Development of a highly sensitive immunoassay to measure ataxin2 as a target engagement marker in Spinocerebellar Ataxia Type 2

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Research Article

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Abstract

Background Spinocerebellar Ataxia Type 2 (SCA2) belongs to a group of neurodegenerative diseases, inherited as an autosomal dominant trait. SCA2 is a trinucleotide repeat disease with a CAG repeat expansion in exon 1 of the *ATXN2*gene resulting in an ataxin-2 protein with an expanded polyglutamine (polyQ)-stretch. The disease is late manifesting leading to premature death. Today, therapeutic interventions to cure the disease or even to decelerate disease progression are not available yet. Furthermore, primary readout parameter for disease progression and therapeutic intervention studies are limited. Thus, there is an urgent need for quantifiable molecular biomarkers such as ataxin-2 becoming even more important due to numerous potential protein reducing therapeutic intervention strategies.

Objective Aim of this study was to establish a sensitive technique to measure the amount of polyQ-expanded ataxin-2 in human biofluids to evaluate ataxin-2 protein levels as prognostic and/ -or therapeutic biomarker in SCA2.

Methods Time-Resolved Fluorescence Energy Transfer (TR-FRET) was used to establish a polyQ-expanded ataxin-2-specific immunoassay. Two different ataxin-2 antibodies and two different polyQ-binding antibodies were validated in three different concentrations and tested in cellular and animal tissue as well as in human cell lines, comparing different buffer conditions as well as total protein concentrations to evaluate the best assay conditions.

Results We established a TR-FRET-based immunoassay for polyQ-expanded ataxin-2 and validated measurements in human cell lines including primary skin fibroblasts, induced pluripotent stem cells (iPSCs) and iPSC-derived cortical neurons. Additionally, our immunoassay was sensitive enough to monitor small ataxin-2 expression changes by siRNA or starvation treatment.

Conclusion We successfully established the first sensitive ataxin-2 immunoassay to measure specifically polyQ-expanded ataxin-2 in human biomaterials.

Introduction

Spinocerebellar ataxia type 2 (SCA2) is an autosomal dominantly inherited neurodegenerative disease which is caused by the expansion of the triplet CAG in exon 1 of the *ATXN2* gene localized on chromosome 12q24 [1, 2]. The triplet CAG encodes for the amino acid glutamine (Q) and therefore, SCA2 belongs to the group of polyglutamine (polyQ) expansion diseases like Huntington´s disease (HD), SCA1, 3, 6, 7, 17, dentatorubral-pallidoluysian atrophy (DRPLA) and spinal and bulbar muscular atrophy (SBMA). Normal repeat length varies between 14–29 glutamines (most individuals have 22Q) [3], intermediate length alleles (27-33Q) are associated with amyotrophic lateral sclerosis (ALS) [4] and a length of more than 32Q results in SCA2 [5], while most SCA2 patients show expanded repeats between 36 to 52, respectively [6, 7] and rare expansions beyond 800Q were observed [8].

For SCA2, mean age of onset is about 32 years. Age of onset, disease duration but also clinical manifestation show substantial variability. Key features include cerebellar ataxia and slowing of saccadic eye movements in variable combination with cerebellar dysarthria, dysphagia, peripheral neuropathy, and postural tremor. Degeneration of Purkinje cells in the cerebellum is the main neuropathological hallmark of SCA2 [7, 9–21].

The protein ataxin-2 (ATXN2) is predominantly localized in the cytosol [22] and has a role in posttranscriptional RNA modification, quality control and translation [23–27]. Under stress conditions, the expression and translation of ATXN2 is enhanced, and the protein relocalizes into stress granules [28].

So far there is no curative therapy for SCA2 [29]. Clinical studies are difficult as primary readout parameters reflecting disease activity or key pathogenic processes are limited. Clinical scores like the Scale for the Assessment and Rating of Ataxia (SARA) are commonly used to monitor disease progression [30]. For other polyQ diseases like HD [31–33] or SCA3 [34, 35], TR-FRET-based immunoassays for quantification of polyQ-expanded disease proteins, like huntingtin or ataxin-3, have been established. Additionally, to detect very low protein concentrations in peripheral blood or CSF, ultrasensitive single molecule counting (SMC) immunoassays were validated [36, 37] and used as prognostic and/or therapeutic readout parameters. In SCA2, the disease protein ATXN2 could also represent a potential prognostic and/or therapeutic biomarker, as the amount of polyQ-expanded ATXN2 in e.g., blood or CSF may reflect the course of the disease and potential therapeutic success. Therefore, the aim of this study was to develop a sensitive assay for the quantitative determination of the polyQ-expanded ATXN2 protein in patient derived cell lines and mammalian biomaterials.

Materials And Methods

Ethical use of animals

All mice were maintained by animal care staff and veterinarians of the University of Frankfurt/Main Zentrale Forschungs-Einrichtung (ZFE). All procedures were performed according to the German Animal Welfare Act and the guidelines of the Federation of European Laboratory Animal Science Associations, based on European Union legislation (Directive 2010/63/EU). Animal experiments were approved by the local ethics committee (Regierungs-Präsidium Darmstadt V54-19c18-FK/1083).

Ethical use of human tissue

All the work involving human tissue has been carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) and with national legislation as well as our institutional guidelines. Experiments were approved by the local ethics committee (ethical vote Tübingen, 598/2011BO1 and 911/2019BO2).

