Structural basis for recognition of the third SH3 domain of full-length R85 (R85FL)/ponsin by ataxin-7

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Ataxin-7 (Atx7) is a component of the nuclear transcription co-activator complex; its polyglutamine (polyQ) expansion may cause nuclear accumulation and recruit numerous proteins to the intranuclear inclusion bodies. Full-length R85 (R85FL) is such a protein sequestered by polyQ-expanded Atx7. Here, we report that Atx7 specifically interacts with the third SH3 domain (SH3C) of R85FL through its second portion of proline-rich region (PRR). NMR structural analysis of the SH3C domain and its complex with PRR revealed that SH3C contains a large negatively charged surface for binding with the RRTR motif of Atx7. Microscopy imaging demonstrated that sequestration of R85FL by the polyQ-expanded Atx7 in cells is mediated by this specific SH3C–PRR interaction, which is implicated in the pathogenesis of spinocerebellar ataxia 7.

Structured summary of protein interactions:
Atx7 PP2 and SH3C bind by isothermal titration calorimetry (View Interaction: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12)
Atx7 binds to SH3C by pull down (View interaction)
Atx7\textsubscript{100Q} and SH3C colocalize by fluorescence microscopy (View interaction)
SH3C and Atx7 bind by nuclear magnetic resonance (View interaction)

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1. Introduction

Spinocerebellar ataxia 7 (SCA7) is an autosomal dominant neurodegenerative disorder characterized by cerebellar ataxia associated with progressive macular dystrophy [1,2]. SCA7 is generally considered to be caused by expansion of a CAG repeat encoding polyglutamine (polyQ) tract in the protein ataxin-7 (Atx7) [3]. Atx7 is a component of mammalian STAGA (SPT3-TAF9-ADA-GCN5 acetyltransferase) transcription co-activator complex, and its polyQ expansion inhibits STAGA histone acetyltransferase activity [4,5]. Moreover, its yeast form regulates ubiquitin levels of histone H2B and links histone deubiquitination with gene gating and mRNA export [6]. Thus, impairments of these normal functions by polyQ expansion of Atx7 may underlie the production of SCA7 phenotype.

Abbreviations: Atx7, ataxin-7; polyQ, polyglutamine; PRR, proline-rich region; R85FL, full-length R85; SCA7, spinocerebellar ataxia 7; SH3, Src homology 3

There are nine inherited neurodegenerative disorders related to polyQ expansion, the hallmark of this kind of disease is the formation of nuclear inclusion bodies [7]. The intranuclear inclusions found in human SCA7 brain contain many other proteins, including transcriptional regulators, ubiquitin/proteasome pathway components, cell death associated proteins, and chaperones and their partners [1]. The function of all these proteins can possibly be influenced by polyQ expansion of Atx7. However, whether these proteins are recruited to inclusions by polyQ expanded Atx7 through specific or non-specific protein interactions remains largely unknown. One such protein sequestered by polyQ expanded Atx7 to intranuclear inclusions is full-length R85 (R85FL), a splice variant of ponsin or Cbl-associated protein (CAP). R85FL/ponsin belongs to the SoHo adaptor protein family that regulates cytoskeletal organization and signal transduction [8]. As known, SH3 domain specifically recognizes proteins with proline-rich region (PRR) [9–11]. The canonical binding sites for SH3 domains usually include the amino-acid sequences like R/KxxPxxP (class I) or PxxPxR/K (class II) [10,12]. Like huntingtin (Htt), Atx7 contains a PRR just downstream of the polyQ tract [13].
We herein report identification and structural analysis for the interaction of Atx7 PRR with the third SH3 domain (SH3C) of R85FL. Moreover, R85FL can be sequestered to the nuclear inclusion bodies by polyQ expanded Atx7 through specific SH3C–PRR interactions.

