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The interaction of small heat shock molecular chaperone proteins with a-synuclein

Dezerae Cox
University of Wollongong
The interaction of small heat shock molecular chaperone proteins with $\alpha$-synuclein

Dezerae Cox

A thesis submitted in fulfilment of the requirements for the award of the degree

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DECLARATION

This thesis is submitted in accordance with the University of Wollongong guidelines in fulfilment of the requirements for the award of Doctor of Philosophy. This thesis does not contain material that has been previously published by another person except where referenced or acknowledged, and has not been submitted for the award of any degree at any other academic institution.

____________________
Dezerea M. Cox
September, 2016
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ABSTRACT

Protein homeostasis, or proteostasis, is the process of maintaining the conformational and functional integrity of the proteome. Proteostasis is preserved in the face of stress by a complex network of cellular machinery which acts to maintain proteins in their native state, enabling them to perform their biological function. Failure of the proteostasis network can result in the accumulation of non-native (misfolded) proteins, leading to their aggregation and deposition. For example, the amyloid fibrillar aggregation of the protein α-synuclein (α-syn) into Lewy bodies and Lewy neurites is associated with neurodegenerative diseases, classified as α-synucleinopathies, which include Parkinson's disease and dementia with Lewy bodies.

The small heat shock proteins (sHsps) are a family of molecular chaperones that are one of the cell's first lines of defence against protein aggregation. They act to stabilise partially-folded protein intermediates, in an ATP-independent manner, to maintain cellular proteostasis under stress conditions. Thus, the sHsps are ideally suited to protect against α-syn aggregation, however, they fail to do so in the context of the α-synucleinopathies. Therefore, this project aimed to characterise the ability of the canonical sHsps, αB-crystallin (αB-c) and Hsp27, to prevent α-syn aggregation, both in vitro and in a neuronal cell model.

Experiments investigating the mechanism of interaction between the sHsps and monomeric, aggregation-prone α-syn are described in Chapter 3. Analytical size exclusion chromatography and ultracentrifugation were used to demonstrate that the interaction between αB-c or Hsp27 and monomeric α-syn is transient, yet sufficient to prevent α-syn aggregation. This work also sought to address potential mechanisms by which aggregating α-syn may evade sHsp chaperone action. By using two methods (monomeric concentration and disease-related mutations) to alter the kinetics of α-syn aggregation, it was demonstrated that the rate of aggregation (and in particular the length of the lag phase) had a significant effect
on the chaperone efficacy of αB-c and Hsp27. The more rapid the rate of α-syn aggregation, the less effective these sHsps were in terms of their ability to prevent aggregation.

Given the ability of αB-c to bind mature α-syn fibrils, the interaction of the sHsps with other α-syn aggregation intermediates was also examined. Single molecule techniques were employed in Chapter 4 to study the interaction between Hsp27 and oligomeric α-syn species formed early during its aggregation. It was found that Hsp27 inhibits the growth and structural conversion of α-syn oligomers into amyloid fibrils. In addition, Hsp27 binds to mature α-syn fibrils with micromolar affinity. Using super resolution microscopy, the interaction of Hsp27 with α-syn fibrils was found to reduce the surface hydrophobicity of the fibrils. Furthermore, by measuring the production of reactive oxygen species generated by a murine neuronal cell line (Neuro-2a) in response to fibrillar α-syn, this work shows that by binding to α-syn fibrils, both αB-c and Hsp27 inhibit fibril-associated toxicity. Taken together, these data suggest that binding to fibrils is a generic property of sHsps and, by doing so, the sHsps prevent the toxicity associated with amyloid aggregates.

Finally, the ability of the sHsps, αB-c and Hsp27, to prevent α-syn aggregation in cells was examined. As part of the work presented in Chapter 5, a robust cellular model of α-syn aggregation was established using Neuro-2a cells. Overexpression of αB-c and Hsp27 was shown to significantly reduce the formation of intracellular α-syn inclusions in this model. This suggests that over-expressing or boosting the activity of sHsps may be a valid way to prevent amyloid fibrillar aggregation of α-syn in cells. In contrast, the sHsps were ineffective at preventing the accumulation of another disease-associated amyloidogenic protein, huntingtin, highlighting the substrate specificity of the sHsp-target protein interaction.

The work presented in this thesis highlights the multi-faceted nature of the chaperone mechanisms used by sHsps to prevent protein aggregation. It also reveals, for the first time, a
specific factor (i.e. the rate of aggregation) which may contribute to the failure of the sHsps to prevent α-syn aggregation in the context of the α-synucleinopathies. Elucidating the mechanistic details of interactions between the sHsps and disease-relevant proteins such as α-syn provides essential insight into sHsp chaperone action. Future work establishing the physiological relevance of these interactions in the cellular environment, and in the context of the α-synucleinopathies, will be crucial in pursuing potential avenues for therapeutic intervention that target the sHsp molecular chaperones.
CONTENTS

DECLARATION ........................................................................................................................................... ii

ACKNOWLEDGEMENTS ......................................................................................................................... iii

ABSTRACT ................................................................................................................................................ iv

CONTENTS ............................................................................................................................................... vii

LIST OF FIGURES .................................................................................................................................. xii

LIST OF TABLES ....................................................................................................................................... xiv

LIST OF ABBREVIATIONS ....................................................................................................................... xv

LIST OF PUBLICATIONS AND PRESENTATIONS .................................................................................... xviii

CHAPTER 1: INTRODUCTION .................................................................................................................. 1