Mouse and iPSC sample description

Different mouse tissues (liver, hemisphere, cerebellum) isolated from young (2.5–3 months) and old (14–18 months) knock-in mouse lines modeling SCA2, including *ATXN2*-CAG42 [38] and *Atxn2*-CAG100 knock-in mice [39] were used during the establishment of the assay conditions. The generation of mouse embryonic fibroblast (MEF) lines from *Atxn2*-CAG100 knock-in mice was reported before [39]. Human fibroblasts were isolated from skin biopsies of three SCA2 patients and three neurologically healthy controls after written informed consent. Fibroblasts were reprogrammed into induced pluripotent stem cells (iPSCs) as specified earlier [40] and differentiated into cortical neurons (CNs) as described before [41]. Specification of analyzed human cell lines are represented in Table 1.

Table 1
Specification of human cell lines used in this study:

Line	Number of iPSC clones	*ATXN2* CAG repeat length	Age at biopsy	Sex
Co-1	1	not determined	74 years	male
Co-2	1	14/21	69 years	female
Co-3	1	14/23	46 years	female
Co-4	1	21/23	46 years	male
Ax-1	2	23/36	31 years	male
Ax-2	1	22/42	36 years	female
Ax-3	1	22/38	45 years	female

Cell culture and transfection

MEF and human embryonic kidney (HEK293T) cells were maintained in DMEM (Thermo Fisher Scientific, Waltham, USA) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics-antimycotics (both Thermo Fisher Scientific, Waltham, USA) at 5% CO_2 and 37°C. For MEF, experiments were carried out only within the first 5 passages. Different ATXN2 plasmids including GFP-ATXN2-22Q/myc-ATXN2-22Q, GFP-ATXN2-79Q/myc-ATXN2-79Q or GFP-empty/myc-empty as internal control were used for transfection experiments.

HEK293T cells were transfected with GFP or myc ATXN2 plasmids with different polyQ length (GFP-22Q/myc-22Q, GFP-79Q/myc-79Q or GFP-empty/myc-empty as internal control) with Attractene transfection reagent (Qiagen, Hilden, Germany) using the traditional transfection protocol. Shortly, 24 h before transfection, 400 000 HEK293T cells per well were seeded on a six-well tissue culture plate in DMEM medium. For transfection, 1.2 µg plasmid DNA, 100 µl OptiMEM (Thermo Fisher Scientific, Waltham, USA) and 4.5 µl Attractene Transfection Reagent were mixed, incubated for 15 min at room temperature (RT), and afterwards dropped carefully on the seeded cells. 72 h after transfection, HEK293T cells as well as MEF cells were harvested with DPBS (Thermo Fisher Scientific, Waltham, USA), centrifuged for 5 min at 300*g* and directly lysed in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% IGEPAL) supplemented with cOmplete (EDTA-free) Protease Inhibitor

(Roche, Mannheim, Germany) followed by incubation on ice for 30 min and vortexing every 10 min. For lysate preparation, the cell homogenates were centrifuged for 30 min at 4°C and 16 100*g*. The Bradford protein assay was used to determine the total protein concentration [42].

Cell culture starvation and siRNA experiments

For the starvation experiments, 400 000 HEK293T cells per well were seeded on a six-well tissue culture plate and transfected with ATXN2 GFP-22Q, GFP-79Q or GFP-empty as control, as described above.

72 h after transfection, the medium was changed to Hanks` Balanced Salt Solution (Thermo Fisher Scientific, Waltham, USA) and incubated for either 0 h, 1 h or 2 h, respectively. After incubation, cells were harvested as described.

For esiRNA experiments, knockdown of ATXN2 was achieved using endoribonuclease-prepared siRNA (esiRNA) directed against human ATXN2 (MISSION® esiRNA EHU104101, Sigma-Aldrich) or control esiRNA against Renilla luciferase (MISSION® esiRNA EHURLUC, Sigma-Aldrich). 400 000 HEK293T cells per well were seeded on a six-well tissue culture plate. The transfection followed the traditional Attractene transfection protocol (Qiagen, Hilden, Germany) with 1.2 µg plasmid DNA (ATXN2 GFP-22Q, ATXN2 GFP-79Q or GFP-empty as control), 18 pmol esiRNA and 4.5 µl Attractene Transfection Reagent. 72 h after transfection, cells were harvested with DPBS, centrifuged for 5 min at 300*g* and lysed in RIPA for 30 min on ice and vortexing every 10 min.

Protein extraction

For the establishment of the TR-FRET, mouse tissues and cells were homogenized in three different lysis buffers: RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% IGEPAL), TES buffer (20 mM Tris, pH 7.5, 2 mM EDTA, 100 mM NaCl) supplemented with TNES buffer (50 mM Tris pH 7,5, 2 mM EDTA, 100 mM NaCl, 1% IGEPAL) or PBS buffer (DPBS (1X) Dulbecco's Phosphate-Buffered Saline with 1% Triton™ X-100). All buffers were supplemented with cOmplete (EDTA-free) Protease Inhibitor (Roche, Mannheim, Germany). For each buffer conditions, 10 mg tissue or cells were homogenized mechanistically in 100 µl of the respective buffer using the VDI 12 homogenisator (VWR, Darmstadt, Germany). Afterwards, homogenates were incubated on ice for 30 min vortexing every 10 min. Homogenates were stored at -80°C for later TR-FRET analyzes. Part of the homogenates were centrifuged at 13 200*g* for 30 min. Lysates were transferred to new collection tubes supplemented with 10% glycerol and stored at -80°C for later western blot analyses. The Bradford protein assay was used to determine the total protein concentration from homogenates and lysates [42].

