 37°C while stirring for 3-4 hours (preferred) or at 4°C overanight.
3) Monitor the reaction by SDS-PAGE. Take aliquot every 1-hour and mix it with the SDS-PAGE sample buffer.
4) Add the covalent sortase inhibitor, AAEK2, to a final concentration of 500 μM once sortase reaction is complete (usually after 3-4 hours at 37°C).
5) Incubate the reaction for another 30 min at room temperature while gently shaking.
6) Apply (3 times) the reaction mixture onto NiNTA equilibrated with 20 mM Tris-HCl, pH 7.5, 500 mM NaCl buffer. **Please note** that we perform this Ni purification step to remove TEV, sortase, cleaved C-terminal His tag and the un-circularized NWs. We recommend that you use excess Ni resin in this step.
7) Wash with 3 CV of buffer containing 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 10 mM imidazole.
8) Combine flow-through and wash fractions (containing the circularized products) then add 50-100 mM EDTA (optional).
9) Concentrate the circularized products using centricon concentrators. Use 10 kDa MW cutoff concentrators for NW9 and NW11, and use 30 and 50 kDa MW cutoff for NW30 and NW50 respectively.
10) Inject cNWs into a Superdex 75 16/60 column equilibrated in buffer containing 20 mM Tris-HCl, pH 7.5, 500 mM NaCl plus 50 mM sodium cholate or 1 mM dodecyl-β-D-maltoside (DDM).
11) Collect fractions containing cNWs and check the purity using SDS-PAGE.
12) Exchange the buffer into buffer A_{ix} (20 mM Tris, pH 8.2, 1 mM DDM) using centricon concentrators or dialysis.
13) Concentrate the protein solution then apply it to a Resource Q column equilibrated with buffer A_{ix}. A linear salt gradient from 0-60 % buffer B_{ix} (20 mM Tris, pH 8.2, 1 mM DDM, 1M NaCl) was applied. Circularized proteins usually elute around 150-200 mM NaCl.
14) Collect the fractions containing cNWs and concentrate. Use fresh preparation to make nanodiscs (preferred) or store at -80°C for future use.

E. Example of nanodiscs assembly reaction using circularized NW9 (cNW9)

V = 300 μL

- 135 μL of 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 70 mM sodium cholate buffer. Final cholate concentration: 31.5 mM.
- 45 μL POPC/POPG (3:2) (80 mM stock) → final concentration: 12 mM.
- 120 mL cNW9 (500 μM stock) → final concentration: 0.2 mM.
- Incubate for 1 hour on ice.
- Add ~ 0.3-0.5 g of wet Biobeads SM-2 and continue incubation over ice for another 30-60 min.
- Shake on orbital shaker for 4-12 h at 4°C.
- Remove Biobeads by filtration (or use the centrifuge tube filters Spin-X (Costar)) and then apply reaction mixture to Superdex 200 size exclusion column.

F. Example of nanodiscs assembly reaction using circularized NW11 (cNW11)

V = 300 μL

- 159 μL of 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 60 mM sodium cholate buffer. Final cholate concentration: 31.8 mM.
- 45 μL POPC/POPG (3:2) (80 mM stock) → final concentration: 12 mM.
- 96 μL cNW11 (500 μM stock) → final concentration: 0.16 mM.
- Incubate for 1 hour on ice.
- Add ~ 0.3-0.5 g of wet Biobeads SM-2 and continue incubation over ice for another 30-60 min.
- Shake on orbital shaker for 4-12 h at 4°C.
- Remove Biobeads by filtration and then apply reaction mixture to Superdex 200 size exclusion column.

G. Example of nanodiscs assembly reaction using circularized NW30 (cNW30)

V = 300 μL

- 175 μL of 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 60 mM sodium cholate buffer. Final cholate concentration: 35 mM.

- 25 μL POPC/POPG (3:2) (60 mM stock) → final concentration: 5 mM.
- 100 μL cNW30 (100 μM stock) → final concentration: 33.3 µM.
- Incubate for 1 hour on ice.
- Add ~ 0.4-0.5 g of wet Biobeads SM-2 and continue incubation over ice for another 30-60 min.
- Shake on orbital shaker for 3-4 h at 4°C.
- Remove Biobeads by filtration and then apply reaction mixture to Superose 6 size exclusion column.

  - **Please note** that we also performed the assembly reaction successfully in the presence of excess lipids at NW30: lipids ratio of 1:1000 (**Supplementary Figure 7**)

H. Example of nanodiscs assembly reaction using NW50

V = 300 μL

- 183 μL of 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 60 mM sodium cholate buffer. Final cholate concentration: 36.6 mM.
- 42 μL POPC/POPG (3:2) (80 mM stock) → final concentration: 11.25 mM.
- 75 μL cNW50 (10 μM stock) → final concentration: 2.5 µM.
- Incubate for 1 hour on ice.
- Add ~ 0.4-0.5 g of wet Biobeads SM-2 and continue incubation on ice for another 30-60 min.
- Shake on orbital shaker for 3-4 h at 4°C.
- Remove Biobeads by filtration then apply reaction mixture to Superose 6 size exclusion column.