Background and Purpose—Mutations in NOTCH3 cause cerebral autosomal–dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), the most common monogenic cause of stroke and vascular dementia. Misfolding and aggregation of NOTCH3 proteins triggered by cysteine-affecting mutations are considered to be the key disease mechanisms. However, the significance of cysteine-sparing mutations is still debated.

Methods—We studied a family with inherited small vessel disease by standardized medical history, clinical examination, MRI, ultrastructural analysis of skin biopsies, and Sanger sequencing of all NOTCH3 exons. In addition, we performed in vitro characterization of NOTCH3 variants using recombinant protein fragments and a single-particle aggregation assay.

Results—We identified a novel cysteine-sparing NOTCH3 mutation (D80G) in 4 family members, which was absent in a healthy sibling. All mutation carriers exhibited a CADASIL typical brain imaging and clinical phenotype, whereas skin biopsy showed inconsistent results. In vitro aggregation behavior of the D80G mutant was similar compared with cysteine-affecting mutations. This was reproduced with cysteine-sparing mutations from previously reported families having a phenotype consistent with CADASIL.

Conclusions—Our findings support the view that cysteine-sparing mutations, such as D80G, might cause CADASIL with a phenotype largely indistinguishable from cysteine mutations. The in vitro aggregation analysis of atypical NOTCH3 mutations offers novel insights into pathomechanisms and might represent a tool for estimating their clinical significance. (Stroke. 2015;46:00-00. DOI: 10.1161/STROKEAHA.114.007472.)

Key Words: CADASIL ■ cerebral small vessel disease ■ genetic testing ■ NOTCH3 protein, human ■ recombinant proteins

Cerebral autosomal–dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is the most common monogenic disease leading to stroke and vascular dementia.1 This hereditary cerebral small vessel disease (SVD) is caused by mutations in the NOTCH3 gene that encodes for a large type I transmembrane receptor mainly expressed in vascular smooth muscle cells and pericytes.2 Accumulation and deposition of the NOTCH3 extracellular domain within vessel walls have been recognized as key pathological features in CADASIL and occur both in patients and in a transgenic mouse model of the disease.3,4 NOTCH3 deposits are believed to promote the formation of granular osmiophilic material (GOM) on the surface of vascular smooth muscle cells and pericytes. GOM is pathognomonic for CADASIL, and therefore, it has a diagnostic value when observed in ultrastructural analyses of skin biopsies.5

The vast majority of CADASIL-related NOTCH3 mutations result in the gain or loss of a cysteine residue,6 leading to an odd number of cysteines. It has therefore been proposed that mutant proteins may promote aggregate formation via their unpaired sulfhydryl groups.5 We were recently able to reproduce the spontaneous multimerization of mutant NOTCH3...
in vitro using recombinant NOTCH3 proteins and a single-particle detection assay. This technique allows studying the propensity to form multimers on a molecular level in a highly controlled setting.

Importantly, there are several reports on NOTCH3 mutations that do not alter the number of cysteine residues. There is an ongoing debate whether these sequence variants also cause CADASIL, are associated with another form of cerebral SVD, or are not pathogenic and therefore represent rare single-nucleotide polymorphisms occurring in the general population. Incomplete diagnostic workup of reported cases or discrepancies in the clinical phenotype so far precluded a final appraisal on the pathogenicity of these variants. Of note, to date, no data are available from biochemical analysis or animal models.

We here describe several members of a family with a CADASIL-archetypical clinical and imaging phenotype carrying a novel cysteine-sparing NOTCH3 mutation (D80G). In vitro single-particle analysis of D80G and several other cysteine-sparing mutations demonstrated a multimerization behavior comparable with cysteine-affecting CADASIL mutations, thus providing biochemical support for their pathogenicity.

**Methods**

**Clinical Assessment of the Family**

Each sibling of the index patient (Figure 1, II.1) underwent a systematic clinical interview with a focus on the typical symptoms of CADASIL. We also obtained structured information from patient III.3 who was known to show symptoms of the disease. Because of logistic and ethical reasons, further family members were not contacted directly, but they were assessed indirectly through questioning the members of generation II.

