Table S1, related to Figure 1

The protein sequences (accession numbers given in Figure S1) were pair-wise aligned with the program EMBOSS Stretcher, using the EBLOSUM62 matrix, with gap penalty set to 12, and the extend penalty set to 2. The alpha crystallin domain (ACD) was identified by the NCBI's conserved domain database utility (CDD).

Organism	ACD		NTD (including P1)		P1 alone	
	% Identity	% Similarity	% Identity	% Similarity	% Identity	% Similarity
Pan troglodytes	100	100	100	100	100	100
Macaca mulatta	100	100	100	100	100	100
Mus musculus	94.2	97.7	77	85.1	52.9	58.8
Rattus norvegicus	94.2	97.7	75.9	83.9	47.1	52.9
Bos Taurus	94.2	97.7	83.3	84.5	57.1	57.1
Sus scrofa	93	98.8	89.4	89.4	80	80.0
Danio rerio	75.6	90.7	54.9	65.9	13.6	18.2

Table S2, related to Figure 1

Average molar mass and average hydrodynamic radius moments of the HSP variants

HSP	Average Molar mass ($x10^6$ g/mol)	Average Hydrodynamic radius (nm)
P1	0.80	8.2
Var-1	0.86	8.3
Var-2	0.84	8.2
Var-3	0.58	7.0
Var-4	0.60	7.4
Var-5	0.64	7.4
Var-6	0.69	7.7
Var-7♦	0.53	7.0
	0.40	6.2
Var-8♦	0.59	7.2
	0.44	6.4
Var-9♦	0.56	7.1
	0.40	6.1
WT	0.39	6.1

The molar masses and the radii were measured by SEC-MALS of 100µg proteins eluted at 25 ºC using Astra software (Wyatt). ♦These variants segregate into two distinct populations.

Table S3, related to Figure 3, and Figures S5, S6

Relative abundances of individual oligomeric states of Hsp16.5 variants determined by native mass spectrometry.

HSP	24	28	30	32	34	36	38	28-38	48
Var-2 [1]	-	0.07	0.03	0.09	0.07	0.22	0.15	0.62	0.37
Var-3 [1]	0.04	0.03	0.45	0.06	0.16	0.26	-	0.96	-
Var-6 [1]	0.03	0.10	0.05	0.20	0.17	0.44	-	0.97	-
Var-7 [2]	0.74	0.00	0.05	0.00	0.15	0.06	-	0.26	-
Var-8 [2]	0.86	0.01	0.02	0.08	0.02	-	-	0.14	-

Oligomeric distributions were fit to the intact spectrum using oligomeric masses determined from tandem mass spectrometry (1), as described in the methods. Unresolved regions of Var-7 and -8 were subjected to tandem MS and the oligomeric intensities were reconstructed (2) from the weighted data, as described in the methods. The columns shown in gray are shown for comparison with Table 2 and EM particle distributions (Table 1).

Table S4, related to Figure 7

Dissociation constants of Hsp16.5 variants to T4L-L99A

HSP variant	K_D (μM)
P1	1.28±0.09
Var-1	0.19±0.04
Var-3	2.65±0.29
Var-5	1.58±0.23
Var-6	2.22±0.13
Var-8	9.47±0.48
WT	11.96±0.84

5μM T4L-L99A labeled with monobromobimane was titrated with increasing concentrations (0-25 fold molar excess) of sHsp at 37 °C in pH 7.2 buffer. The number of binding sites parameter, n, was constrained to 0.5 in all fits. The K_D are reported ± the standard deviation (s.d.) of the fit.

Figure S1. **Sequence alignment of the P1 peptide and the evolution of Hsp27/HspB1, related to Figure 1**.