SDS-PAGE and Western Blot

20 to 30 µg of total protein from cell culture or mouse tissue lysates were supplemented with 4 × LDS sample buffer (1 M Tris pH 8.5, 2 mm EDTA, 8% LDS, 40% glycerol, 0.075% CBB G, 0.025% phenol red) in a ratio 3:1 and 0.1 M dithiothreitol. Samples were heat denatured for 10 min at 70°C and afterwards, electrophoretically separated [43] using 8 to 10% Bis-Tris gels with the electrophoresis MOPS buffer (50 mM MOPS, 50 mM Tris pH 7.7, 0.1% SDS, 1 mM EDTA) at 100 V, 250 mA for 2–2.5 h. Proteins were

blotted [44] on nitrocellulose membranes (Amersham Protran Premium 0.2 µm, GE Healthcare) using transfer buffer (25 mM Bicine, 25 mM Bis-Tris pH 7.2, 1 mM EDTA, 15% methanol) at 80 V and 250 mA for 1.5 h. After transfer, the membranes were blocked with 5% skim milk powder in Tris-buffered saline (TBS) for 1h, washed with TBS-T (TBS with 0.1% Tween 20) and incubated overnight at 4°C with the following primary antibodies diluted in TBS-T: ataxin-2 polyclonal antibody, (1:1000, 21776-1-AP, rabbit, Proteintech Group, Rosemont, USA); mouse anti-β-actin monoclonal antibody (1:500, clone AC-15, Sigma-Aldrich, Darmstadt, Germany). Afterwards, membranes were washed with TBS-T and incubated at RT for 1 h with secondary IRDye antibodies goat anti-mouse 800CW or goat anti-rabbit 800CW (both 1:5000, Li-Cor Biotechnology GmbH, Bad Homburg, Germany), respectively. After washing with TBS-T, fluorescence signals were detected using the LI-COR ODYSSEY FC and quantified with Image Studio 4.0 software (both Li-Cor Biotechnology GmbH, Bad Homburg, Germany).

Time resolved fluorescence energy transfer (TR-FRET)

To establish a polyQ-expanded ATXN2 specific TR-FRET-based immunoassay, two different ATXN2 antibodies and two different polyQ-specific antibodies were compared. Therefore, the two ATXN2 specific antibodies ataxin-2 polyclonal antibody (21776-1-AP, Proteintech Group, Rosemont, USA) and purified mouse anti-ataxin-2 monoclonal antibody (AB_398900, Becton, Dickinson and Company, Sparks, USA) were labelled with the fluorophore Tb by the company CisBio Inc. (PerkinElmer, Waltham, USA). Additionally, the two polyQ-specific antibodies clone MW1 (AB 528290, Development Studies Hybridoma Bank, Iowa, USA) and clone 5TF1-1C2 (MAB1574, Sigma-Aldrich, Darmstadt, Germany) received the acceptor fluorophore D2 by the same company. For establishment of the TR-FRET-based immunoassay, the labelled antibodies were diluted in detection buffer (50 mM NaH_2PO_4, 400 mM NaF, 0.1% BSA, 0.05% Tween-20) in different concentrations (Tb-labelled antibodies: 0.3 ng, 0.5 ng, 1 ng and D2-labelled antibodies: 1 ng, 3 ng, 10 ng). Mouse tissue or cell culture homogenates were diluted to a total protein concentration of either 2 µg/µl or 1 µg/µl in one of the following buffers (RIPA, TES/TNES or PBS) supplemented with cOmplete (EDTA-free) Protease Inhibitor. 5 µl per diluted sample were incubated in duplicates with 1 µl antibody-mix (tb-antibody with d2- antibody, ratio 1:1) in a low-volume white ProxiPlate 384 TC Plus plate (PerkinElmer, Waltham, USA) for 24 h at 4°C. Signals were detected at 620 nm and 665 nm with the Multimode Plate Reader Envision (PerkinElmer, Waltham, USA) and the ratio between 665/620 was normalized to the total protein concentration and over the background signal (deltaF).

Statistical analysis

The program GraphPad Prism 8 (GraphPad Software Inc., San Diego, USA) was used for the statistical evaluation of all data and for data visualization. Normality of the data sets were evaluated by Shapiro-Wilk test. Due to the non-normally distributed data, the non-parametric Mann-Whitney U-test was used to compare the different groups. P-values with less than 0.05 were considered statistically significant with * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. All values are shown as mean ± standard error of the mean, SEM.

Results

Establishment of antibody combination and concentration

To establish a TR-FRET-based immunoassay to quantify specifically polyQ-expanded ATXN2 protein, two different Tb-labelled ATXN2 specific antibodies (polyclonal ATXN2 antibody (Ataxin2poly-Tb, aa 251–600) and monoclonal ATXN2 antibody (Ataxin2mono-Tb, aa 713–904)) as well as two different D2-labelled polyQ-specific antibodies (MW1-D2 (≥ 15Q) and 1C2-D2 (≥ 37Q)) were tested in combination with each other, each at three different concentrations (for Tb 0.3 ng, 0.5 ng and 1 ng/µl, for D2 1 ng, 3 ng and 10 ng/µl) (schematic representation in Fig. 1a). If both antibodies bind specifically and in close proximity, an energy transfer takes place between the donor (Tb) and the acceptor (D2). The measured signal is proportional to the ATXN2 protein concentration of the tested sample and needs to be normalized to the total protein concentration and over the background signal. To evaluate the different antibody combinations, liver homogenates from *Atxn2*-CAG100 knock-in mice [39] (blue; KI) compared to wildtype mice (red; WT) and homogenates of MEF from *Atxn2*-CAG100 knock-in mice [39] compared to wildtype mice were generated in RIPA buffer. For the antibody combination Ataxin2poly-Tb x MW1-D2, the best discrimination between WT and KI was achieved with the concentration of Ataxin2poly-Tb 0.3 ng/µl x MW1-D2 3 ng/µl (labelled in green, Fig. 1b). Antibody combination Ataxin2poly-Tb x 1C2-D2 showed the best discrimination between WT and KI with the concentration of Ataxin2poly-Tb 0.3 ng/µl x 1C2-D2 10 ng/µl (labelled in green, Fig. 1c). Evaluation of MW1-D2 or 1C2-D2 in combination with the monoclonal ATXN2 antibody (Ataxin2mono-Tb) showed the best discrimination between WT and KI at the concentration of Ataxin2mono-Tb 0.5 ng/µl x MW1-D2 3 ng/µl (labelled in green, **supplementary information** Fig. 1a) or with Ataxin2mono-Tb 0.5 ng/µl x 1C2-D2 10 ng/µl (labelled in green, **supplementary information** Fig. 1b). In general, TR-FRET signals were lower when using the monoclonal ATXN2 antibody compared to the polyclonal ATXN2 antibody.