In addition, we collected MRI data from routine examinations in digital form. Where available, we evaluated axial fluid-attenuated inversion recovery images or T2-weighted images (when fluid-attenuated inversion recovery not available) for the pattern of white matter hyperintensities.

**Electron Microscopy of Skin Biopsies**

The skin biopsy of the index patient (II.1) was performed in-house via a 5-mm skin punch from the upper arm. The tissue was immediately fixed in 6.25% glutaraldehyde, 1% osmiumtetroxide in 0.1 mol/L sodium phosphate buffer, pH 7.4, and embedded in epon. Ultrathin sections, stained with uranyl acetate and lead citrate, were observed in a Zeiss Libra 120 transmission electron microscope (Carl Zeiss AG, Oberkochen, Germany). In case II.1, tissue already embedded in epon blocks was sent to us for re-evaluation using electron microscopy. In case II.2, photographs from electron microscopy performed at another laboratory were re-evaluated at our institute.

**Molecular Genetic Testing**

All siblings of generation II and patient III.3 underwent molecular genetic testing of the entire NOTCH3 coding region. Genomic DNA was extracted from peripheral blood leukocytes using standard protocols. All exons and flanking intron regions were amplified by polymerase chain reaction and bidirectionally sequenced on an automated Sanger sequencer. The human NOTCH3 mRNA sequence (GenBank accession number, NM_000435.2) was used as a reference.

**DNA Constructs**

An overview of all variants is provided in Table 1. Allele frequencies in the general population were checked against data from the Exome Sequencing Project (ESP6500) using Ensemble release 75. The cloning procedure is described in the online-only Data Supplement.

**Expression and Purification of Recombinant NOTCH3 Protein**

We used transient transfection of human embryonic kidney 293T cells and the HaloTag Protein Purification System (Promega GmbH, Mannheim, Germany) for the purification of recombinant NOTCH3 fragments consisting of the first 5 epithelial growth factor–like repeat domains (online-only Data Supplement).

**Single-Particle Assay**

The multimerization assay is based on the measurement of green and red fluorescent dye–labeled proteins in a confocal dual-laser setup and has previously been described in detail. Labeled protein fragments were incubated for 5 days at 37°C with gentle agitation (500 rpm). We conducted 26 incubations (from different protein aliquots) for each construct as independent experiments. The measurement set-up and parameters are described in the online-only Data Supplement. Data were quantified in 2-dimensional (2D) intensity distribution histograms with 8-bit intensity values from 0 to 255 photons per bin and bin width 40 μs. The multimer signal was quantified as the number of high-intensity dual color bins (Figure 1 in the online-only Data Supplement) using a custom 2D software module (Evotec Technologies, Hamburg, Germany).

**Statistical Analysis**

Because of the relatively low number of independent measurements (n=6), we applied nonparametric statistical tests to ensure robust results. For each time point (baseline, 3 days, and 5 days), we performed a Kruskal–Wallis test for differences between the NOTCH3 constructs. When significant, we used the Wilcoxon signed-rank tests as post hoc analysis to test for a difference between each variant and the wild-type. False discovery rate control with a q value of 0.05 was used to account for multiple testing.
Results

Medical History and Clinical Examination
A 79-year-old woman (index patient; Figure 1, II.1) was referred to our specialized neurovascular outpatient clinic because of recurrent falls and progressive gait impairment. Past medical history comprised memory deficits over the preceding months, episodes of presyncope and hemicrania for several years, and a major depressive disorder for >30 years. Recurrent episodes of hemiparesis and tetraparesis had so far been interpreted as a psychosomatic disorder. Clinical examination showed gait apraxia and a mild right-sided hemiparesis. Neuropsychological evaluation demonstrated impaired memory and executive functions.

Family history revealed neurological symptoms in 2 siblings and a niece (Figure 1; Table 2). Sibling II.2 suffered a first ischemic stroke at 53 years and then presented >10× with small subcortical infarcts or transient focal neurological episodes. He developed mild cognitive impairment in multiple domains. Clinically, he remained with aphasia and a mild right-sided hemiparesis. Neuropsychological evaluation demonstrated impaired memory and executive functions.

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Blood tests and cerebrospinal fluid analyses (only II.2 and II.4) remained without signs of vasculitis, thrombophilic state, or other causes of dementia and stroke. Taken together, symptoms were strongly indicative for a familial SVD.