2. Materials and methods

2.1. Cloning, expression, peptide synthesis, and protein purification

The R85FL gene was cloned from human brain cDNA library (Invitrogen), and inserted into pcDNA3.0 vector with a FLAG tag in N-terminus of the protein. The Atx7 gene with 10 or 100 glutamine repeats in the vector pEGFPN1 was a gift from Dr. A. Brice. The SH3 domains and their mutants were subcloned into pGEX-NH2 [14] and pGEX-4T-3. The peptides were purified by Ni²⁺–NTA or GST affinity column, followed by gel filtration chromatography. ¹³N/¹³C-labeled proteins were prepared using the M9 minimal medium containing ¹⁵NH₄Cl and/or ¹²C₀-d-glucose as the sole nitrogen and/or carbon resource, respectively.

2.2. GST pull-down experiments

The GST-peptide fusions were added to the glutathione Sepharose 4B beads (Amersham Biosciences) in a PBS buffer (140 mM sodium chloride, 2.7 mM potassium chloride, 10 mM sodium phosphate, 1.8 mM potassium phosphate, pH 7.3), and the suspensions were agitated at 4°C for 30 min. The beads were washed three times with the same buffer to remove any unbound proteins. An equivalent amount of different SH3 domains were added and incubated at 4°C for an additional 4 h. Finally, the beads were recovered by centrifugation, and then the samples were resuspended in the sample buffer and subjected to SDS–PAGE (15% gel), followed by Coomassie staining.

2.3. NMR spectroscopy and structure determination

The sample containing ¹⁵N/¹³C-labeled SH3C protein (~1.0 mM) with or without Atx7_PP2 peptide (4 mM) in a phosphate buffer (20 mM NaH₂PO₄, 50 mM NaCl, pH 6.5) was used for the structural determination by NMR. All data were acquired at 25 °C on a Bruker Avance 600-MHz spectrometer. The spectra including HNCO, HNCACB, CBCA(CO)NH, H(CO)NH, C(CO)NH and HCCH-TOCSY were obtained for the backbone and side-chain assignments. NOE restraints were obtained from ¹⁵N- and ¹³C-edited NOESY spectra with a mixing time of 100 ms. The NMR data were processed using NMRPipe and analyzed with SPARKY. Backbone dihedral restraints were derived from TALOS program. The structures were calculated using ARIA2.0, assessed with PROCHECK, and displayed with MOLMOL.

2.4. MTS labeling and paramagnetic relaxation enhancement (PRE)

A Cys residue was introduced to the N- or C-terminus of Atx7_PP2, and the peptide fused with GB1 was obtained with pGBTNH expression vector [14]. After purification, MTS ((1-oxyl-2,2,5,5-tetramethyl pyrroline-3-methyl) methanethiosulfonate) (ALEXIS Biochemicals, San Diego) was incubated with Atx7_PP2 at 4°C overnight. Ten-fold excess of MTS was used for the reaction and the excess MTS was removed by desalting [11]. In the PRE experiment, after collecting a HSQC spectrum, dithiothreitol (DTT, 10 mM) was added to the mixture in the NMR tube and stayed at room temperature for 5 h to remove MTS from the Atx7_PP2 peptide, and then another HSQC spectrum was acquired for reference [15]. The peak intensities with and without MTS in the HSQC spectra of SH3C were compared for identifying the residues that are close to the N- or C-terminus of the peptide.

2.5. Construction of the complex structure

The complex structure was generated by HADDOCK2.1 [16]. The structures of SH3C in the peptide-bound form solved by NMR and Atx7-PP2 generated by homology modeling from the known proline-rich peptide (PDB ID: 2DF6) were used as inputs for structure calculation. The NOE restraints and chemical-shift changes were treated as significantly perturbed residues. The active residues included Y13, Y15, N19, D21, D39, W49, P56 and Y59 of SH3C, and all residues of Atx7-PP2. The PRE-derived intermolecular distances were introduced as unambiguous restraints. Only those residues with resonance peaks significantly broadened or almost disappeared by paramagnetic effect were used as inputs for structure calculation. Normally the distance between the amide nitrogen atoms of SH3C and the paramagnetic oxygen of MTS was set as 10 ± 3 Å. The ambiguous constraints were further modified by the fact that Pro56 of SH3C has intermolecular NOEs with the prolines of Atx7_PP2. The docking protocol included three stages with a final explicit water refinement. At the last iteration, 200 structures were generated and clustered by an RMSD cut-off of 3 Å.