The P1 sequence was queried through the protein suite of the BLAST web engine. Among the Hsp27/HspB1 sequences, those representing diverse taxa were multisequence aligned by the Clustal Omega program. The phylogenetic tree was generated from the alignment by Neighbour-joining clustering and the cladogram was visualized using the Phylodendron web utility. Primates, highlighted by the box in the cladogram, have identical P1 sequence. The residues highlighted in red are identical to P1. Accession numbers of the protein sequences are: *Ailuropoda melanoleuca* (ADM32403.1), *Alligator mississippiensis,* (BAF94137.1), *Bos indicus* (AOO19780.1), *Bos taurus* (AAI02130.1), *Canis lupus familiaris* (NP_001003295.1), *Castor canadensis* (XP_020011984.1), *Columba livia* (XP_005502015.1), *Danio rerio* (AAI64999.1), *Drosophila melanogaster* (AAA28638.1), *Felis catus* (AHZ62764.1), *Gallus gallus* (NP_990621.1), *Homo sapiens* (CAG38728.1), *Macaca mulatta* (XP_001109274.1), *Mus musculus* (AAH99463.1), *Mus pahari* (XP_021042616.1), *Musca domestica* (AQY54361.1), *Pan troglodytes* (XP_003339451.1), *Patagioenas fasciata monilis* (OPJ78725.1), *Rattus norvegicus* (NP_114176.4), *Rana catesbeiana* (ACO51783.1), *Salmo salar* (ACI68354.1), *Sus scrofa* (NP_001007519.1), *Xenopus laevis* (ABF17872.1), *Xenopus tropicalis* (AAI21618.1)

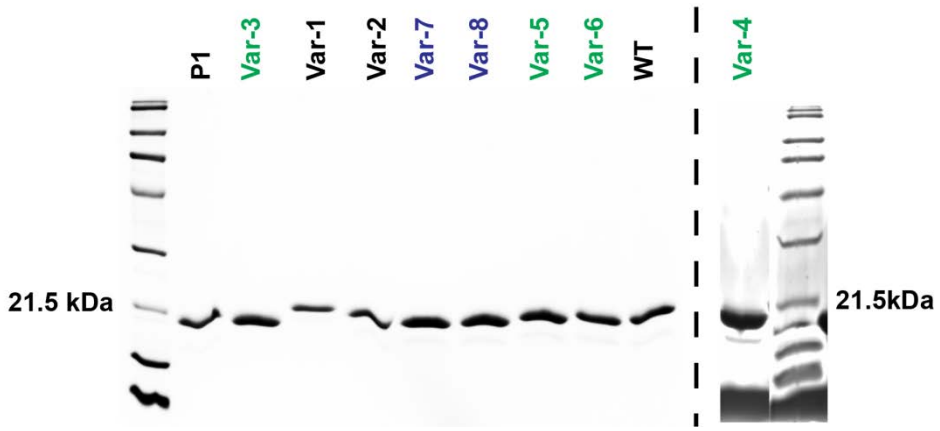

Figure S2. SDS-PAGE of Hsp16.5 variants, related to Figure 1.

20µg of each of the indicated proteins was run on 12% denaturing polyacrylamide gel.