Establishment of buffer conditions

Next, three different lysis buffers (RIPA, PBS and TES/TNES) were compared (Figs. 1d **+ e**) for the antibody combinations Ataxin2poly-Tb x MW1-D2 (Fig. 1d) and Ataxin2poly-Tb x 1C2-D2 (Fig. 1e), each at the best antibody concentrations as established for RIPA buffer in Figs. 1b **+ c**, for PBS buffer in **supplementary information** Figs. 1c **+ d** and for TES/TNES buffer in **supplementary information** Figs. 1e **+ f**. Protein homogenates in RIPA buffer reached the highest signals and the best discrimination between WT and KI. Therefore, all further experiments were carried out in RIPA buffer.

Establishment of protein amount

After establishment of the antibody combinations, concentrations and buffer conditions, the total protein concentration was evaluated using MEF homogenates including 2 µg/µl or 1 µg/µl whole protein concentration isolated from *Atxn2*-CAG100 knock-in mice (blue; KI) or wild-type controls (red; WT) (Fig. 1f). Additionally, homogenates (2 µg/µl or 1 µg/µl) from HEK293T cells transfected with myc ATXN2 plasmids including myc-22Q (red; 22Q) and myc-79Q (blue; 79Q) or empty plasmid (black; Ø) (Fig. 1g) including 2 µg/µl or 1 µg/µl whole protein concentration were used. Samples were lysed in RIPA buffer

and measured with the antibody combination Ataxin2poly 0.3 ng/µl x MW1-D2 3 ng/µl. Results for the antibody combination Ataxin2mono-Tb 0.5 ng/µl x MW1-D2 3 ng/µl are shown in the supplement (**supplementary information** Figs. 2a **+ b**). Overall, measurements demonstrated that a lower total protein concentration of 1 µg/µl showed higher polyQ-expanded ATXN2 specific signals and a better discrimination between WT and KI samples.

In summary, the polyclonal ATXN2 antibody Ataxin2poly-Tb discriminated better between ATXN2 WT and KI samples in comparison to the monoclonal antibody Ataxin2mono-Tb. Therefore, further experiments were performed either in the combination Ataxin2poly-Tb x MW1-D2 with the antibody concentration 0.3 ng/µl x 3 ng/µl or in the combination Ataxin2poly-Tb x 1C2-D2 with antibody concentration of 0.3 ng/µl x 10 ng/µl. Additionally, RIPA buffer and a total protein concentration of 1 µg/µl were used in all further measurements.

SCA2 specificity

As mentioned before, SCA2 belongs to the group of polyQ diseases including SCA1, 3, 6, 7, HD, DRPLA and SBMA. In each of these diseases, neurodegeneration is mainly triggered by translation of disease proteins with an elongated polyQ region that can potentially be detected by the polyQ-specific antibodies MW1 (≥ 15Q) and 1C2 (≥ 37Q). For the development of an immunoassay to quantify of the polyQ-expanded protein ATXN2 in human biomaterials it is therefore essential to demonstrate that no other polyQ proteins are detectable. For that reason, we performed a specificity experiment including cerebellar homogenates from three wildtype controls and SCA2, SCA3, SCA17 and HD mouse models. The antibody combinations Ataxin2poly-Tb 0.3 ng/µl x MW1-D2 3 ng/µl and Ataxin2poly-Tb 0.3 ng/µl x 1C2-D2 10 ng/µl specifically measured polyQ-expanded disease protein only in the SCA2 samples (**supplementary information** Figs. 2c **+ d**). As observed before, ATXN2 values were higher if measured with Ataxin2poly-Tb x MW1-D2, but demonstrated also low background signals in controls and in other polyQ diseases.

Detection of small changes in ATXN2 expression levels using siRNA and starvation experiments

As the primary reason to develop an immunoassay is to monitor therapeutic studies, the assay should be able to detect even small protein changes. Therefore, we downregulated ATXN2 expression in HEK293T cells using siRNA and increased polyQ-expanded ATXN2 levels by starvation experiments. For downregulation, HEK293T cells were transfected with GFP ATXN2 plasmids expressing 22Q or 79Q and treated with either ATXN2 siRNA (ATXN2), or as control reference with luciferase siRNA (Luc), or without siRNA (Ø, untreated). For starvation experiments, HEK293T cells transfected with GFP ATXN2 plasmids expressing 22Q or 79Q were grown in HBSS medium for 1 or 2 hours to induce starvation. Western blot analyses detected the ATXN2 protein at 150 kDa using the polyclonal Ataxin2poly antibody (Fig. 2a **+ b)**. Quantification of the ATXN2 22Q or 79Q protein expression under ATXN2 siRNA treatment revealed a slight downregulation compared to untreated or luciferase siRNA treated cells (Fig. 2c **+ e**). Additionally, inducing starvation revealed increased ATXN2 expression over time (Fig. 2d **+ f**). Measuring the same samples as analyzed by western blot with the TR-FRET immunoassay revealed a clear downregulation of

ATXN2 after ATXN2 siRNA treatment (Fig. 2g). The TR-FRET immunoassay analysis of the starvation experiments with 0, 1 or 2 hours of starvation in HBSS medium detected an upregulation after 2 hours of starvation using the antibody combination Ataxin2poly-Tb 0.3 ng/µl x 1C2-D2 10 ng/µl (Fig. 2h).