2.6. Isothermal titration calorimetry (ITC)

ITC was performed with an ITC200 MicroCalorimeter (MicroCal). Proteins were dissolved at 100 µM in a PBS buffer (140 mM sodium chloride, 2.7 mM potassium chloride, 10 mM sodium phosphate, 1.8 mM potassium phosphate, pH 7.3; or 20 mM sodium phosphate, 50 mM sodium chloride, pH 6.5 or 7.0). Upon reaching the equilibrium temperature of 25°C, different peptides were titrated from a syringe into a sample chamber holding by 20 injections. Data were obtained using χ² minimization on a model assuming a single set of sites to calculate the binding affinity.

2.7. Cell culture and immunofluorescence microscopy

HEK 293T cells were transfected by FuGENE HD transfection reagent (Roche) following the manufacturer’s instructions. Anti-FLAG antibody (Sigma) was used for immuno-fluorescence. For confocal microscopy, HEK 293T cells were grown on glass coverslips for 48 h after transfection. Images were obtained on a Leica TCS SP2 confocal microscope (Leica Microsystems).

3. Results

3.1. Atx7 PRR specifically interacts with the third SH3 domain of R85FL

By using two-hybrid approach to screen human retina cDNA library, a protein named R85 was identified for binding with Atx7 [13]. In addition, the R85FL was also found to co-localize with Atx7 [13]. R85FL is a splice variant of the SH3P12 gene product. All variants have three repeated SH3 domains located at the C-termini (Fig. 1A). As known, SH3 is a modular domain recognizing proline-rich motifs [9,11], while Atx7 contains a PRR motif located at the N-terminus just behind the polyQ tract (Fig. 1A). To get molecular details of the recognition between Atx7 and R85FL, we examined the interactions of Atx7 PRR with different SH3 domains of R85FL by GST pull-down analysis. The results show that Atx7 PRR can interact with the third SH3 domain (as referred to SH3C)
but it neither binds with the first domain (SH3A) (Fig. 1B) nor the second (SH3B) (Fig. 1C). SH3 domain canonically binds with such amino-acid sequence as R/KxxPxxP (class I) or PxxPxR/K (class II) \[10,12\]. So we dissected the Atx7 PRR sequence into two parts, namely Atx7_PP1 and Atx7_PP2 (Fig. 1A), each containing a class-II consensus peptide motif. Intriguingly, SH3C only interacts with Atx7_PP2 but not with Atx7_PP1 (Fig. 1E). ITC experiments indicate that Atx7_PP2 has almost the same affinity (K_D = 6.8 μM) to SH3C when compared with the full-length Atx7 PRR sequence (Atx7_PP12) (Fig. S1), suggesting that the PP2 motif in Atx7 is the major binding moiety for SH3C. NMR titration further corroborates our observation from GST pull-down experiments (Fig. S2). The dissociation constant (K_D) for SH3C binding with Atx7_PP2 is around a range of 50 μM. However, SH3A is incapable of binding with Atx7_PP1 or Atx7_PP2. Altogether, these results demonstrate that the PP2 motif of Atx7 specifically interacts with the third SH3 domain of R85FL.