  1.1 Proteostasis ....................................................................................................................................... 2
    1.1.1 Protein folding ........................................................................................................................... 2
    1.1.2 Protein misfolding and aggregation .......................................................................................... 4
    1.1.3 In vitro versus in vivo: considering the cellular context ............................................................. 6
    1.1.4 The role of molecular chaperones .............................................................................................. 8

  1.2 Proteostasis and disease .................................................................................................................... 10
    1.2.1 The α-synucleinopathies: a collection of aggregation disorders .............................................. 11
    1.2.2 α-Syn in disease ....................................................................................................................... 12
    1.2.3 Aggregation of α-syn .................................................................................................................. 14
    1.2.4 α-Syn pathogenicity .................................................................................................................. 16
    1.2.5 Identifying the toxic species in disease ..................................................................................... 16
    1.2.6 Current cell-based models of α-syn aggregation .................................................................... 22
    1.2.7 Lewy bodies and Lewy neurites: α-syn is not alone ................................................................. 24

  1.3 Small heat shock proteins (sHsps) as molecular chaperones .......................................................... 25
    1.3.1 Structure and function of the sHsps ......................................................................................... 25
    1.3.2 Modulators of sHsp activity ..................................................................................................... 26
    1.3.3 Expression of the sHsps in the brain ......................................................................................... 30
    1.3.4 sHsps interact with multiple species to inhibit aggregation .................................................... 32

  1.4 sHsps interact with α-syn ................................................................................................................... 34
<table>
<thead>
<tr>
<th>1.5</th>
<th>Summary and aims</th>
<th>..........................................................</th>
<th>37</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHAPTER 2: MATERIALS AND METHODS</td>
<td>..........................................................</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>2.1</td>
<td>Materials</td>
<td>..........................................................</td>
<td>40</td>
</tr>
<tr>
<td>2.2</td>
<td>Bacterial and mammalian expression constructs</td>
<td>..........................................................</td>
<td>41</td>
</tr>
<tr>
<td>2.2.1</td>
<td>Plasmids</td>
<td>..........................................................</td>
<td>41</td>
</tr>
<tr>
<td>2.2.2</td>
<td>Transformation of chemically competent <em>E. coli</em></td>
<td>..........................................................</td>
<td>42</td>
</tr>
<tr>
<td>2.2.3</td>
<td>Sequence verification</td>
<td>..........................................................</td>
<td>43</td>
</tr>
<tr>
<td>2.3</td>
<td>Recombinant protein production</td>
<td>..........................................................</td>
<td>44</td>
</tr>
<tr>
<td>2.3.1</td>
<td>Expression and purification</td>
<td>..........................................................</td>
<td>44</td>
</tr>
<tr>
<td>2.3.2</td>
<td>Quantification and storage</td>
<td>..........................................................</td>
<td>46</td>
</tr>
<tr>
<td>2.4</td>
<td>SDS-PAGE</td>
<td>..........................................................</td>
<td>46</td>
</tr>
<tr>
<td>2.5</td>
<td>Immunoblotting</td>
<td>..........................................................</td>
<td>47</td>
</tr>
<tr>
<td>2.6</td>
<td>Fluorescent labelling of recombinant proteins</td>
<td>..........................................................</td>
<td>48</td>
</tr>
<tr>
<td>2.7</td>
<td>Mammalian tissue culture</td>
<td>..........................................................</td>
<td>49</td>
</tr>
<tr>
<td>2.7.1</td>
<td>Passaging and plating</td>
<td>..........................................................</td>
<td>49</td>
</tr>
<tr>
<td>2.7.2</td>
<td>Storage</td>
<td>..........................................................</td>
<td>50</td>
</tr>
<tr>
<td>2.8</td>
<td>Data Analysis</td>
<td>..........................................................</td>
<td>50</td>
</tr>
<tr>
<td>CHAPTER 3: SHSPS INHIBIT THE AGGREGATION OF MONOMERIC α-SYNUCLEIN</td>
<td>..........................................................</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td>3.1</td>
<td>Introduction</td>
<td>..........................................................</td>
<td>52</td>
</tr>
<tr>
<td>3.1.1</td>
<td>Experimental rationale</td>
<td>..........................................................</td>
<td>52</td>
</tr>
<tr>
<td>3.2</td>
<td>Methods</td>
<td>..........................................................</td>
<td>54</td>
</tr>
<tr>
<td>3.2.1</td>
<td><em>In vitro</em> α-syn aggregation assays</td>
<td>..........................................................</td>
<td>54</td>
</tr>
<tr>
<td>3.2.2</td>
<td>Analytical size exclusion chromatography (SEC)</td>
<td>..........................................................</td>
<td>57</td>
</tr>
<tr>
<td>3.2.3</td>
<td>Analytical ultracentrifugation (AUC)</td>
<td>..........................................................</td>
<td>58</td>
</tr>
<tr>
<td>3.2.4</td>
<td>Bulk Förster Resonance Energy Transfer (FRET) analysis of sHsp subunit exchange rate</td>
<td>..........................................................</td>
<td>59</td>
</tr>
<tr>
<td>3.3</td>
<td>Results</td>
<td>..........................................................</td>
<td>61</td>
</tr>
<tr>
<td>3.3.1</td>
<td>sHsp inhibition of α-syn aggregation is concentration dependent</td>
<td>..........................................................</td>
<td>61</td>
</tr>
<tr>
<td>3.3.2</td>
<td>In preventing the fibrillar aggregation of α-syn, the sHsp do not form stable, high molecular mass sHsp-target protein complexes</td>
<td>..........................................................</td>
<td>61</td>
</tr>
<tr>
<td>3.3.3</td>
<td>The ability of sHsp to prevent the aggregation of α-syn is dependent on the kinetics of the aggregation process</td>
<td>..........................................................</td>
<td>67</td>
</tr>
<tr>
<td>3.4</td>
<td>Discussion</td>
<td>..........................................................</td>
<td>71</td>
</tr>
</tbody>
</table>
CHAPTER 4: SHSPS INTERACT WITH MATURE α-SYNUCLEIN FIBRILS ..........77

