

Video Article

Generation, Purification, and Characterization of Cell-invasive DISC1 Protein Species

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Abstract

Protein aggregation is seen as a general hallmark of chronic, degenerative brain conditions like, for example, in the neurodegenerative diseases Alzheimer's disease (A β , tau), Parkinson's Disease (α -synuclein), Huntington's disease (polyglutamine, huntingtin), and others. Protein aggregation is thought to occur due to disturbed proteostasis, *i.e.* the imbalance between the arising and degradation of misfolded proteins. Of note, the same proteins are found aggregated in sporadic forms of these diseases that are mutant in rare variants of familial forms.

Schizophrenia is a chronic progressive brain condition that in many cases goes along with a permanent and irreversible cognitive deficit. In a candidate gene approach, we investigated whether Disrupted-in-schizophrenia 1 (DISC1), a gene cloned in a Scottish family with linkage to chronic mental disease^{1,2}, could be found as insoluble aggregates in the brain of sporadic cases of schizophrenia³. Using the SMRI CC, we identified in approximately 20 % of cases with CMD but not normal controls or patients with neurodegenerative diseases sarkosyl-insoluble DISC1 immunoreactivity after biochemical fractionation. Subsequent studies *in vitro* revealed that the aggregation propensity of DISC1 was influenced by disease-associated polymorphism S704C⁴, and that DISC1 aggresomes generated *in vitro* were cell-invasive⁵, similar to what had been shown for A β ⁶, tau⁷⁻⁹, α -synuclein¹⁰, polyglutamine¹¹, or SOD1 aggregates¹². These findings prompted us to propose that at least a subset of cases with CMD, those with aggregated DISC1 might be protein conformational disorders.

Here we describe how we generate DISC1 aggresomes in mammalian cells, purify them on a sucrose gradient and use them for cell-invasiveness studies. Similarly, we describe how we generate an exclusively multimeric C-terminal DISC1 fragment, label and purify it for cell invasiveness studies. Using the recombinant multimers of DISC1 we achieve similar cell invasiveness as for a similarly labeled synthetic α -synuclein fragment. We also show that this fragment is taken up *in vivo* when stereotactically injected into the brain of recipient animals.

Video Link

The video component of this article can be found at <http://www.jove.com/video/4132/>

Protocol

1. Preparation of Aggresome Donor Cells: Transfection of Monomeric Red Fluorescent Protein (mRFP) or Enhanced Green Fluorescent Protein (eGFP)-tagged Human Full Length (FL) DISC1

1. Seed 1.5×10^6 10 cm dish human neuroblastoma NLF (NLF; Children's Hospital of Philadelphia, Philadelphia, PA) cells in ten 10 cm dishes and grow overnight. NLF cells are cultured in RPMI-1640 medium supplemented with 10% FBS, Penicillin/Strepomycin, L-Glutamine. The next day, cells should be at 70-80% confluency.
2. Transiently transfect each 10 cm dish with 15 μ g plasmid DNA and Metafectene transfection reagent (Biontex, Martinsried, Germany). In brief, for a 10 cm dish, 15 μ g plasmid DNA and 30 μ l Metafectene were added to 750 μ l Opti-MEM, mixed and incubated at RT for 20 min. 5 dishes were transfected with pcDNA3.1 mRFP/eGFP-tagged human (FL) DISC1 and 5 dishes with the eGFP-plasmid alone.

2. Aggresome Purification from Transfected Donor Cells

The protocol described here is a combination of a method to purify Lewy-Bodies from brain tissue¹³ and a protocol to isolate large aggregates and aggresomes¹⁴. Since already existing methods are based on the usage of detergents, the isolation of detergent-free protein for the application in cell-invasiveness assays is critical.