Table 1. Overview of Analyzed NOTCH3 Variants

<table>
<thead>
<tr>
<th>Variant*</th>
<th>cDNA†</th>
<th>SNP-ID‡</th>
<th>Allele Frequency (ESP6500)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Current family</td>
<td>D80G</td>
<td>c.239A&gt;G</td>
<td>n/a</td>
<td>…</td>
</tr>
<tr>
<td>Cysteine-sparing mutations described in the literature</td>
<td>R61W</td>
<td>c.181C&gt;T</td>
<td>rs200595885</td>
<td>0.05% (European American)</td>
</tr>
<tr>
<td>R75P</td>
<td>c.224G&gt;C</td>
<td>n/a</td>
<td>…</td>
<td>…</td>
</tr>
<tr>
<td>Δ88–91</td>
<td>c.263_274del</td>
<td>n/a</td>
<td>…</td>
<td>…</td>
</tr>
<tr>
<td>Typical cysteine mutations (positive controls)</td>
<td>R213K</td>
<td>c.638G&gt;A</td>
<td>n/a</td>
<td>…</td>
</tr>
<tr>
<td>C183R</td>
<td>c.547T&gt;C</td>
<td>n/a</td>
<td>…</td>
<td>…</td>
</tr>
<tr>
<td>C212Y</td>
<td>c.634T&gt;A</td>
<td>n/a</td>
<td>…</td>
<td>…</td>
</tr>
<tr>
<td>Polymorphisms/rare variants (negative controls)</td>
<td>R113Q</td>
<td>c.338G&gt;A</td>
<td>rs143385744</td>
<td>0.07% (European American)</td>
</tr>
<tr>
<td>H170R</td>
<td>c.509A&gt;G</td>
<td>rs147373451</td>
<td>0.15% (European American)</td>
<td>…</td>
</tr>
<tr>
<td>G179V</td>
<td>c.536G&gt;T</td>
<td>rs142778401</td>
<td>0.18% (Black American)</td>
<td>…</td>
</tr>
</tbody>
</table>

ESP indicates Exome Sequencing Project; and SNP, single-nucleotide polymorphism.
*Single letter amino acid code.
†Reference sequence: NM_000435.2; ‡database of SNP (dbSNP).21
MRI Examination
MRI of the index patient revealed confluent T2 hyperintensities predominantly in periventricular regions but also involving the white matter of the temporal poles (Figure 2). The same CADASIL-typical pattern was also found in MRI scans of all clinically affected family members. In particular, the temporal pole was affected in all subjects as a characteristic imaging sign for CADASIL.

Genetic Testing and Skin Biopsy
Sanger sequencing of the entire \textit{NOTCH3} coding region revealed the novel heterozygous missense mutation c.239A>G in all clinically affected family members, leading to the amino acid exchange p.Asp80Gly (D80G). No other sequence alteration apart from already known single-nucleotide polymorphisms was detected. The mutation was absent in the unaffected sibling II.3 and >8000 subjects from the ESP. Moreover, pathogenicity of the D80G mutation is strongly indicated by mutation algorithms that predict D80G as possibly damaging (PolyPhen-2\textsuperscript{27}), disease causing (MutationTaster\textsuperscript{28}), and deleterious (Sorting Intolerant From Tolerant amino acid substitutions).\textsuperscript{29} Human splicing finder (version 3.0)\textsuperscript{30} indicated the creation of an exonic splicing silencer and the disruption of an exonic splicing enhancer, potentially leading to an alteration of splicing. There was no indication for an introduction of new splice sites or branch points.

All clinically affected siblings of generation II underwent ultrastructural analysis of skin biopsies but showed variable results (Figure 1B). In sibling II.2, CADASIL-typical deposits of dense GOM within plasma membrane indentations were present. GOM could not be detected in the index patient (II.1) and sibling II.4, although the quality of biopsies was deemed adequate and a sufficient number of vessels could be analyzed. Because of the high suspicion of CADASIL, the index patient underwent a second biopsy, albeit with the same negative result.