4.1 Introduction .............................................................................................................................................78
  4.1.1 sHsps interact with aggregation intermediates ..............................................................................78
  4.1.2 Single molecule techniques .............................................................................................................79
  4.1.3 Experimental rationale .....................................................................................................................80

4.2 Methods .....................................................................................................................................................82
  4.2.1 Single molecule confocal microscopy .............................................................................................82
    4.2.1.1 Microscope setup ......................................................................................................................82
    4.2.1.2 Preparation of microfluidic devices .........................................................................................83
    4.2.1.3 Single molecule Förster resonance energy transfer (smFRET) .............................................84
    4.2.1.4 Data analysis .............................................................................................................................85
  4.2.2 Seeded aggregation assays .................................................................................................................87
  4.2.3 Preparation of mature, unlabelled α-syn fibrils ...............................................................................88
  4.2.4 Analytical sucrose gradient centrifugation .......................................................................................89
  4.2.5 Fluorescence fibril pelleting assay .....................................................................................................89
  4.2.6 Total internal reflection fluorescence (TIRF) microscopy ...............................................................90
    4.2.6.1 Microscope setup ......................................................................................................................90
    4.2.6.2 Sample preparation ...................................................................................................................91
    4.2.6.3 Image processing .....................................................................................................................92
  4.2.7 Dihydroethidium (DHE) assay ..........................................................................................................92
  4.2.8 Analytical ultracentrifugation (AUC) of fibrils ..................................................................................93

4.3 Results .......................................................................................................................................................94
  4.3.1 Hsp27 inhibits the growth and structural conversion of α-syn oligomers during early aggregation .............................................................94
  4.3.2 Hsp27 inhibits the elongation of α-syn fibril fragments ......................................................................95
  4.3.3 sHsps forms a stable complex with mature α-syn fibrils and this is mediated by the N- and/or C-termini .................................................................................................................................98
  4.3.4 Binding of Hsp27 to α-syn fibrils occurs along the surface, leading to a decrease in hydrophobicity .........................................................................................................................................................101
  4.3.5 sHsps protect against the cellular toxicity of exogenous α-syn fibrils .............................................104
  4.3.6 Binding of sHsps promotes dissociation of mature α-syn fibrils .....................................................107

4.4 Discussion ..................................................................................................................................................110
CHAPTER 5: SHSP CHAPERONE ACTIVITY IN CELLULAR MODELS OF AGGREGATION.............................................................................................................. 118

5.1 Introduction ............................................................................................................. 119
  5.1.1 Modelling cellular aggregation ........................................................................... 119
  5.1.2 Monitoring chaperone action in cells ............................................................... 121
  5.1.3 Experimental rationale .................................................................................... 122
5.2 Methods ................................................................................................................... 124
  5.2.1 Cloning strategy to produce truncated variant of α-synA53T-EGFP .................. 124
  5.2.2 Transfection and treatment with inducers of cellular stress ......................... 127
  5.2.3 Immunocytochemistry ..................................................................................... 127
  5.2.4 Confocal microscopy imaging ........................................................................ 128
  5.2.5 Inclusion quantification .................................................................................. 129
    5.2.5.1 Flow cytometry ......................................................................................... 129
    5.2.5.2 Manual quantification using ImageJ .......................................................... 130
  5.2.6 Cell lysate analysis via filter trap assay ......................................................... 130
5.3 Results .................................................................................................................... 133
  5.3.1 Developing a cell model of α-syn aggregation ................................................. 133
    5.3.1.1 Transfection with fluorescently tagged α-syn constructs ....................... 133
    5.3.1.2 Transfection with α-synA53T* ................................................................. 138
  5.3.2 sHsps inhibit the deposition of α-syn into inclusions in cells ....................... 142
  5.3.3 Hsps do not prevent the accumulation of Htt inclusion bodies .................... 145
5.4 Discussion .............................................................................................................. 150

CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS ........................................... 160

6.1 sHsps interact transiently with aggregation-prone monomeric α-syn ............... 161
6.2 sHsps bind to α-syn fibrils ................................................................................... 163
6.3 sHsps prevent the aggregation of α-syn into inclusions in cells ........................... 165
6.4 A model of how sHsps interact with aggregation-prone α-syn ............................ 166
6.5 Concluding remarks ............................................................................................. 169

CHAPTER 7: REFERENCES ............................................................................................. 170

CHAPTER 8: APPENDICES ........................................................................................... 201

Appendix I: .................................................................................................................... 202
Appendix II: .................................................................................................................... 203
Appendix III: ............................................................................................................ 213
Appendix IV: ............................................................................................................. 219
Appendix V: ............................................................................................................... 223
Appendix VI: .............................................................................................................. 224
Appendix VII: ............................................................................................................ 226
Appendix VIII: .......................................................................................................... 232
Appendix IX: ............................................................................................................. 233
LIST OF FIGURES

Figure 1.1: Cellular proteostasis mechanisms. .................................................................3