1. 48 hr after transfection, monitor the expression of eGFP and mRFP/eGFP-DISC1 and confirm the formation of aggresomes under a fluorescence microscope (**Figure 1**).
2. Wash the cells with PBS pH 7.4, scrape with 400 μ l PBS each, add 1X Protease Inhibitor cocktail (Roche, Indianapolis, IN) and disrupt the cells using a Precellys24 homogenizer (Bertin technologies, France). Briefly, add 1 ml cell suspension to a 2 ml lysis tube and add 10 ceramic beads. Lyse the cells with 3 x 60 sec pulses. The mechanical force of the process may increase the temperature within the sample above 37 $^{\circ}$ C so care should be taken to chill the sample on ice after the lysis procedure.
3. Cool the cells on ice and add 10 mM MgCl₂, 40 U/ml DNase A and incubate for 60 min at 37 $^{\circ}$ C.
4. Prepare solutions of 80%, 50%, 20% sucrose (w/v) in PBS pH 7.4 (10 ml each).
5. Prepare the sucrose gradient in a 15 ml falcon tube, starting with 2 ml 80% sucrose, followed by 50% and 20%. Pour the gradient slowly and be careful not to disturb already poured layers. Layer the digested lysate on top of the gradient and centrifuge for 15 min at 1000 x g at 4 $^{\circ}$ C.
6. Carefully collect the interphase between the 50-80% sucrose layer with a pipette and mix with 5 volumes of PBS pH 7.4. Centrifuge for 15 min at 1000 x g at 4 $^{\circ}$ C. Repeat washing two additional times. In this interphase, aggresomes are fluorescent and can be visualized in a microscope under UV light.
7. Discard the supernatant and resuspend the pellet in 250-500 μ l PBS pH 7.4. This pellet contains the purified aggresomes (**Figure 2**).

3. Treatment of Recipient SH-SY5Y Cells with mRFP/eGFP-DISC1 Aggresomes

1. Seed 5 x 10⁴ recipient SH-SY5Y human neuroblastoma cells constitutively expressing GFP-DISC1(598-854) on sterile glass coverslips in each well of a 24 well plate. SH-SY5Y cell are cultured in DMEM/F-12 medium supplemented with Penicillin/Streptomycin, non-essential amino acids (NEAA) and 10% FBS.
2. 24 hr later, add 10 μ l to each well and incubate for 48-72 hr.
3. Wash the cells 3 x with PBS pH 7.4 and quench extracellular fluorescence with 0.04% Trypan Blue for 5 min.
4. Fix the cells with 4% PFA in PBS pH 7.4 for 10 min on ice, wash once with sterile H₂O and mount the coverslips on glass slides with ProLong Gold with DAPI mounting medium (Invitrogen, USA).
5. Confirm the uptake/invasion of exogenously applied aggresomes by colocalization with recruited proteins expressed by recipient cells in Z-stack images.

Yet, uptake/invasion of exogenous protein might not essentially be detected in parallel with recruitment of host cell protein. In our example mRFP-tagged DISC1 aggresomes recruit soluble GFP-tagged DISC1(598-854) protein expressed by the host cell (see **Figure 3**). Pictures were taken on a Zeiss LSM 510 confocal- or a Zeiss Axiovision Apatome2 Microscope.

4. Generation of Recombinant DISC1(598-785)

In a previous publication we analyzed the ability of various DISC1 protein fragments to self-interact based on the presence of self-association domains. Among all protein fragments tested human DISC1(598-785) displayed the strongest multimerization propensity⁴ (**Figure 4**). Therefore, human DISC1(598-785) was cloned into pET15b (Novagen, Madison, Wisconsin) containing an N-terminal 6-histidine tag, expressed in *E. coli* BL21-(DE3) Rosetta (Novagen, US), and purified under denaturing conditions in 8 mol/l urea as described⁴. In short, the protocol is outlined below.