ATXN2 protein level in human material

To assess if the developed immunoassay also measures human polyQ-expanded ATXN2 specifically in human cell lines, different human cell lines including human fibroblasts (Fig. 3a) from SCA2 patients n = 3 (red; F-AX) and controls n = 4 (blue; F-Co), human iPSCs (Fig. 3b) from SCA2 patients n = 4 (red; iPSC-AX) and healthy controls n = 4 (blue; iPSC-Co) and human CNs (Fig. 3c) from SCA2 patients n = 2 (red; iCN-AX) and healthy controls n = 4 (blue; CN-Co) were diluted in RIPA buffer and tested with a total protein concentration of 1 µg/µl with the antibody combination Ataxin2poly-Tb 0.3 ng/µl x MW1-D2 3 ng/µl. Our data showed that the newly developed TR-FRET immunoassay is capable to detect specifically polyQ-expanded ATXN2 in patient-derived SCA2 biomaterials compared to respective controls. Western blot analyses confirmed ATXN2 expression in different cell lines (**supplementary information** Fig. 1e).

Discussion

In this study, we developed the first highly sensitive TR-FRET-based immunoassay to measure the polyQ-expanded protein ATXN2 in cellular and animal models as well as human neuronal cell culture.

There is currently no curative therapy for the trinucleotide repeat disease SCA2. One main reason for this is the lack of easily accessible, objective and sensitive outcome parameters to track disease progression but also the lack of effective molecular target treatments. Unfortunately, clinical read outs such as the Scale for the Assessment and Rating of Ataxia (SARA) need long observation periods due to slow disease progression, show substantial day to day variability and interrater differences and rare prone to unspecific changes e.g., due to injuries. For that reason, molecular markers that respond quickly to therapeutics and are readily detectable in biomaterials such as CSF or peripheral blood are of major importance for monitoring on-target effects for upcoming clinical trials. Recently, it has been shown that the protein tau has increased levels in CSF of SCA2 patients [45] and the protein neurofilament light (NfL) chain is suitable to indicate axonal degeneration in many neurodegenerative diseases, including SCA2 [46, 47]. Therapeutic strategies that reduce specifically polyQ-expanded protein levels are currently being applied to slow or stop disease progression in polyQ diseases like SCA1 [48, 49], SCA2 [50], HD [51, 52] and SCA3 [53–56]. Therefore, the disease proteins itself could serve as an excellent and primary potential therapeutic biomarker to monitor disease protein lowering by e.g. antisense oligonucleotides. In HD [31–33] and SCA3 [34, 35], immunoassays based on the TR-FRET technology were already established to quantify the respective disease protein in human leukocytes, mononuclear cells (PBMCs) or human tissue samples. Later, the TR-FRET-based immunoassays were further developed into ultra-sensitive single molecule counting (SMC) immunoassays in order to be able to quantify fmol protein amounts in CSF and peripheral blood of HD and SCA3 mutation carrier, too [36, 37]. Following it's successful establishment in HD and SCA3, the polyQ-expanded ATXN2 protein should also be validated as potential prognostic and therapeutic biomarker in SCA2.

To establish a new TR-FRET-based immunoassay for ATXN2, two antibodies which bind specifically to the disease protein are required. As shown for HD and SCA3, specific detection of the polyQ-expanded disease protein can be achieved by using one polyQ-specific antibody, like MW1. In our study, two different polyQ antibodies were tested as acceptor antibodies: MW1 (AB 528290, Development Studies Hybridoma Bank, Iowa, USA) and 1C2 (MAB1574, Sigma-Aldrich, Darmstadt, Germany). To achieve SCA2 specificity, two ATXN2 specific antibodies were tested as donor antibody including a polyclonal ATXN2 antibody (Ataxin2poly, 21776-1-AP, rabbit, Proteintech Group, Rosemont, USA) and a monoclonal ATXN2 antibody (Ataxin2mono, AB_398900, Becton, Dickinson and Company, Sparks, USA). Determination of best antibody pair and conditions revealed better results for the polyclonal ATXN2 antibody in combination with the polyQ-specific antibodies MW1 and 1C2 using antibody concentration of 0.3 ng/µl x 3 ng/µl for Ataxin2poly-Tb x MW1-D2 and 0.3 ng/µl x 10 ng/µl for Ataxin2poly-Tb x 1C2-D2. Following further optimizations, all biomaterials were subsequently lysed in RIPA buffer, biomaterial prediluted to a total protein concentration of 1 µg/µl and measurements performed after an incubation of biomaterial to antibody mixture for 24 h at 4°C.

Since the polyQ-specific antibodies MW1 or 1C2 can also detect other proteins with an expanded polyQ region, we proved in the next step that the newly established ATXN2 immunoassay quantifies specifically the polyQ-expanded ATXN2 protein. As shown already similarly for HD and SCA3, our newly established ATXN2 immunoassay specifically quantifies polyQ-expanded ATXN2 if the polyQ-specific antibody MW1 is combined with an ATXN2 specific donor antibody [34].

In order to use the protein ATXN2 as a molecular readout parameter for disease progression and potentially in clinical trials, the immunoassay must also be adapted to special requirements. If e.g. the progression of the disease correlates with the amount of polyQ-expanded ATXN2 in the respective biomaterials, the aim of various therapeutic approaches can be to reduce specifically the disease protein (“protein lowering therapies”). To further validate if our newly established immunoassay is sensitive enough to detect also small changes in the protein concentration, we down- and upregulated ATXN2 using siRNA and starvation experiments. Our results demonstrated that already after 2 h of starvation the increased ATXN2 concentration was successfully measured. Additionally, downregulation of ATXN2 was confirmed by TR-FRET analyses in ATXN2 siRNA experiments. These results confirm that the established and validated polyQ-expanded ATXN2 immunoassay can reliable detect small protein changes.