Figure 1.2: Schematic representation of the funnel-shaped energy landscape explored by proteins during folding and aggregation. .................................................................5

Figure 1.3: Amyloid fibrillar aggregation of α-syn. .........................................................15

Figure 1.4: A schematic model for the potential mechanisms by which α-syn aggregation is toxic to neuronal cells. .........................................................................................21

Figure 3.1: Schematic illustration of the Boltzmann sigmoidal curve used to describe the increase in ThT fluorescence upon α-syn fibril formation. ........................................55

Figure 3.2: sHsps inhibit α-syn aggregation in a concentration-dependent manner ...........62

Figure 3.3: sHsps prevent α-syn aggregation but, in doing so, do not form a stable high molecular mass sHsp-target protein complex .................................................................63

Figure 3.4: Analysis of the interaction between sHsps and aggregation-prone α-syn by absorbance-based AUC .................................................................................................65

Figure 3.5: Aggregation-prone α-syn induces the dissociation of oligomeric Hsp27. .........66

Figure 3.6: The ability of αB-c to inhibit the fibrillar aggregation of α-syn is dependent on the kinetics of aggregation ..............................................................................................68

Figure 3.7: The ability of the sHsps αB-c and Hsp27 to prevent the aggregation of disease-associated mutant forms of α-syn. .............................................................................70

Figure 4.1: Hsp27 inhibits the growth of oligomers formed during α-syn aggregation .......95

Figure 4.2: Hsp27 inhibits the elongation of α-syn fibril fragments. ....................................96

Figure 4.3: Hsp27 interacts with α-syn fibril fragments ......................................................98

Figure 4.4: sHsps bind to α-syn fibrils and this is mediated by the N- and/or C-termini ....100

Figure 4.5: Hsp27 binds mature α-syn fibrils with µM affinity ............................................101

Figure 4.6: Hsp27 binds along the surface of α-syn fibrils ................................................102

Figure 4.7: Hsp27 decreases the relative hydrophobicity at the surface of α-syn fibrils .....104

Figure 4.8: sHsps reduce the generation of ROS by N2a cells exposed to exogenous α-syn fibrils. .........................................................................................................................106
Figure 4.9: sHsps decrease the apparent size of mature α-syn fibrils. ............................................. 108

Figure 4.10: sHsps promote the dissociation of α-syn fibrils into smaller oligomers. ............... 109

Figure 5.1: Cloning strategy used to generate α-synA53T* construct for mammalian expression. .................................................................................................................. 126

Figure 5.2: Fluorescently tagged α-syn variants produce visible inclusions. ......................... 134

Figure 5.3: Fluorescently tagged α-syn variants do not produce inclusions detectable via PulSA ........................................................................................................................................ 135

Figure 5.4: Expression of fluorescently tagged α-syn isoforms does not result in robust inclusion formation in N2a or HEK293 cells. .................................................................................................. 137

Figure 5.5: Cloning and expression of α-synA53T*. ................................................................. 139

Figure 5.6: α-synA53T* forms punctate inclusions in N2a cells. ............................................. 142

Figure 5.7: αB-c co-localises with α-synA53T* in some intracellular inclusions.................... 143

Figure 5.8: sHsps inhibit the formation of α-syn inclusions in cells. ....................................... 145

Figure 5.9: Hsps do not prevent the cellular deposition of Htt into inclusions. ..................... 149

Figure 6.1: sHsps interact with various species formed during the fibrillar aggregation of α-syn........................................................................................................................................... 168

Figure 8.1: sHsp variants inhibit α-syn aggregation................................................................. 202

Figure 8.2: Hsp27 interacts with α-syn fibril fragments.......................................................... 223

Figure 8.3: Quantification of α-synA53T* expression in whole cell lysate......................... 232

Figure 8.4: The concentration of DNA used to transfect N2a cells influences the proportion of transfected cells with Htt46Q inclusions. ........................................................................ 233
LIST OF TABLES

Table 1.1: A summary of studies that have investigated the interaction between sHsp and α-syn........................................36
LIST OF ABBREVIATIONS