1. BL21-(DE3) Rosetta *E. coli* were grown in 2 x 500 ml 2YT containing 5 mM-arginine-HCl, 5mM MGSO₄, 100 μ g/ml carbencillin, 35 μ g/ml chloramphenicol up to an OD₆₀₀: 0.6-0.8 and DISC1(598-785) expression was induced with 1 mM IPTG.
2. DISC1(598-785) was expressed for 4 hr at 37 $^{\circ}$ C, the expression was terminated by harvesting the bacteria by centrifugation.
3. The bacterial pellet was resuspended in 50 ml TE buffer (50 mM TRIS-HCl pH 8.0, 5 mM EDTA) plus 2 mM PMSF, 1% TX-100, 250 μ g/ml lysozyme, 20 mM MgCl₂ and 400 U/50 ml DNase I. To ensure complete lysis, the reaction was incubated for 30 min at RT with gentle stirring.
4. Add 10 mM β -mercaptoethanol (ME) and 500 mM NaCl and incubate for another 30 min.
5. Spin down bacterial inclusion bodies at 20.000 g for 30 min at 4 $^{\circ}$ C.
6. Resuspend pellet in 50 ml extraction buffer containing 50mM Tris pH 8.0, 5 mM imidazole, 500 mM NaCl, 8 M urea and 10 mM β -ME and remove debris by centrifugation at 20.000 g for 30 min at 4 $^{\circ}$ C.
7. Wash the Ni-NTA agarose matrix with extraction buffer. Incubate the resuspended, precleared protein extract with Ni-NTA matrix for 2 hr at RT.
8. Wash the Ni-NTA matrix with extraction buffer containing 12 mM imidazole.
9. Elute the protein slowly with 15 ml elution buffer containing 50 mM Tris, 500 mM NaCl, 300 mM imidazole, 8 M urea, 1 mM PMSF, 5 mM EDTA and 10 mM β -ME.
10. Dialyze the protein stepwise to PBS pH 7.4 containing 10 mM β -ME.

From 1 L bacterial starting culture it is possible to isolate up to 50 mg of recombinant DISC1(598-785) protein.

α -synuclein refolding

For experiments with α -synuclein, 500 μ g of the recombinant protein (Sigma-Aldrich, USA) was dissolved in PBS at a concentration of 1 mg/ml. To obtain oligomers, refolding was performed overnight at 37 $^{\circ}$ C as described in a previous publication¹⁰.

5. Labeling of Recombinant DISC1(598-785) with DyLight594

1. Prior the subsequent labeling process, DISC1(598-785) protein has to be freed from β -ME. Therefore, the protein is dialyzed 3 times to PBS pH 7.4 at a dilution of 1:2,000.
2. Label 1 mg DISC1(598-785) with DyLight 594 maleimide (Thermo Scientific, USA) according to the manufacturers instructions. In short, dissolve 1 mg/ml protein in PBS pH 7.4 and add 5 mM TCEP to recover free thiol groups. Add 20 μ l of the DMF dissolved dye to the reaction and incubate for 2 hr at RT.

- Dialyze the protein 3 x to PBS pH 7.4 at a ratio of 1:2000 for 2 hr each.

To further increase the purity of the labeled protein a His₆-tagged based affinity-purification on a Ni-NTA column is performed.

- Wash 1 ml Ni-NTA matrix (Qiagen, Germany) with 10 ml PBS pH 7.4.
- Apply the labeled, dialyzed protein to the column and let it run over the column slowly.
- Wash the protein with 3 x 20 ml PBS pH 7.4
- Elute the protein with PBS pH 7.4, 500 mM imidazole dropwise while monitoring the colored band on the Ni-NTA column to reduce the eluate volume.
- Dialyze the eluate 3 x to 10 mM NaPi pH 7.4 at a dilution of 1:2000 for 2 hr each.
- Use a sterile 0.45 µm syringe filter to filter the eluate, aliquot in 5 x 100 µl samples and snap freeze in liquid nitrogen. The total amount of protein should be 0.5 - 1 µg/ml in each 100 µl aliquot.

optional concentration step

- For stereotactical rat injections, the protein was concentrated 10-fold in a speed-vac (Eppendorf Concentrator 5301). The final buffer condition was 100 mM NaPi.