To further confirm that our immunoassay is also working in human biomaterials, patient-derived cell lines including human fibroblasts, induced pluripotent stem cells and iPSC-derived cortical neurons were used. In all patient-derived cell lines the immunoassay perfectly discriminated between SCA2 patients and healthy controls. However, the aim is to adapt the immunoassay to human biomaterials like cerebrospinal fluid (CSF) or peripheral blood. As known for HD and SCA3, significantly lower concentrations of respective disease proteins are presumably present in the CSF or in peripheral blood [36, 37] Therefore, with the successfully established polyQ-expanded ATXN2 TR-FRET-based immunoassay we have now the tool available to adapt the assay to an ultrasensitive platform like SMC in the future.

In summary, we described the first ATXN2 immunoassay to measure specifically polyQ-expanded ATXN2 in cellular and animal tissue as well as in human cell lines. Further validation in human biomaterials like CSF, peripheral blood or PBMCs and correlation to clinical data is needed to confirm polyQ-expanded atatxin-2 as new important molecular readout parameter in further patient-based studies.

Declarations

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Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the study conception and design especially done by Olaf Riess, Jeannette Hübener-Schmid and Jessica Bux. Material preparation, data collection and analysis were performed by Jessica Bux and Isa-Maria Klink. Nesli Ece Sen, Stefan Hauser, Ludger Schöls and Georg Auburger provided resources and prepared biomaterial for this study. The first draft of the manuscript was written by Jessica Bux and Jeannette Hübener-Schmid and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data availability

All data generated during this study are included in this article or are available on reasonable request from the corresponding author.

Ethics approval

Ethical use of animals: All mice were maintained by animal care staff and veterinarians of the University of Frankfurt/Main Zentrale Forschungs-Einrichtung (ZFE). All procedures were performed according to the German Animal Welfare Act and the guidelines of the Federation of European Laboratory Animal Science Associations, based on European Union legislation (Directive 2010/63/EU). Animal experiments were approved by the local ethics committee (Regierungs-Präsidium Darmstadt V54-19c18-FK/1083).

Ethical use of human tissue: All the work involving human tissue has been carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) and with national legislation as well as our institutional guidelines. Experiments were approved by the local ethics committee (ethical vote Tübingen, 598/2011BO1 and 911/2019BO2).

Consent to participate

All patients gave written informed consent before enrollment.

Consent to publish

All study participants agreed in publication of the data with the informed consent.

Statements and Declarations

Conflicts of interest: The authors declare that they have no conflict of interest.