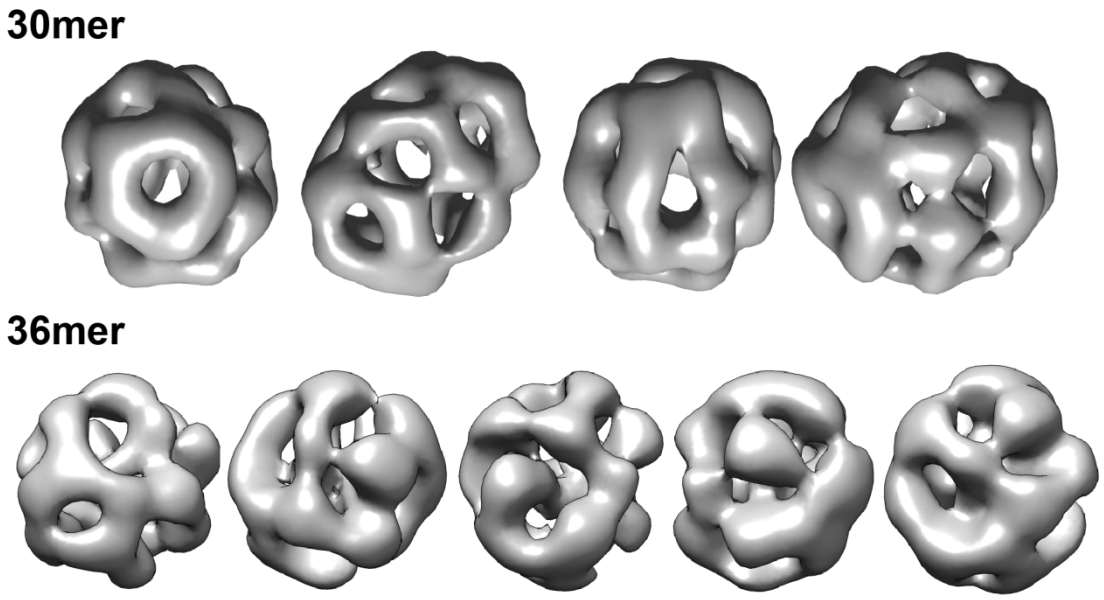

Figure S3, related to Figure 2.

Various views of the EM reconstruction of the 30- and 36-subunit oligomers of variants Hsp16.5