α-synuclein Labeling

The labeling and purification (Ni-NTA column) of the recombinant α-synuclein was performed in parallel to DISC1(598-785). The total starting material was 500 µg instead of 1 mg for DISC1(598-785).

6. Treatment of Recipient SH-SY5Y Cells with Labeled Recombinant DISC1 (598-785) Protein

- Seed 5x10⁴ recipient SH-SY5Y human neuroblastoma cells constitutively expressing GFP-DISC1(598-854) on sterile glass coverslips in each well of a 24 well plate.
- Add labeled protein to the cell culture medium at a concentration of 5-10 µg/ml and incubate for 48-72 hr.
- Wash the cells 3 x with PBS pH 7.4 and quench extracellular fluorescence with 0.04% Trypan Blue for 5 min.
- Fix the cells with 4% PFA in PBS pH 7.4 for 10 min on ice, wash once with sterile H₂O and mount the coverslips on glass slides with ProLong Gold with DAPI mounting medium (Invitrogen, USA).
- Confirm the uptake/invasion of exogenously applied recombinant protein by colocalization with recruited proteins expressed by recipient cells in Z-stack images generated by laser scanning confocal microscopy. Yet, recruitment of endogenous protein might not essentially be detected in parallel with invasion of host cell protein, Z-stack images can still confirm the invasive nature of the protein.

In our example rec. DISC1(598-785)* at least in part recruits soluble GFP-tagged DISC1(598-854) protein expressed by the host cell (see **Figure 5A**). For recombinant α-synuclein invasion but no recruitment is shown (**Figure 5B**). Pictures were taken on a Zeiss LSM 510 confocal- or a Zeiss Axiovision Apotome2 Microscope.

The injection of the concentrated (ca. 4 µl of 2.5 µg/µl), labeled DISC1(598-785)* into the mPFC results in the spreading of the protein around the injection site which can be detected even after perfusion of the animal with 4% PFA in PBS pH 7.4 with a rhodamine filterset. In our example DISC1(598-785) is taken up by a distinct number of neurons and can be monitored with Z-stack imaging (**Figure 6**).

In general, the injection of proteins other than the ones used here do not necessarily lead to cell-invasion. The invasion event is essentially dependent on the nature of the protein, nevertheless a clean purification is a prerequisite for further analysis.

7. Representative Results

Aggresomes consisting of recombinant FL DISC1-eGFP purified from NLF cells invaded recipient SH-SY5Y cells at low efficiency (approximately 0.3%⁵) as seen by colocalization with confocal microscopy (**Figure 3**). As previously reported, DISC1(598-785) expressed and purified from *E. coli* formed multimers⁴ were equally cell-invasive at an efficiency of approximately 20%⁵ (**Figure 5A**) similar to that of α-synuclein that was used in parallel as positive control (**Figure 5B**). Labeled recombinant DISC1(598-785) expressed and purified from *E. coli* was also cell-invasive to neurons *in vivo* when the multimeric protein was stereotactically injected into the medial prefrontal cortex of a rat (**Figure 6**; Pum, Bader, Huston, Korth, unpublished).