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Figures

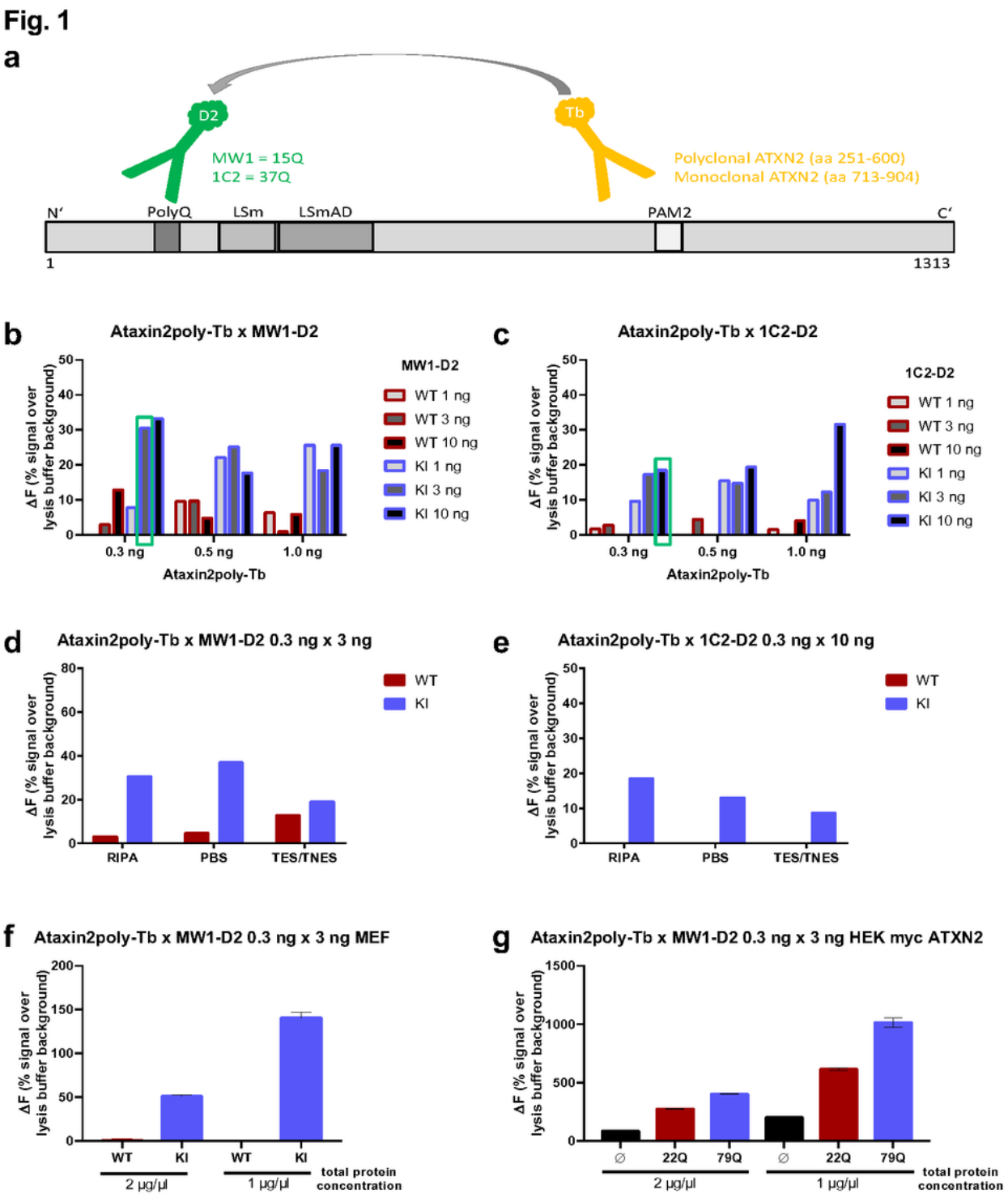

Figure 1

Establishment of a polyQ-expanded ATXN2 TR-FRET-based immunoassay

a) Schematic illustration of the functional mechanism of a TR-FRET-based immunoassay to detect polyQ-expanded ATXN2. The ATXN2 protein with its four most important domains (PolyQ, LSm, LSmAD and PAM2) is shown to visualize the binding sites of the tested antibodies. Antibodies used as donor

were labelled with the luminophore terbium cryptate (Tb) and the acceptor antibodies with the luminophore D2. Two D2-labeled antibodies (MW1-D2 and 1C2-D2) and two ATXN2 specific antibodies (Ataxin2poly-Tb and Ataxin2mono-Tb) were tested. **b+c)** Combination of the antibodies Ataxin2poly-Tb and MW1-D2 (**b**) or Ataxin2poly-Tb and 1C2-D2 (**c**) each in three different concentrations: Ataxin2poly-Tb with 0.3 ng, 0.5 ng and 1 ng/µl, MW1- D2 or 1C2-D2 with 1 ng, 3 ng and 10 ng/µl. Conduction of the TR-FRET measurement with homogenates from wildtype mouse liver (red; WT) and Atxn2-CAG100 knock-in mouse liver (blue; KI) lysed in RIPA buffer, using a total protein concentration of 1 µg/µl. The highest discrimination between WT and KI (green box) was achieved with the concentrations of Ataxin2poly-Tb 0.3 ng/µl x MW1-D2 3 ng/µl (**b**) and Ataxin2poly-Tb 0.3 ng/µl x 1C2-D2 10 ng/µl (**c**). **d+e**) Comparison of three different lysis buffers including RIPA, PBS and TES/TNES. TR-FRET measurement with homogenates from wildtype mouse liver (red; WT) and Atxn2-CAG100 knock-in mouse liver (blue; KI) in the established antibody concentrations: Ataxin2poly-Tb 0.3 ng/µl x MW1-D2 3 ng/µl (**d**) and Ataxin2poly-Tb 0.3 ng/µl x 1C2-D2 10 ng/µl. (**e**) **f+g)** To establish the optimal antibody to total protein ratio, homogenates from MEF wildtype (red; WT) or ATXN2 KI (blue; KI) (**f**) or homogenates from HEK293T (HEK) transfected with myc ATXN2 plasmids with a length of 22 glutamines (red; 22Q) or 79 glutamines (blue; 79Q) as well as empty plasmid as transfection controls (black; Ø) (**g**) were diluted in RIPA buffer to a total protein concentration of 2 µg/µl or 1 µg/µl and measured with the antibody combination Ataxin2poly-Tb 0.3 ng/µl x MW1-D2 3 ng/µl.