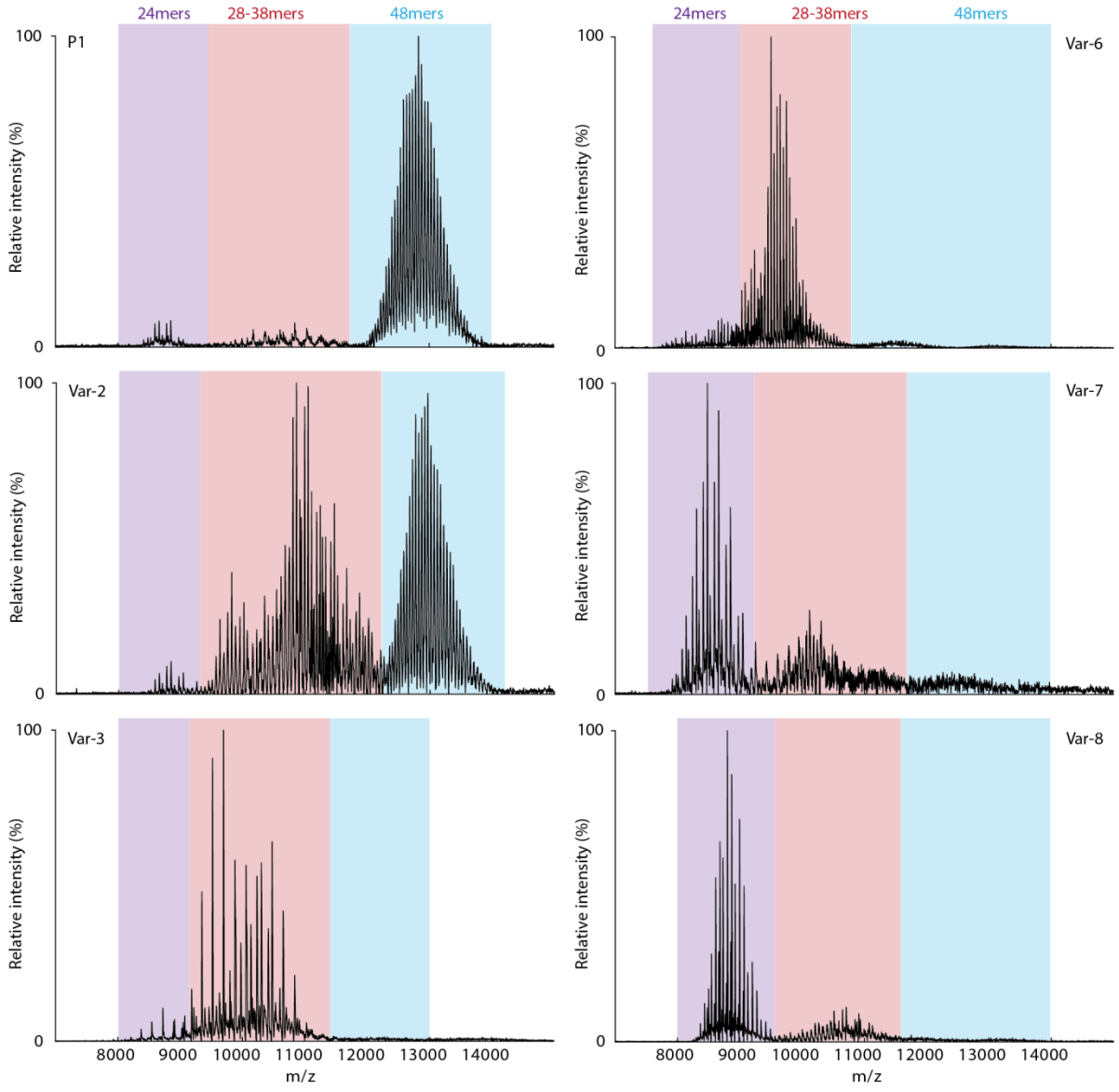

Figure S4, related to Table 2

Native mass spectra of different Hsp16.5 variants, showing the regions integrated to obtain relative abundances of the different oligomers, as summarized in Table 2.