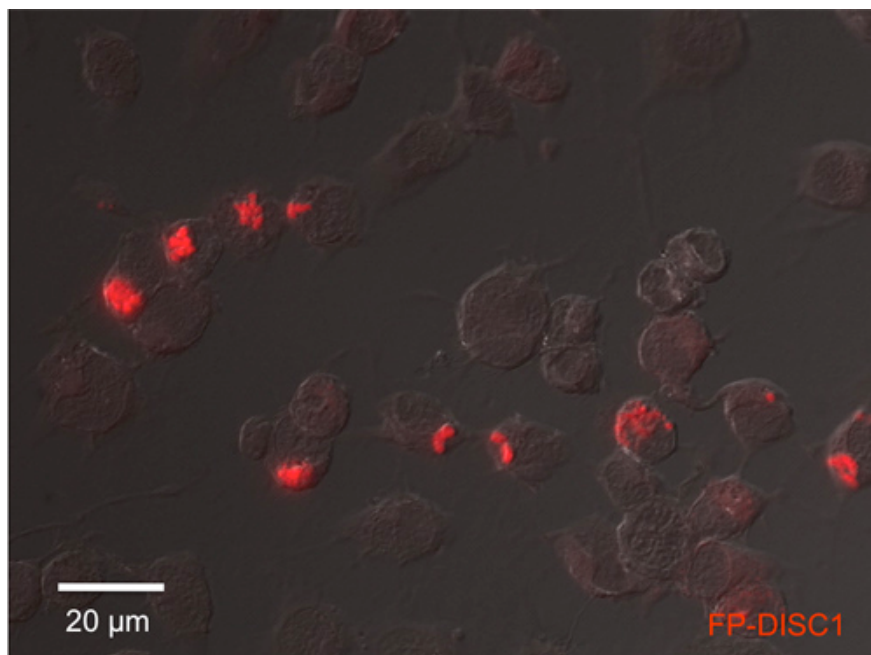


Figure 1. NLF neuroblastoma cells transiently expressing FP-DISC1 (red). Red fluorescent aggresomes can be detected within the cells.

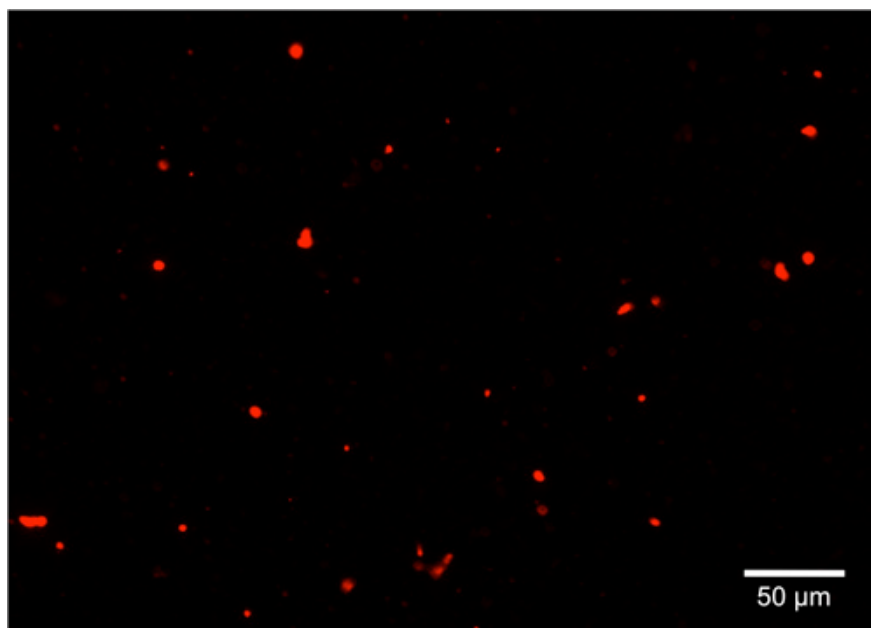


Figure 2. Purified mRFP-tagged DISC1 aggresomes after the purification in a sucrose gradient.