αA-c                      αA-crystallin
αB-c                      αB-crystallin
αB-c_{3D}                 phosphomimicking variant of αB-c (i.e. S15D/S45D/S59D αB-c)
αB-c_{core}               core domain variant of αB-c (i.e. residues 68 – 153)
α-lac                     α-lactalbumin
α-syn                     α-synuclein
A.U.                      arbitrary units
AF                         alexafluor
AFM                        atomic force microscopy
ALS                        amyotrophic lateral sclerosis
apoC-II                    apolipoproteinC-II
AUC                        analytical ultracentrifugation
Aβ                         amyloid-β
BSA                        bovine serum albumin
DHE                        dihydroethidium
DMEM/F12                   Dulbecco's modified eagle's medium/Ham's nutrient mixture F-12
DMSO                       dimethyl sulfoxide
DTT                        dithiothreitol
EDTA                       ethylenediaminetetraacetic acid
EGFP                       enhanced green fluorescent protein
EGFP^{inv}                 non-fluorescent (invisible) mutant form of EGFP
EM                         electron microscopy
ER                         endoplasmic reticulum
FACS                       fluorescence activated cell sorting
FBS                        foetal bovine serum
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>FRET</td>
<td>Förster Resonance Energy Transfer</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>HEK</td>
<td>human embryonic kidney</td>
</tr>
<tr>
<td>Hsp</td>
<td>heat shock protein</td>
</tr>
<tr>
<td>Hsp27&lt;sub&gt;3D&lt;/sub&gt;</td>
<td>phosphomimicking variant of Hsp27 (i.e. S19D/S79D/S82D Hsp27)</td>
</tr>
<tr>
<td>Hsp27&lt;sub&gt;core&lt;/sub&gt;</td>
<td>core domain variant of Hsp27 (i.e. residues 84 – 176)</td>
</tr>
<tr>
<td>Htt</td>
<td>huntingtin</td>
</tr>
<tr>
<td>IPOD</td>
<td>insoluble protein deposit</td>
</tr>
<tr>
<td>i&lt;sub&gt;pop&lt;/sub&gt;</td>
<td>inclusion population</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>IRES</td>
<td>internal ribosome entry site</td>
</tr>
<tr>
<td>JUNQ</td>
<td>juxta nuclear quality control compartment</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani broth</td>
</tr>
<tr>
<td>N2a</td>
<td>Neuro-2a</td>
</tr>
<tr>
<td>n&lt;i&gt;pop&lt;/i&gt;</td>
<td>non-inclusion population</td>
</tr>
<tr>
<td>NMR</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<td>PBS</td>
<td>phosphate-buffered saline</td>
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<td>PBS-T</td>
<td>PBS containing 0.05% (v/v) Tween-20</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>PuLSA</td>
<td>pulse shape analysis</td>
</tr>
<tr>
<td>Q</td>
<td>association quotient</td>
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<tr>
<td>RFP</td>
<td>red fluorescent protein</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>S.E.M.</td>
<td>standard error of the mean</td>
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<tr>
<td>Abbreviation</td>
<td>Explanation</td>
</tr>
<tr>
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</tr>
<tr>
<td>( S_{av} )</td>
<td>weight-average sedimentation coefficient</td>
</tr>
<tr>
<td>SAVE</td>
<td>single aggregate visualisation by enhancement</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEC</td>
<td>size exclusion chromatography</td>
</tr>
<tr>
<td>sHsp</td>
<td>small heat shock protein</td>
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<td>smFRET</td>
<td>single molecule Förster Resonance Energy Transfer</td>
</tr>
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LIST OF PUBLICATIONS AND PRESENTATIONS

REFEREED PUBLICATIONS:


NON-REFEREED PUBLICATIONS:


CONFERENCE PRESENTATIONS:

1. “RELATIONSHIP STATUS: IT’S COMPLICATED. The small heat shock proteins interact with several stages of α-synuclein aggregation”
   *The PhD Series, University of Wollongong, Australia. November 2015*
   Oral Presentation, awarded Second Place Academic Prize

2. “THE LONG & THE SHORT OF IT: The interaction of small heat shock proteins with α-synuclein”
   *Neurodegeneration and Dementia Conference, Melbourne, Australia. August 2015*
   Poster Presentation

3. “TO BIND OR NOT TO BIND: The small Heat shock proteins interact with several stages of α-synuclein aggregation”
   *East Coast Protein Meeting, Coffs Harbour, Australia. July 2015*
   Oral Presentation, awarded Bruker Travel Award for outstanding student presentation
4. “RELATIONSHIP STATUS: It’s complicated”  
   Sydney Protein Group, Sydney, Australia. November 2014  
   Oral Presentation, awarded Thompson Prize for best student presentation

5. “OVERCOMING THE FRONTLINE DEFENSE: The role of sHsps in preventing α-synuclein aggregation”  
   International Proteostasis and Disease Symposium, Wollongong, Australia. November 2012  
   Poster Presentation

6. “AVOIDING THE STRAIGHT AND NARROW: the role of sHsps in preventing disease-associated amyloid fibril formation”  
   Lorne Protein Structure and Function Conference, Lorne, Australia. February 2012  
   Poster Presentation
Chapter 1:
Introduction

Portions of this chapter have been previously published in the following work:

Author Contributions: DC wrote the manuscript, composed the figures and generated the table; HE and JAC edited the manuscript for submission.
1.1 Proteostasis

Proteostasis, or the maintenance of protein homeostasis, operates both inside and outside the cell (for recent comprehensive reviews see Gidalevitz et al. (2011) and Wyatt et al. (2013)) and is dependent on numerous integrated pathways that control the lifecycle and fate of a protein (Figure 1.1). These pathways include those involved in gene transcription, mRNA translation and protein synthesis on the ribosome, through to protein folding, trafficking and compartmentalisation in cellular organelles, and finally to protein degradation by autophagy or the proteasome (Yerbury et al. 2005). As such, the macromolecular elements comprising the cellular proteostasis network include transcription factors, RNA processing and translocation factors, folding enzymes, trafficking components, molecular chaperones and degradation components. The capacity to maintain proteostasis varies between cell types (Chen and Brown 2007), and is thought to be reflective of the composition and concentration of components of the proteostasis network that arise as a result of differences during cellular differentiation and development (Powers et al. 2009). Whilst this capacity is finite at any point in time, it can be spatially and temporally altered through varying the amount and/or activity of individual components. Thus, an appropriate analogy for proteostasis is a see-saw; there is an intricate balance required in the use of energy and resources to maintain the functional integrity of the proteome and thus avoid protein aggregation and disease.