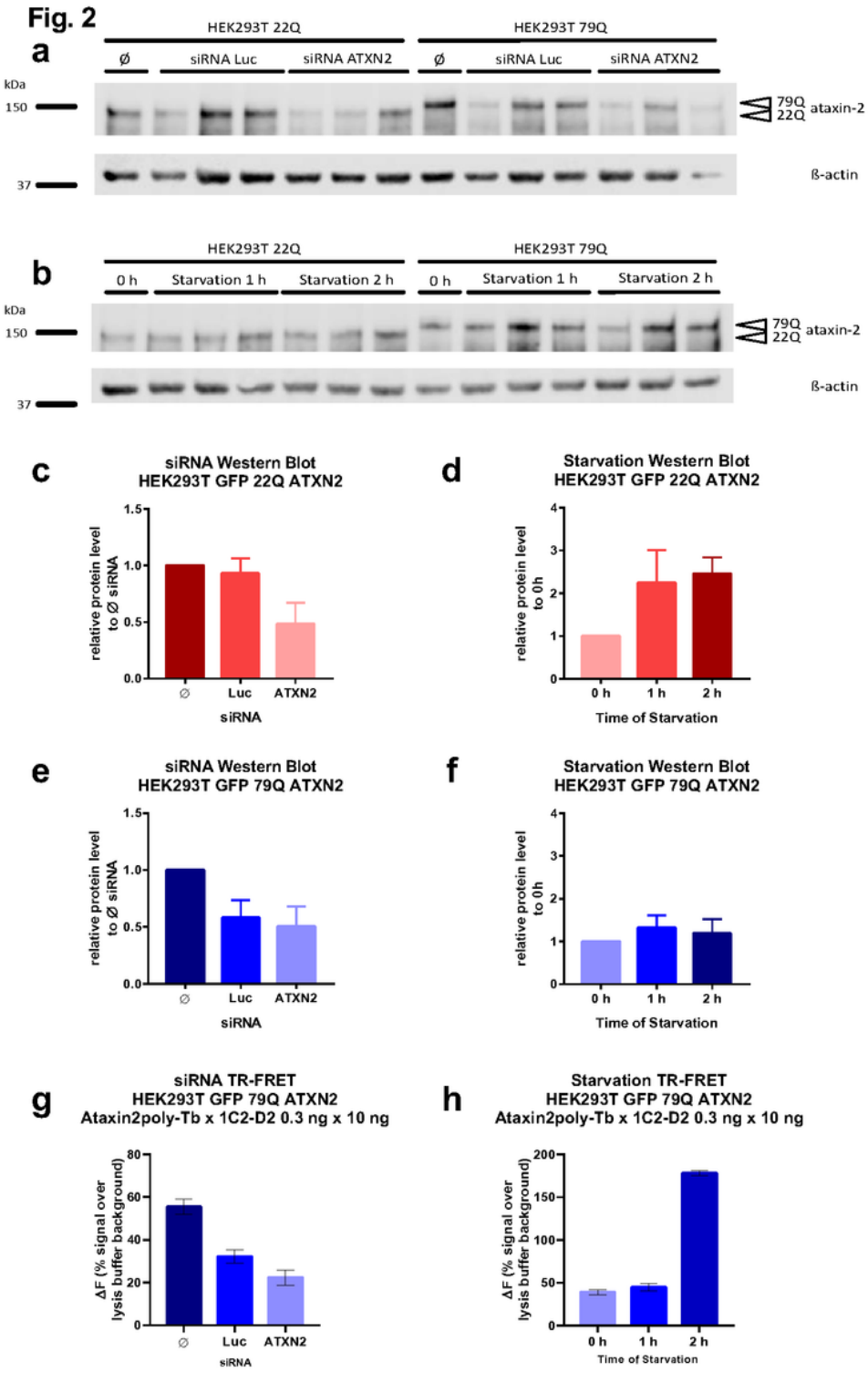

Figure 2

siRNA and starvation experiments demonstrated that the TR-FRET-based immunoassay detects small changes in ATXN2 expression levels

To lower ATXN2 expression, HEK293T cells were transfected with GFP ATXN2 plasmids with 22Q or 79Q and treated either with ATXN2 siRNA (ATXN2), luciferase siRNA (Luc) as a control or without siRNA (Ø)

(siRNA experiments). Additionally, GFP ATXN2 transfected HEK293T cells were incubated in HBSS for 0, 1 or 2 hours to induce starvation and therefore, ATXN2 upregulation **a+b)** Western blot of siRNA experiments **(a)** and western blot of starvation experiments **(b)** using the polyclonal Ataxin2poly antibody detected the ATXN2 protein at 150 kDa. β-actin is shown as loading control. (Western Blot analysis with 30 µg of total protein and 8% Bis-Tris gel) **c-f)** Western Blot quantification of ATXN2 expression after lowering by siRNA (**c+e**) or ATXN2 increased expression by starvation (**d+f**). The bars show the relative protein levels compared to treatment without siRNA (Ø) or 0h of starvation, respectively. **g+h)** TR-FRET analysis of the siRNA and starvation experiments using the antibody combination Ataxin2poly-Tb 0.3 ng/µl x MW1-D2 10 ng/µl, diluted in RIPA buffer.

Fig. 3

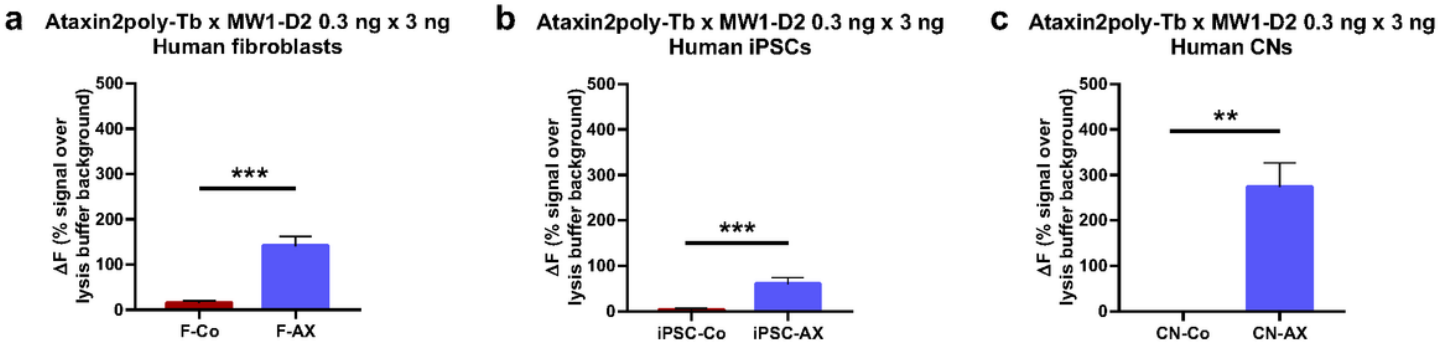

Figure 3

TR-FRET analysis in human cell culture materials

a) Determination of polyQ-expanded ATXN2 by TR-FRET based immunoassay in homogenates of human fibroblasts isolated from SCA2 patients n=3 (red; F-AX) and healthy controls n=4 (blue; F-Co). **b)** Homogenates of human iPSCs from SCA2 patients n=4 (red; iPSC-AX) compared to n=4 healthy controls (blue; iPSC-Co). **c)** Homogenates of human CNs from SCA2 patients n=2 (red; CN-AX) and healthy controls n=4 (blue; CN-Co). All samples were diluted in RIPA buffer, evaluated with a total protein concentration of 1 µg/µl and analyzed using the antibody combination Ataxin2poly-Tb 0.3 ng/µl x MW1-D2 3 ng/µl. ** P≤0.01; *** P≤0.001.

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