3.2. Solution structures of SH3C in free and peptide-bound states

The SoHo family adaptor proteins include three members, vinexin, Arg-binding protein 2 (ArgBP2) and R85FL (ponsin or CAP). Each protein has one SoHo domain in N-terminus and three SH3 domains in C-terminus. The SH3 domain is highly conserved among the family members with an identity up to 60% (Fig. S3), whereas only 17% identity among the three SH3 domains of R85FL (Fig. 2A). The structures of SH3A (2DL3) and SH3B (2O9S) are available in the Protein Data Bank. By using heteronuclear NMR approach, we solved the solution structures of the SH3C domain of R85FL in free and PP2-bound states. The best 15 final structures, as judged from energetic and geometrical considerations, have a backbone RMSD of 0.44 ± 0.08 Å for SH3C alone or 0.61 ± 0.14 Å for it binding with...
the PP2 peptide (Table S1). Both structures of the free and PP2-bound forms display a β-barrel fold of five β-strands and a 310-helix (Fig. 2B and C), showing a typical SH3 domain architecture. In comparison of the two average structures in free and PP2-bound states, it appears that binding of the PP2 peptide does not cause any significant conformational change (Fig. 2D), because the backbone RMSD of the two structures is only 1.22 Å. There is only a small structural difference in the RT-loop and n-Src loop regions, which are the most flexible regions of SH3 domains but engaged in the peptide binding. The binding surface for PRR on a typical SH3 domain can be divided into two areas, one is the PxxP binding pocket and the other is located in between the RT-loop and n-Src loop[17]. The second region is contributable to the ligand specificity of SH3 domain. The significant difference among the three SH3 domains of R85FL is that SH3C contains more negatively charged residues (e.g. D20, D21 and D39) on its surface (Fig. 2E). This negatively charged area lies in between the RT-loop and n-Src loop. In comparison, the corresponding surface of SH3A is roughly hydrophobic almost without negative charge, while the surface of SH3B contains less negative charge than that of SH3C. This charge difference might provide molecular basis for the specificities of the SH3 domains binding with partners. Actually, SH3B of R85FL binds with non-canonical proline-rich motif[18], while SH3A of vinexin, a highly conserved homolog of SH3A of R85FL, can also bind with a non-canonical proline-rich motif[19].

3.3. Structural insights into the specific interaction between SH3C and Atx7_PP2

Since SH3C has a negatively charged surface and Atx7_PP2 contains a RRTR motif with positive charges, the electric charges on the surface may play important roles in mediating the electrostatic

Table 1

<table>
<thead>
<tr>
<th>Atx7 PRR Peptide</th>
<th>PP12</th>
<th>PP2</th>
<th>PP2-R6A</th>
<th>PP2-R7A</th>
<th>PP2-T8A</th>
<th>PP2-R9A</th>
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<td><strong>R85L_SH3C</strong></td>
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<td>(K_D) (μM)</td>
<td>46.5 ± 7.1</td>
<td>68.0 ± 4.6</td>
<td>980 ± 19</td>
<td>253 ± 45</td>
<td>862 ± 44</td>
<td>588 ± 96</td>
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<td><strong>Atx7_PP2</strong></td>
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<tr>
<td>(K_D) (μM)</td>
<td>&gt;1000</td>
<td>139 ± 24</td>
<td>D21A</td>
<td>D39A</td>
<td>T54A</td>
<td>Y59A</td>
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<td><strong>SH3C Mutant</strong></td>
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<td>Y13A</td>
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<td>(K_D) (μM)</td>
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interaction between the two peptide chains, thus ion strength may also influence the complex. Indeed, ITC experiments show that the dissociation constant ($K_D$) for SH3C binding with PP2 is significantly increased with an increase of salt concentration in the buffer (Fig. 3A), suggesting that the electrostatic interactions are contributable to the formation of the SH3C–PP2 complex. NMR chemical-shift perturbation exhibits that some residues like Y15, D21, D39, W41 and Y59, experience significant chemical-shift changes upon PP2 binding (Fig. 3B). These residues are also contributable to constructing the xP groove and the specificity pocket of SH3 domain [9,10,12]. Thus, both the proline-mediated hydrophobic interaction and the arginine-conducted electrostatic interaction are crucial to forming the complex between Atx7_PP2 and SH3C. More evidence comes from the NOE signals between Atx7_PP2 and SH3C (Fig. 3C). The $\beta$-methylene group of Pro56 of SH3C has only intra-residue NOE signals in the free state, whereas some intermolecular NOE signals appear in the complex, though they are still unsigned due to 5 continuous prolines in Atx7_PP2. The intermolecular NOE data from $^{13}$C filtered/edited NOESY spectra are confirmable for this assumption (Fig. 3C, right panel). According to the chemical-shift pattern, these intermolecular NOE signals may come from one of the proline residues of Atx7_PP2 by PRE experiments. The cross peaks of Y13, S14, Y15 and I16 of SH3C are significantly broadened when it binds with the N-terminally MTSL-labeled peptide (Fig. 3D), indicating that the N-terminus of Atx7_PP2 is seated around the xP groove of SH3C formed by Y13 and Y15. Meanwhile, C-terminally labeled Atx7_PP2 causes large peak broadening of D38, D39 and G40 (Fig. 3E), suggesting that the C-terminus of Atx7_PP2 interacts with the acidic residues in the N-Src loop of SH3C. The ITC experiments on various mutants of both Atx7_PP2 and SH3C further confirm the viewpoint that the extended RRTR motif of Atx7_PP2 and the key residues forming the xP groove and specificity pocket of SH3C are important for the specific interaction (Table 1). The dramatic decrease of the binding affinities in the D21A and D39A mutants can account for the different recognition modes for polyproline peptides among the three SH3 domains of R85FL (Fig. 1 and Fig. S2). Taken together, these results suggest that both the xP groove and specificity pocket of SH3C are engaged in the interaction with Atx7_PP2, while both the PPPPP and RRTR motifs in Atx7_PP2 are also contributable to the specific interaction.