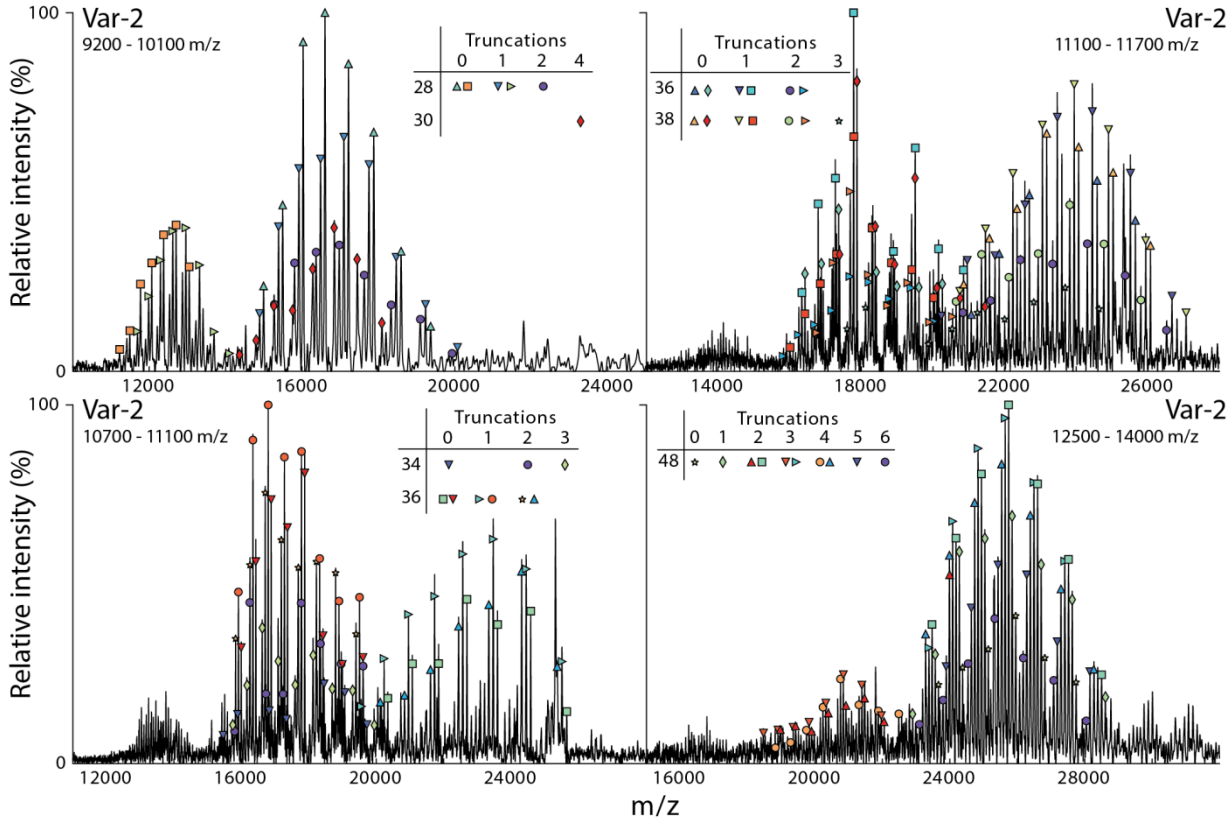

Figure S5, related to Table S3 and Native mass spectrometry in STAR Methods

Representative native tandem MS spectra for Hsp16.5 insertion variant Var-2. Precursor ions were isolated according to the m/z range shown and subjected to collision induced dissociation to determine the identity of the precursor. Truncated monomer incorporated into the oligomeric precursor is also assigned and the number of incorporations was determined for each oligomeric species.