1.1.1 Protein folding

The biological function of most proteins is inextricably linked to them obtaining the correct (native) conformation. Whilst some proteins are intrinsically disordered, remaining unfolded throughout their life span or acquiring structure upon binding to other proteins (Wright and Dyson 1999, Dunker et al. 2001, Uversky 2002), most exhibit defined secondary and tertiary structures which require ‘folding’ of the polypeptide chain. Protein folding is defined as the
Figure 1.1: Cellular proteostasis mechanisms. In the endoplasmic reticulum (ER), molecular chaperones assist newly synthesised protein intermediates to fold into their native conformation for transport into the cytosol. Persistent or misfolded protein intermediates can be proteolytically degraded within the ER or transported to the cytosol. Once in the cytosol, protein intermediates are recognised by molecular chaperones and targeted for refolding or degradation via lysosomal or proteasomal pathways. When these mechanisms fail to clear protein intermediates, insoluble aggregates can accumulate within the ER or cytosol as aggresomes. Adapted from Yerbury et al. (2005).

process by which the three-dimensional conformation of a functional protein results from the linear sequence of information contained in the amino acid chain (Anfinsen et al. 1961, Hartl 1996). In a cellular context, folding may be co-translational (i.e. initiated during completion of protein synthesis whilst the nascent chain is still associated with the ribosome) or occur post-translationally, either in the cytoplasm or in specific compartments, such as the endoplasmic reticulum (ER), following trafficking and translocation through membranes (Dobson 2003). Unfolding is also important in the life cycle of many proteins; it is required in various biological processes including protein trafficking, secretion and translocation across membranes, as well as regulation of the cell cycle (Radford and Dobson 1999). In all cases, attaining the correct native state, whether folded or disordered, is essential for protein function and often relies on the presence of molecular chaperones.
Folding reactions are highly complex given the sheer number of potential conformations available to a protein chain following synthesis. The current model posits a funnel-shaped energy landscape in which polypeptide chains explore a range of energy states whilst progressing along downhill routes in search of the native structure (Figure 1.2). Soluble proteins rely on hydrophobic forces, which encourage chain collapse thereby facilitating burial of non-polar residues (Hartl et al. 2011). The potential conformations populating the folding landscape are rapidly restricted by this chain collapse, and an increasing number of native interactions. However, there are often substantial energetic barriers that must be overcome in the search toward a native conformation, resulting in partially-folded intermediate states which can become kinetically trapped (Figure 1.2). An important function of molecular chaperones in the folding process is assisting a protein to overcome these energetic barriers and preventing non-native, but energetically favourable, interactions. In particular, for larger proteins, hydrophobic collapse often results in the formation of kinetically trapped, partially-folded intermediates which may be stabilised by non-native interactions (Hartl et al. 2011). In this case, these conformations are now considered ‘misfolded’.

1.1.2 Protein misfolding and aggregation

The persistence of partially-folded or misfolded protein states becomes problematic in the cellular context, as they expose hydrophobic and unstructured regions which would otherwise be buried in the native conformation (Dobson 2001, Hartl et al. 2011). These features often lead to the association of non-native proteins, resulting in them leaving the ‘on-folding’ pathway and entering ‘off-folding’ pathways, eventually leading to amorphous (disordered) or amyloid fibrillar (ordered) aggregates (Figure 1.2). These aggregated protein forms are generally defined by the presence of non-native secondary structures which are stable under
physiological conditions, have poor solubility and aberrant subcellular or extracellular localisation (Kopito 2000). The propensity of any given protein to aggregate is a function of its chemical properties, governed by the amino acid sequence, the conformational stability of its folded states, and its relative cellular concentration (Hipp et al. 2014). For example, although some proteins are expressed at levels exceeding their solubility (referred to as supersaturated), they may not be at risk of becoming insoluble unless their intrinsic aggregation propensity is high (Ciryam et al. 2015). In addition, the total cellular concentration of proteins is commonly in excess of 300 g/L, resulting in macromolecular crowding, and this significantly increases the propensity of non-native proteins to aggregate (Hipp et al. 2014).

Whether a protein undergoes amorphous or fibrillar aggregation is similarly governed by the structure and stability of the precursor partially-folded intermediate. Native-like folding
intermediates that have a relatively high degree of exposed hydrophobicity, while retaining elements of secondary structure, are observed to preferentially lead to the rapid formation of amorphous aggregates (Khurana et al. 2001). Conversely, intermediates which are relatively unfolded, display little secondary structure and have a relatively low degree of exposed hydrophobicity, commonly form fibrils via ordered self-assembly (Khurana et al. 2001). Importantly, the formation of fibrillar aggregates is characterised by the initial formation of soluble, oligomeric species which must undergo rearrangement to form amyloid fibrils. In this way, oligomeric states may be comparable to kinetically trapped monomeric intermediates, as their conversion to fibrils requires overcoming energetic barriers, making this a key process for chaperones in order to prevent protein aggregation and deposition (Figure 1.2).

1.1.3 In vitro versus in vivo: considering the cellular context

Many studies examine protein aggregation in isolation, often using reactions containing only the protein(s) of interest (an aggregation-prone target protein) and inducers or inhibitors of aggregation. These experiments provide useful mechanistic details of a protein’s aggregation pathway, allowing researchers to consider the evolution of aggregating species and their relationship with a range of aggregation modulators. These approaches have allowed the characterisation of amyloid as a generic conformation accessible by most, if not all, proteins (Chiti and Dobson 2006). It is now routine to investigate the kinetics of amyloid formation using amyloidophilic dyes such as Thioflavin-T (ThT) or Congo red, characterise aggregate morphology via Transmission Electron Microscopy (TEM) or Atomic Force Microscopy (AFM), determine the secondary structure of aggregates using Circular Dichroism (CD) and Fourier-transform infrared (FTIR) spectroscopy, and identify the protease-resistant core via limited protease digestion (Gregoire and Kwon 2012). In addition, the advent of confocal and
total internal reflection fluorescence (TIRF) microscopy-based single-molecule techniques, such as single molecule Förster Resonance Energy Transfer (smFRET), two-colour coincidence detection (TCCD), Stochastic Optical Reconstruction Microscopy (STORM), single aggregate visualisation by enhancement (SAVE) and spectrally-resolved Point Accumulation for Imaging in Nanoscale Topography (sPAINT), now enable examination of rare species present during aggregation that are otherwise undetectable using traditional ‘bulk’ methods. These techniques can therefore provide both qualitative and quantitative information regarding the formation of oligomers and fibrils, their surface properties and potential interactions with other proteins of interest.