3.4. Structure model for the complex of SH3C and Atx7_PP2

Due to the relatively weak interaction and difficulty in NMR assignment of the Atx7_PP2 peptide, we have not obtained enough unambiguous intermolecular NOEs for direct computation of the complex structure, so we generated a rational complex structure by HADDOCK method [16], using the information from chemical shift perturbation (Fig. 3B), intermolecular NOEs (Fig. 3C), PRE restraints (Fig. 3D and E) and binding affinities of various mutants (Table 1). With the experimental structure constraints as an input, a major cluster of the initial complex structures were generated, and a refined structure was finally obtained (Fig. 4). In the structure model, the PPPPP motif is docked on the hydrophobic xP groove of SH3C and the RRTR motif is directed to the negatively charged specificity pocket (Fig. 4A). The prolines of Atx7_PP2 are contacting with the xP groove of SH3C formed by Y13, Y15, W41,
to dysfunction [7]. Using confocal microscopy, we investigated the c0-localization of Atx7 with R85FL in cells. Normal Atx7 (Atx7100Q) is a nucleus localized protein, while R85FL is distributed mainly in cytosol (Fig. 5, 1st row). The polyQ expanded Atx7 (Atx71000Q) forms nuclear inclusion bodies, which can sequester R85FL into the nucleus (Fig. 5, 2nd row). As a control, double mutation (I16A/Q18A) in the SH3C moiety of R85FL, which does not alter the interaction with Atx7_PP2, can also be sequestered into the nucleus by Atx71000Q (Fig. 5, 3rd row). However, the D39A mutant in the SH3C moiety of R85FL that significantly reduces the binding affinity to the PP2 motif of Atx7 cannot be sequestered by Atx71000Q (Fig. 5, 4th row). Moreover, deletion of SH3C in R85FL significantly abolishes sequestration of R85FL into the nucleus by Atx71000Q (Fig. 5, 5th row). This observation indicates that the SH3C–PP2 interaction mediates the association of Atx7 and R85FL in cells. The polyQ expanded Atx7 is prone to formation of inclusion bodies and may significantly alter the localization of R85FL from cytosol to nucleus. This kind of abnormal interactions of the polyQ expanded Atx7 with other partners may be implicated in the pathological process of SCA7.

4. Discussion

SH3 is a modular domain commonly found in all eukaryotic genomes [10]. R85FL/ponsin is an adaptor protein that harbors three SH3 domains in the C-terminus. Adaptor proteins are usually composed of multiple modular domains for linking different parts of enzymatic cascade or signal transduction pathway [20,21]. So the three SH3 domains of R85FL must have evolved unique properties to accommodate different partners. Comparing the structures of these three SH3 domains from R85FL reveals that the major difference for binding diverse PRR partners comes from the negatively charged region between the RT loop and n-Src loop (Fig. 2E). This structural difference among these three SH3 domains may provide a possibility for R85FL to function both specifically and versatility in living cells.