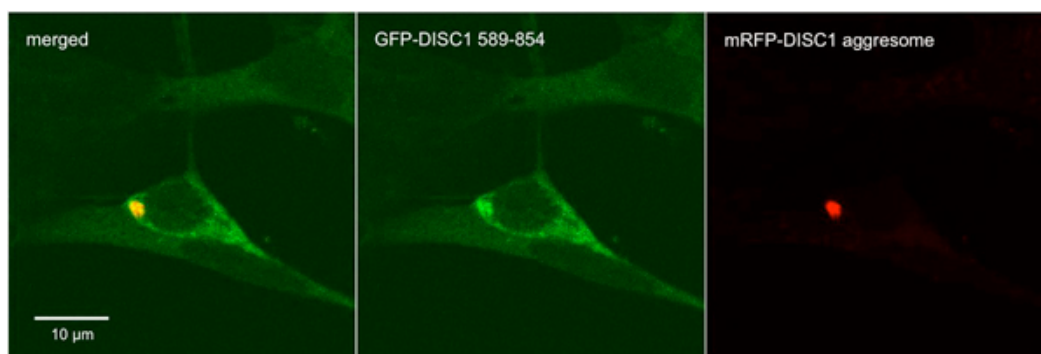


Figure 3. Fluorescent picture of invaded aggregates. mRFP-tagged fDISC1 aggregates were purified and incubated with SH-SY5Y neuroblastoma cells overexpressing soluble GFP-tagged DISC1(598-854). An uptake of labeled aggregates is monitored by Z-stack imaging on a Laser-Scan microscope (Zeiss LSM 510).

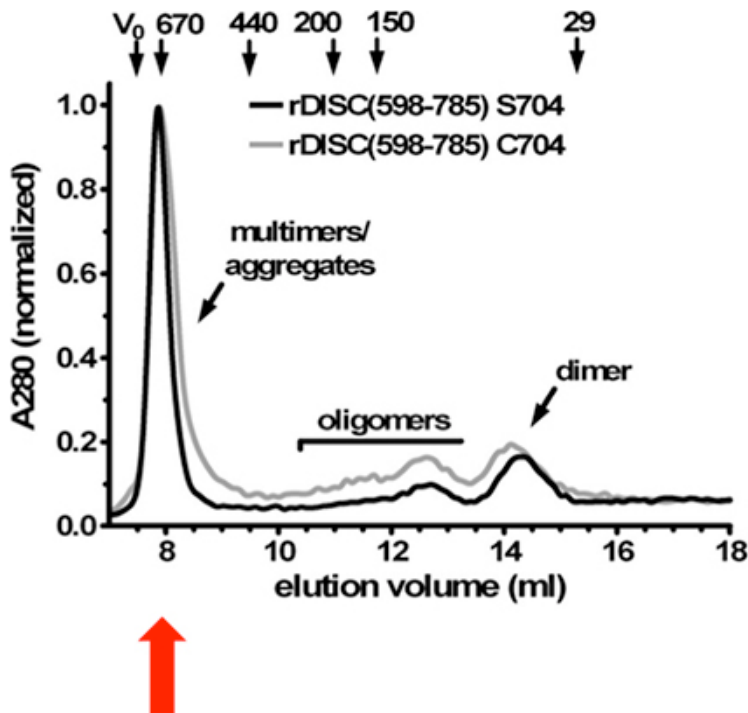


Figure 4. SEC-profile of rec. DISC1(598-785) containing the S704 and the C704 single nucleotide polymorphisms (SNP). Both variants show a disruption of ordered oligomerization and the formation of high molecular weight multimers (red arrow). This picture is modified from the publication of Leliveld *et al.*, *Biochemistry* 2009.