Single-Particle Aggregation Analysis of Cysteine-Sparing Mutations
We used our previously established single-particle in vitro multimerization assay to study the aggregation behavior of the different constructs. D80G and 4 additional cysteine-sparing mutations described as causative in the literature were analyzed in comparison with wild-type NOTCH3, 3 known polymorphisms, and 3 typical cysteine-affecting CADASIL mutants (Table 1).

Differences in multimer signal between analyzed variants were observed after 3 days ($\chi^2=45.31; df=11; P=4.27\times10^{-6}$, Kruskal–Wallis test) and 5 days of incubation ($\chi^2=55.98; P=5.12\times10^{-8}$). There was also a marginally significant difference at baseline ($\chi^2=19.8601; P=0.047$), but this was not significant in post hoc tests. Results of post hoc group comparisons at days 3 and 5 are depicted in Figure 3 and can be summarized as follows: as expected and previously described, in vitro multimer formation was found for all cysteine-affecting CADASIL mutations but not for wild-type NOTCH3 or the polymorphisms. Within the group of cysteine-sparing mutations, D80G showed the strongest effect. R75P and R88–91, albeit less pronounced, still showed good multimerization behavior within the range of typical cysteine mutations. In contrast, R61W and R213K variants showed no increased multimerization.

Discussion
We provide combined support for the clinical significance of a novel cysteine-sparing \textit{NOTCH3} mutation (D80G) from the assessment of a family and in vitro characterization using a single-particle multimerization assay. To our knowledge, this is the first study to provide biochemical data on the pathogenicity of cysteine-sparing \textit{NOTCH3} mutations.

Importantly, mutation carriers demonstrated an archetypal clinical phenotype\textsuperscript{1} in the absence of vascular risk factors. MRI findings were also fully consistent with the diagnosis. CADASIL has distinct imaging features that are not frequent in other types of SVD. The presence of T2-hyperintense lesions in the temporal pole, located directly below the cortex, is the most prominent and typical finding.\textsuperscript{11} This feature was present in all mutation carriers. The D80G mutation was absent in the healthy brother and control genomes from the ESP further supporting its clinical significance. Still, the possibility remains that the D80G variant is not pathogenic...
Analysis of the D80G mutant in our in vitro multimerization assay revealed an aggregation behavior comparable with that of typical cysteine mutants. Multimerization of 3 known polymorphisms was not significantly different from wild-type proteins, confirming the specificity of our assay. The analysis of other reported cysteine-sparing mutations showed inconsistent results at first, which can however be adequately explained when re-examining the clinical data. On one hand, R75P and Δ88–91 showed significantly enhanced aggregation behavior within the range of cysteine mutations. R75P was described in multiple Asian families11,16,17 and can be considered the best characterized cysteine-sparing NOTCH3 mutation to date. Δ88–91, although identified so far only in a single Italian family, can be considered as sufficiently documented based on the available genetic, clinical, and histological data.18 On the other hand, multimerization was absent for R61W and R213K. Intriguingly, the clinical significance of these mutations can be questioned. R61W was reported in only 2 siblings, which, although displaying clinical symptoms compatible with CADASIL, had atypical MRI findings and no family history for neurological deficits.15 NOTCH3 deposits were identified in one of the brothers by light and electron microscopy, but the absence of cysteine-affecting NOTCH3 mutations via sequencing of the complete coding region was not reported. Thus, the genetic cause of this case could not be clearly defined. Recent data from the ESP revealed a frequency of 0.05% for the R61W variant in European Americans (Table 1), which is 10 fold higher than the CADASIL prevalence at ≈ 5 of 100,000.32,33 Thus, we consider R61W a polymorphism rather than a disease-causing mutation, a conclusion in agreement with the results from our multimerization analysis. Similarly, the R213K variant was reported in a single autopsy case. Available genetic, clinical, and histological evidence supporting CADASIL as diagnosis was sparse.19,20 Hence, the authors explicitly mentioned the possibility of an arteriopathy other than CADASIL.19 Based on our in vitro analysis, the R213K variant likely represents a rare polymorphism, although it was so far not picked up in the large sequencing projects.