By combining each of the techniques discussed above, it is possible to build a comprehensive understanding of the aggregation of a given protein, including pathway intermediates, morphology, structure and kinetics. However, these studies fail to replicate the complex factors present in the intra- or extracellular environments in which aggregation takes place. As such, extending the characterisation of aggregating proteins from the ‘test tube’ to the cellular context is crucial. Studying protein aggregation in situ helps develop our understanding of the interplay between intracellular components, such as chaperones and proteases, and processes including protein expression, deposition and degradation, in the crowded cellular environment (Ami et al. 2013).

The aggregation of intracellular proteins is most commonly monitored in situ using fluorescence techniques. Methods of covalently attaching fluorescent molecules through genetically encoding specific tag sequences at the N- or C-terminals of the protein of interest form the basis of many cellular aggregation studies (Crivat and Taraska 2012). Tracking cellular proteins was initially made routine through the in-frame genetic fusion of inherently fluorescent proteins such as green fluorescent protein (GFP), enhancedGFP (EGFP), yellow
fluorescent protein (YFP) or red fluorescent protein (RFP), to the protein of interest (Crivat and Taraska 2012). Extensive customisation of these fluorescent proteins has resulted in a suite of potential protein tags, covering a range of excitation and emission wavelengths, brightnesses, pH sensitivities and photophysical properties (Day and Davidson 2009). Fluorescently tagged proteins are especially useful when characterising the real-time distribution, trafficking and aggregation of proteins within a cell. Alternatively, cellular proteins may be detected via immunohistochemistry, in which antibodies specific for the protein and conformation of interest are introduced to the cell following fixation and permeabilisation of the cell membrane. Together, these techniques enable protein aggregation, and factors that regulate this process, to be studied and measured in cells.

1.1.4 The role of molecular chaperones

Molecular chaperones protect and stabilise non-native regions of proteins, or assist in proteins acquiring their native state, without contributing conformational information or forming part of the final native structure (Hartl 1996, Hartl et al. 2011). Chaperones achieve this by interacting with (and stabilizing) partially-folded protein intermediates in order to prevent improper associations that could otherwise lead to misfolding and aggregation (Ellis 1997). In addition, chaperones can also facilitate the folding of multi-domain proteins, through transient sequestration of the folding intermediates (Powers et al. 2009).

Due to their role in assisting proteins to acquire and maintain their native conformation, molecular chaperones are key components of the proteostasis network. They participate in protein folding, complex assembly, protein trafficking, protein stabilisation and protein degradation. As their name implies, the class of molecular chaperones referred to as heat shock proteins (Hsps) are most commonly expressed as part of the cellular response to stress, although some members are constitutively expressed and play important roles under non-
stress conditions (Feder and Hofmann 1999). The Hsps have been classified into families based on the mass of their monomeric subunits; these include Hsp100, Hsp90, Hsp70, Hsp60 and the small Heat shock proteins (sHsps; HSPB) (Sarkar et al. 2011). In addition, chaperones have also been classified as having either a ‘foldase’ or ‘holdase’ type action. For example, Hsp70 and Hsp60 are classified as ‘foldase’ type chaperones as they actively facilitate the folding of protein intermediates to their native folded state, often acting in tandem. Their mechanism of action is dependent on ATP hydrolysis, which results in cycles of high- to low-affinity target protein binding, to promote folding of the target protein (Beissinger and Buchner 1998, Hoffmann et al. 2004). Foldase chaperones also encompass the so-called ‘unfoldase’ action attributed to some chaperones, including Hsp100 and Hsp70, in which ATP hydrolysis is used to unfold or disaggregate misfolded or aggregated proteins to provide folding-competent intermediates (Hubbard and Sander 1991, Slepenkov and Witt 2002). In contrast, ‘holdase’ chaperones, which includes the sHsps, interact with partially-folded intermediate states of proteins to stabilise them and prevent their mutual association. The mechanism of action of holdase chaperones is ATP-independent since they do not have an active role in folding proteins; their association with target proteins is driven primarily through hydrophobic interactions. This enables holdases to function in the ATP-depleted environment that occurs in the cell when it is under stress, ensuring their function is not compromised at the time cell viability is threatened. When energy levels permit, holdase chaperones can deliver target proteins to foldases for refolding, or to the proteasome or autophagy for degradation (Beissinger and Buchner 1998, Hoffmann et al. 2004, Rikhvanov et al. 2007).