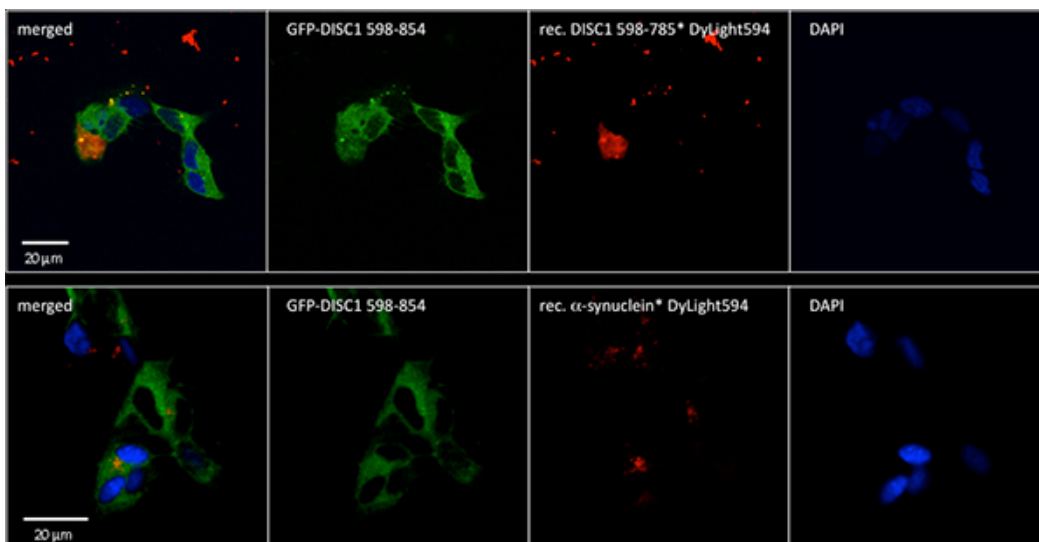


Figure 5. Fluorescent Z-stack picture of invasive DyLight-labeled recombinant DISC1(598-785). SH-SY5Y neuroblastoma cells expressing soluble GFP-DISC1(598-854) were incubated with labeled, recombinant protein at a concentration of 5 μg/ml. (A) Red aggregates shows the invasion of rec. DISC1(598-785) protein and yellow dots indicate a recruitment of soluble GFP-DISC1(598-854) into aggregates. (B) Recombinant α-synuclein (red) invades the cells without recruitment with a frequency of about 20%. [Click here to view larger figure.](#)