Taken together, our in vitro data provide new insights on the potential disease-causing role of cysteine-sparing NOTCH3 mutations. More specifically, we demonstrate their propensity to form higher order multimers offering interesting molecular insights given that these mutations result in NOTCH3 proteins without an a priori unpaired cysteine residues. Two possible mechanisms might be responsible for the multimerization of these proteins. In one scenario, cysteine-sparing amino acid exchanges might lead to the disruption or reorganization of disulfide bonds, thereby creating unpaired cysteine residues. Alternatively, they could induce a conformational change promoting multimerization by disulfide-independent mechanisms. Both mechanisms might also contribute to the aggregation of cysteine-affecting mutants once unpaired sulphydryl groups have been engaged in disulfide bonds. More studies are required to elucidate the exact mechanisms of NOTCH3 aggregation.

Another potential mechanism of the mutation could be the alteration of splicing. Bioinformatics analysis suggests...
the creation of an exonic splicing silencer and the disruption of a splicing enhancer through the mutation in exon 3. This could lead to alternate splicing, in particular to skipping of exon 3.\(^3,4\) However, the resulting splicing variant would introduce an open reading frameshift with a premature stop codon at the beginning of exon 4. The resulting truncated protein would be 6.8 kDa in size and carry an even number of cysteines (4). This protein would most likely exhibit a loss-of-function and is unlikely to contribute to the CADASIL phenotype.

The presence of GOM in ultrastructural analysis of skin biopsies is a pathognomonic finding in CADASIL and has therefore diagnostic values. Interestingly, GOM deposits were present only in 1 mutation carrier, although skin biopsy was performed by an experienced pathologist and the biopsy quality was deemed adequate as assessed by the presence of medium-sized arteries/arterioles with vascular smooth muscle cells in the specimen.\(^5\) We are therefore confident not to have missed GOM in the 2 negative patients. We can only speculate on the reasons for this finding: first, although GOM is apparently absent in skin vessels, we cannot entirely rule out its presence in cerebral vessels of cysteine-sparing mutation carriers. Second, the formation of large deposits detectable as GOM might not represent a direct determinant but rather an end point of the NOTCH3 aggregation process, without a key role in disease pathogenesis. As demonstrated in neurodegenerative diseases, such as Alzheimer disease, intermediates in the aggregation process, not the end product, might represent the toxic agents.\(^6\)

The lack of GOM in some CADASIL patients has also important implications on disease diagnosis. False-negative skin biopsies in carriers of cysteine-sparing mutations may lead to unnecessary examinations and wrong diagnoses. Thus, for example, our index patient had been diagnosed with a psychosomatic conversion disorder and patient II.4 underwent invasive muscle biopsy in search for an alternate diagnosis. Skin biopsy has been proposed as a gold standard to assess the clinical relevance of atypical NOTCH3 mutations.\(^12\) Our single-particle in vitro aggregation assay might represent a more reliable tool to evaluate their clinical significance.

A potential limitation of our study is the small family size with only 1 healthy sibling. We tried to compensate for this by including data from thousands of control genomes from the ESP. The missing MRI data for the healthy control can also be considered a limitation. Furthermore, we were not able to perform anti-NOTCH3 immunostaining on skin biopsies because of the immediate glutaraldehyde fixation and epon embedding during the clinical routine procedure. Immunostaining could have provided additional information about the aggregation of NOTCH3 proteins in these patients.

In conclusion, causative cysteine-sparing NOTCH3 mutations in CADASIL might shed new light onto possible pathogenic mechanisms. The in vitro molecular characterization of NOTCH3 mutations might provide a tool to assess their clinical significance.

Acknowledgments

We thank Dr Christian Dohmen (Neurology, University of Cologne) for providing patient information.

Sources of Funding

This work was supported by the Dr Werner Jackstädt-Stiftung, the German Research Foundation (GZ:OP 212/1-1), the Fondation Leducq (Transatlantic Network of Excellence on the Pathogenesis of Small Vessel Disease of the Brain), and the Vascular Dementia Research Foundation.

Disclosures

None.