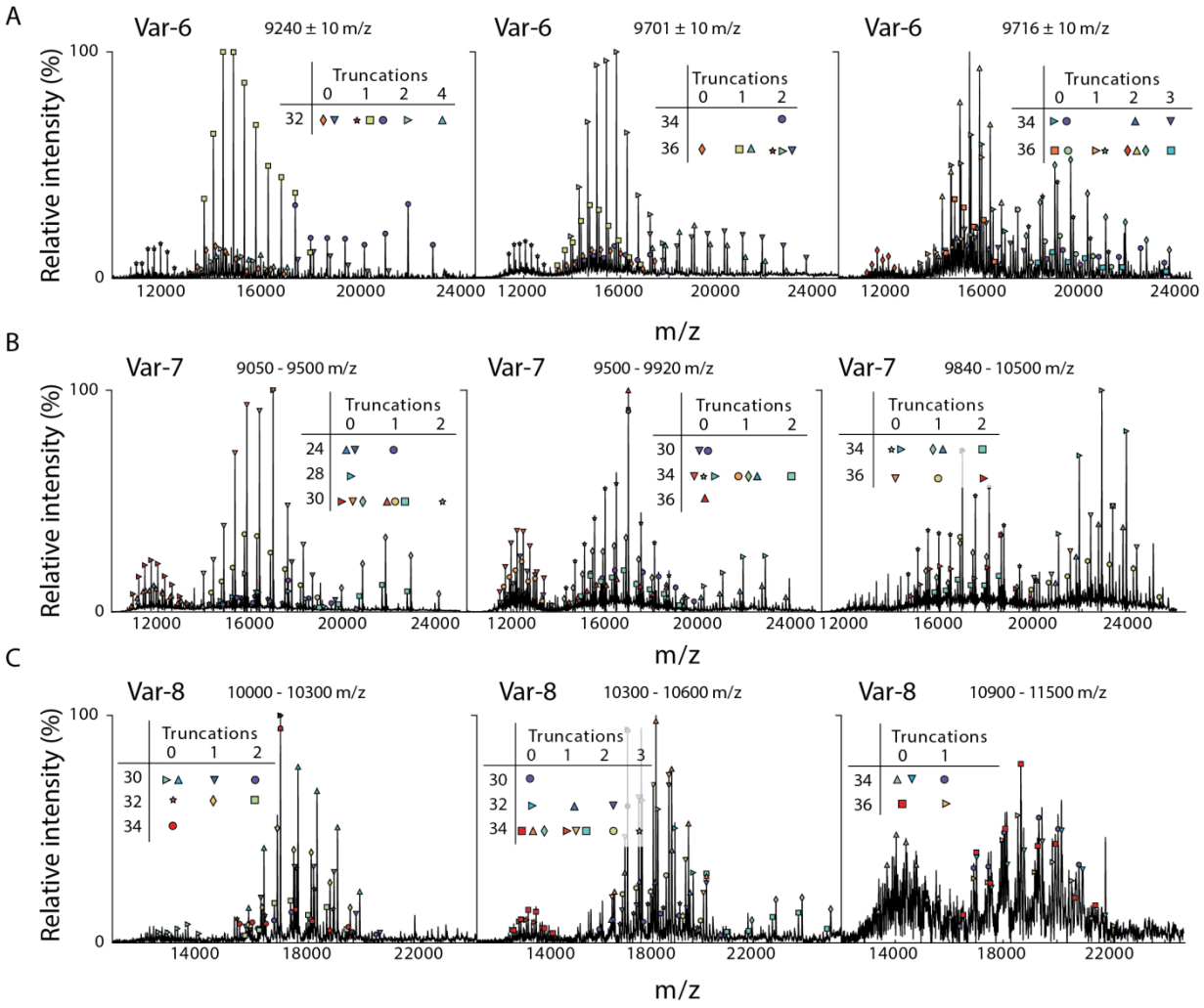

Figure S6, related to Table S3 and Native mass spectrometry in STAR Methods

Representative native tandem MS spectra for Hsp16.5 insertion variants: (A) Var-6, (B) Var-7, (C) Var-8. Precursor ions were isolated according to the m/z range shown and subjected to collision induced dissociation to determine the identity of the precursor. Truncated monomer incorporated into the oligomeric precursor is also assigned and the number of incorporations was determined for each oligomeric species.

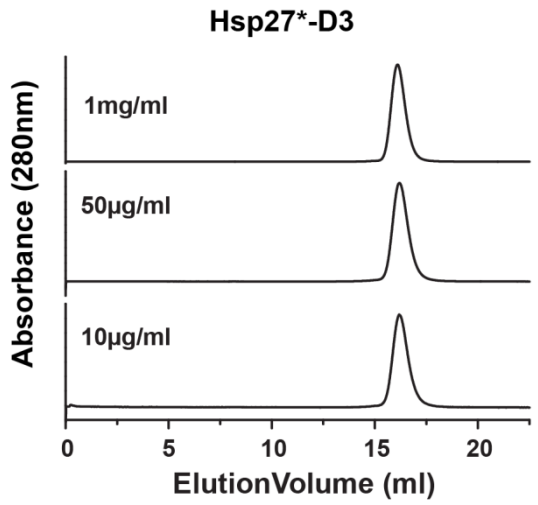

Figure S7, related to Figure 8.

Phosphorylation leads to dissociation of Hsp27* into dimers and abolishes the concentration dependent reassembly of oligomers. SEC profiles of the Hsp27*-D3, the phosphomimetic mutant of Hsp27*. 100µl of the proteins were injected at the indicated concentrations and eluted from SEC column at pH 7.2.

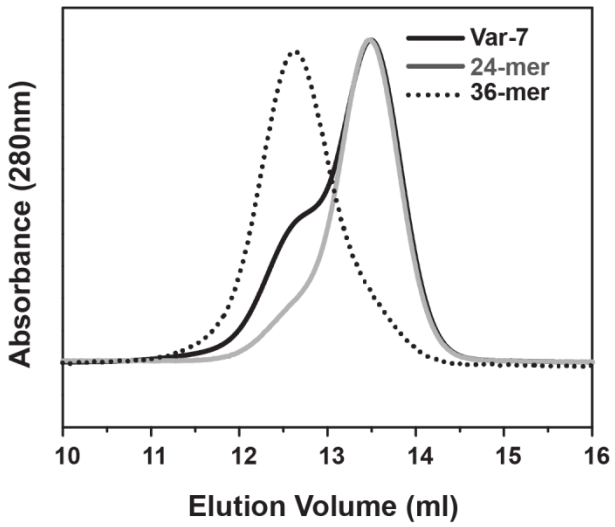

Figure S8. Isolation of the oligomeric subpopulations of Var-7, **related to Figure 5**.

A. The SEC profile of the Var-7 oligomers; the 36-subunit (dotted trace) and the 24-subunit oligomers (gray trace) after the oligomers were isolated and re-characterized by another SEC run; the thick black trace shows the equilibrium distribution before isolation.