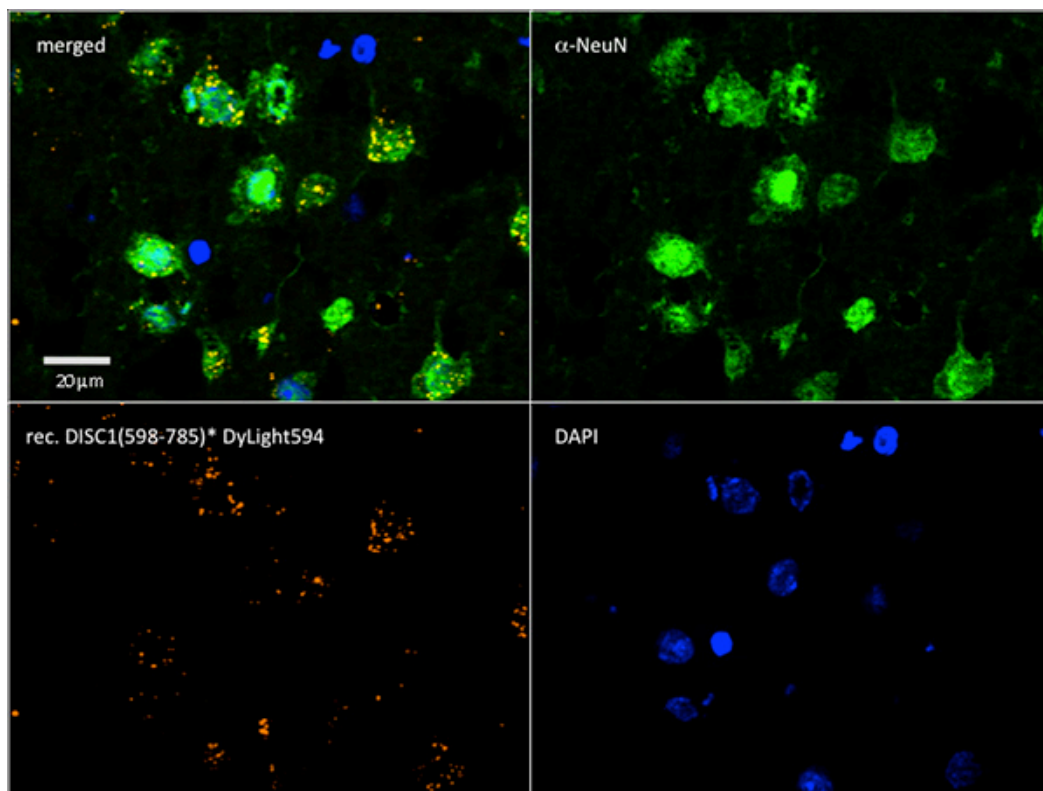


Figure 6. Immunofluorescent picture of the injected labeled, recombinant DISC1(598-785) protein. Z-stack imaging confirms the presence of rec. DISC1(598-785) protein in rat cortical neurons stained with an antibody against neuronal nuclei (NeuN, green).

Discussion

In this study we describe the purification of mRFP/eGFP-DISC1 aggresomes from a transfected neuroblastoma cell line, the preparation and labeling of a recombinant DISC1 protein species and their application in cell-invasion experiments of recipient cells *in vitro* and *in vivo*.

The purification of native mRFP/eGFP-DISC1 aggresomes was developed from protocols to isolate Lewy body-like structures and larger aggregates^{13, 14}, but modified to avoid the use of detergents and to minimize the risk of false positive cell-invasion that might occur due to detergent-facilitated membrane penetration.

One possible future improvement might be the in to further increase the purity of the aggresomes by sonication to completely separate remaining membranes and cytoskeleton from the aggresomes. By increasing overall purity, the number of total invasion events that recorded for large aggresomes may be increased⁵. Whether the limited definition of our suggested 3-phase sucrose is sufficient for aggresomes of other protein species has to be tested, in this sense the protocol outlined here should be considered as a starting point for individual optimization. The purified aggresomes proved to be quite robust in our hands, additional washing steps (without detergent) to eliminate traces of sucrose did not significantly reduce the overall yield.

In a previous publication we described that oligomer assembly of DISC1 is dependent on distinct multimerization domains in the C-terminus⁴ (**Figure 4**). We chose this fragment for recombinant soluble expression in *E. coli* and subsequent and Ni-NTA purification. To monitor its multimerization and to compare its cell invasiveness with a described cell-invasive protein like α -synuclein, we labeled DISC1(598-785) with the fluorescent dye DyLight594, and compared its cell-invasiveness with that of equally labeled α -synuclein. The cell-invasiveness of the recombinant DISC1 fragment was dramatically increased compared to that of native, full length DISC1 aggresomes, likely due to its smaller size and much higher purity. Again, purity seems to play a decisive role in cell-invasiveness.

In our example the invasive aggresomes recruited soluble homologue protein of the target cell line that was recombinantly expressed in a soluble, *i.e.* cell-dispersed form. The expression of a fluorescent protein (*e.g.* GFP or RFP) in the recipient cell line is an advantage since it allows the colocalization of invasive aggresomes and recombinant proteins within the recipient cell limit via Z-stack imaging.

Cell-invasive DISC1 aggresomes and multimeric fragments expressed and purified from *E. coli* could be a defining feature of a protein conformational diseases relating to DISC1, DISC1opathies¹⁵.

Trouble shooting:

Low aggresome yield after sucrose gradient purification:

The sucrose gradient introduced in this protocol works well with eGFP/mRFP-DISC1(FL) aggresomes. Aggresomes consisting of other proteins might be of variable dimensions therefore the sucrose concentrations should be optimized by other users.

Insufficient purification of recombinant protein:

Any bacterial contaminants in the purification process of the recombinant protein will also be labeled in later steps of the protocol. To avoid contaminations, run the protein on a SDS-PAGE and confirm that the recombinant protein is at least 95% pure by staining with Coomassie-Blue. To ensure highest quality and yield, the protocol has to be optimized for each individual protein.

Low labeling efficiency of recombinant proteins:

Any contaminations that contain free SH-groups will decrease efficient labeling of the protein. Therefore, extensive dialysis and Ni-NTA based purification is mandatory.

Disclosures

No conflicts of interest declared.

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