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Cysteine-Sparing CADASIL Mutations in NOTCH3 Show Proaggregatory Properties In Vitro

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Stroke. published online January 20, 2015;
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/early/2015/01/20/STROKEAHA.114.007472

Data Supplement (unedited) at:
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Cysteine-sparing CADASIL mutations in NOTCH3 show pro-aggregatory properties in vitro

SUPPLEMENTAL METHODS

Constructs
Constructs of the human N3-ECD containing only the first 5 EGF-repeats in pTT5 vectors optimized for transient expression have been described previously. We substituted the myc-6xHis-tag at the C-terminus with a Halo-tag. The HaloTag system (Promega GmbH, Mannheim, Germany) offers the possibility to obtain tag-free protein at the end of the purification. The Halo tag coding sequence was amplified from the template vector pFC14A (Promega GmbH) with primers introducing appropriate restriction enzyme sites (AsiSI and XhoI). Single nucleotide mutations were inserted using a megaprimer PCR cloning strategy. The deletion was achieved using a loop-deletion PCR. All PCR steps were performed using the Pfu DNA polymerase (Agilent Technologies, Waldbronn, Germany). Fragments containing the mutation/deletion were inserted into the pTT5 hN3-EGF1-5 HaloTag vector using appropriate restriction sites.

Expression and purification of recombinant NOTCH3 protein
The protein expression system has been described previously. Briefly, we used polyethylenimine transient transfection of pTT5 plasmid constructs into adherently growing human embryonic kidney 293-E cells (Y. Durocher, Quebec, Canada). Conditioned medium was collected for 5 days. Protein purification was performed with the components of the HaloTag Protein Purification System (Promega GmbH) using a modified protocol: Dialyzed medium of four 500 cm² flasks (~350 ml) was incubated with 3 ml resuspended HaloLink resin (equilibrated with purification buffer: 1x phosphate-buffered saline, 0.01% NP-40, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) overnight at 4°C under agitation. HaloLink resin was collected by centrifugation and washed three times with purification buffer. For elution 1 ml cleavage solution (60 µl TEV protease in 1 ml purification buffer) was added to the settled beads and incubated for 1 h at room temperature under agitation. Supernatant was collected and TEV protease removed by adding 50 µl HisLink resin (30 min at room temperature). HisLink resin was removed by centrifugation and supernatant was analyzed by SDS-PAGE with silver and Coomassie gel stain and by western blot analysis using Anti-NOTCH3 antibodies. Purified protein fragments were labeled with amino-reactive dyes Alexa Fluor® 488 or Alexa Fluor® 647 carboxylic acid succinimidyl ester (Invitrogen, Darmstadt, Germany) at 3-fold molar excess overnight at 4°C in 100 mmol/L sodium bicarbonate buffer pH 8.5. Unbound dye was removed by repeated gel filtration with spin desalting columns (Thermo Fisher Scientific, Schwerte, Germany) as documented by fluorescence correlation spectroscopy.

Single-particle assay
Incubations for aggregation experiments were performed at a protein concentration of 80 nmol/l at 37°C with gentle agitation (500 rpm) on a thermomixer (Eppendorf, Hamburg, Germany). We conducted at least 6 incubations (from different protein aliquots) for each construct as independent experiments. For analysis, samples were drawn and diluted 1:7 into 384-well optic measurement plates (Sensoplate, Greiner Bio-One, Frickenhausen, Germany). Within one experiment, we performed three 1:7 dilutions as technical replicates for each incubation and time point. For analysis, these technical replicates were later combined by averaging within one experiment. Single-particle analysis (‘scanning for intensely fluorescent targets’) was performed on an Insight reader (Evotec Technologies, Hamburg, Germany) equipped with 488nm (excitation power 200 µW) and 633 nm (excitation power 300 µW) lasers. Measurement time was 10 s with five repeats per well (50 Hz beam scanner, 100 µm scan path length, 2000 µm positioning table movement).
SUPPLEMENTAL REFERENCES


**Visualization and quantification of data from the single-particle *in vitro* assay.**

Axes in the 2D histogram represent the intensity of photon counts per bin in the two detector channels. Monomers are picked up in the lower left corner. Multimers show high-intensity signals: While multimers comprised of only one protein appear as mono-colored multimers along the axes (e.g. preformed aggregates after thawing), *de novo* multimers from both proteins (green and red labeled) can be detected as dual-color signal. For quantification, we counted dual-color bins above the monomer threshold (grey area).