Most SH3 domains recognize linear polyproline motif through two pockets; one binds to the canonical PxxP core motif and the other binds to the extended motif beyond the PxxP core [17]. Both the canonical and non-canonical peptides associate with SH3 domains through these two pockets. Although lots of SH3 domain structures are available, it is still very hard to predict the interaction partners of a SH3 domain, largely because of the high versatility of SH3 domains. Previous studies showed that the SH3 domain of R85FL can interact with c-Cbl [22], 1-Afadin [23] and c-Abl [24]. The SH3C domain interacts with the PRR-containing partners with a novel consensus motif of PxPPxRxSSL pattern at their binding sites [25]. Besides the PxxP motif, the extended motif also plays a crucial role in SH3 domain recognition [26–29]. However, these SH3 domains bind with different polyproline peptides with the dissociation constants around 1 μM, whereas the binding affinity for R85FL SH3C with Atx7_PP2 is much weaker, till to an extent of about 50 μM. The RxxK motif of Gab2 was reported to recognize the SH3C domain of Grb2 [30]; both side-chains of Arg and Lys residues point to the negatively charged region of the RT loop (Fig. S4A). The interaction between Atx7 and R85FL is a little different from that between Gab2 and Grb2. The negatively charged region of R85FL_SH3C includes both RT loop and n-Src loop, and each residue of the RKR motif in Atx7 contributes to the interaction (Table 1). Another example is from the structural study of Mona/Gads SH3C [27], which recognizes a PxxPxK/kxxK motif. The PxxP motif binds to the two xP grooves of SH3C, while the K/kxxK motif points to the negatively charged region of the RT loop (Fig. S4B).

Fig. 5. PolyQ expanded Atx7 (Atx71000Q) can sequester R85FL in cells. Normal Atx710Q-GFP and polyQ expanded Atx71000Q-GFP were co-transfected with either wild-type R85FL or its mutants in the SH3C moiety into HEK 293T cells, and performed imaging with confocal fluorescence microscopy. Nuclei were stained with DAPI. I16A/Q18A is a double mutant of R85FL in the SH3C moiety, while D39A is a point mutant of R85FL in the SH3C domain. ASH3C stands for the SH3C deletion mutant of R85FL. Scalar bar = 10 μM.

P56 and Y59 (Fig. 4B). The positively charged RRTR motif is located in the negatively charged specificity pocket formed by D21, E22, D38 and D39. On the Atx7_PP2 side, R6 is pointed to the acidic pocket of SH3C formed by D21 and E22, while both K7 and R9 located at the pocket formed by D38 and D39. This is different from the complex structure of SH3B of R85FL, in which the paxillin peptide forms a type-II polyproline helix conformation [18]. The specificity pocket of SH3B is comprised of Val, Asp, Asn and Ile residues, corresponding to the residues of C37, D38, G40 and T54 in SH3C (Fig. 2A), but there is no any charge involved in the interaction. In essence, this structure model can explain the binding specificity for Atx7_PP2 and the unique property of SH3C among the SH3 domains of R85FL.

3.5. PolyQ expanded Atx7 (Atx71000Q) can sequester R85FL through specific interaction with SH3C

Formation of inclusion bodies is the hallmark of polyQ expanded neurodegenerative diseases, and the cellular inclusions can sequester various kinds of proteins and probably lead them...
partners for an SH3 domain is expanded by combination of the PxxP motif and an extended motif.

PolyQ disorders share several common pathological features, including the nuclear accumulation and aggregation of the disease proteins [7,31]. Numerous proteins can be sequestered by the nuclear inclusions, so that their functions must be abolished, which can also be recognized as a pathogen for the polyQ diseases. Our previous work has shown that polyQ expanded huntingtin (Htt) can sequester Htt two-hybrid protein A (HYPA) through its PRR interaction, and thus impairs the function of HYPA in pre-mRNA splicing [32]. It is quite possible to be a similar case in R85FL. The R85FL splicing variant ponsin/CAP regulates cytoskeleton organization [33,34], insulin signal pathway [22,35,36], and receptor-mediated endocytosis [37]. Actually, R85FL colocalizes with Atx7 in the brain of SCA patients [13]. The sequestration of R85FL by polyQ expanded Atx7 possibly leads to impairment of the normal function of R85FL, which may partially contribute to the pathology of the SCA7 disease.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.07.021.

References