When a protein can no longer maintain its correct conformation, the cell utilises degradation pathways such as the ubiquitin-proteasome machinery and autophagic-lysosomal trafficking systems to remove it. Misfolded proteins are recognised by the ubiquitin-proteasome system,
which labels and degrades them through a highly regulated pathway. Using a series of ubiquitin ligase enzymes, ubiquitin polypeptide chains are covalently linked to misfolded proteins, marking them as substrates for selective degradation within the proteasome (Schwartz and Ciechanover 1999, Cook et al. 2012). Lysosomal mechanisms, such as macroautophagy and microautophagy, are less selective; membrane-bound vesicles capture a selection of the cytosol, which is then targeted for degradation. By contrast, chaperone-mediated autophagy provides a level of targeted degradation as it relies on the recognition of a target motif in cytosolic proteins by specific chaperones, which then deliver the proteins to the membrane of the lysosome for internalisation and degradation (Kaushik and Cuervo 2012). The sHsps may play a role in all of these degradation pathways; for instance, αB-crystallin (αB-c; HSPB5) stimulates ubiquitination of insoluble proteins which marks them for ubiquitin-dependent degradation (Engelsman et al. 2003), Hsp22 (HSPB8) in cooperation with Bag3, promotes degradation of mutant Huntingtin (Htt) protein through induction of macroautophagy (Carra et al. 2008) and Hsp27 (HSPB1) targets misfolded cystic fibrosis transmembrane conductance regulator protein for degradation in the proteasome (Ahner et al. 2013). Whether these activities are specific to individual sHsps or represent more generic mechanisms of action of sHsp family members is yet to be determined, however it is clear from this work that as a chaperone class, sHsps function not only to maintain proteins in their biologically active conformation, but also to facilitate their degradation when misfolded (Parcellier et al. 2003).

1.2 Proteostasis and disease

A number of factors can influence the ability of a cell, tissue or organism to maintain proteostasis. Changes in cellular ATP levels, amino acid pools, metabolites, lipid homeostasis and ion balance can all disrupt the protein folding and degradation capabilities of the cell
(Powers et al. 2009). Signalling pathways can be exploited to control transcriptional, translational and post-translational mechanisms in the cell in order to regulate protein synthesis, folding, trafficking and degradation (Powers et al. 2009). However, disruption of any element within this integrated network can result in proteostasis dysfunction. Aberrant protein folding and protein aggregation are now recognised as key factors in many diseases, collectively termed protein misfolding or conformational diseases (Chiti and Dobson 2006). These diseases, which include type II diabetes, cataract, and neurological disorders such as Alzheimer’s disease and Parkinson’s disease (PD) (Barral et al. 2004), are associated with the aggregation and precipitation of misfolded protein into either amorphous or fibrillar aggregates. Those associated with amyloid aggregation represent the largest group of misfolding disorders (Chiti and Dobson 2006). Their prevalence, and the prediction that this will increase dramatically over the next decade as a consequence of the ageing population in many countries (de Lau and Breteler 2006), underlies the importance of understanding the network of pathways that maintain proteostasis.

1.2.1 The α-synucleinopathies: a collection of aggregation disorders

Despite the common pathway of pathogenic protein aggregation and deposition which leads to disease, in many cases the underlying proteins associated with individual disorders are unique (Chiti and Dobson 2006). In some circumstances, however, several disorders may be linked by the aggregation of a single protein. The α-synucleinopathies are a group of neurodegenerative diseases characterised by the amyloid fibrillar aggregation and intracellular deposition of α-synuclein (α-syn) (Martí et al. 2003). Deposition of α-syn occurs in selected populations of neurons or glia, and may be localised to the cytoplasm or neurite processes, giving rise to clinically distinct disorders with a common underlying pathology (Goedert 2001). These diseases include multiple system atrophy, dementia with Lewy bodies

The most well recognised α-synucleinopathy is PD. It is the second most prevalent neurological disorder, after Alzheimer’s disease, with its world-wide incidence projected to reach at least 8.7 million individuals over 50 years of age by 2030 (de Lau and Breteler 2006, Dorsey et al. 2007, Massano and Bhatia 2012). Typically, PD is characterised by motor manifestations including tremor, rigidity, bradykinesia and postural instability, which can also be accompanied by non-motor symptoms such as sleep impairment, neuropsychiatric disorders and olfactory deficits (Braak et al. 2004, Chaudhuri et al. 2006). All of these symptoms are linked to a gradual reduction in dopamine content associated with the progressive loss of dopaminergic neurons in the substantia nigra pars compacta, a region located in the midbrain.

The histological hallmark of PD is the presence of protein inclusions, known as Lewy bodies or Lewy neurites, localised to the cell body or cell processes, respectively (Wakabayashi et al. 2007). These distinctive spherical protein inclusions, of which the principal protein is α-syn (Spillantini et al. 1997), are found in the cytoplasm of nigral neurons and are characterised by a dense protein core surrounded by a halo of fibrils and auxiliary proteins (Fink 2006).

1.2.2 α-Syn in disease

α-Syn, a 140 amino acid protein, was initially identified as a neuron-specific protein localised within the nucleus and presynaptic terminals (Maroteaux et al. 1988). From a functional perspective, there is still no definitive evidence for the role α-syn plays in cells. It has been implicated in modulating synaptic activity through membrane processes, including neurotransmitter release, trafficking and biogenesis (Davidson et al. 1998, Jenco et al. 1998).
In addition to the full-length form of the protein, there are two shorter isoforms, which are 126 and 112 amino acids in length, and result from in-frame deletions of exon 3 and 5, respectively (Ueda et al. 1994, Campion et al. 1995). However, the full length isoform is by far the most abundant in the brain (Ueda et al. 1994, Campion et al. 1995, Halliday and McCann 2008). Truncated versions, derived from proteolytic cleavage, have been identified as significant components of Lewy body inclusions (Baba et al. 1998, Campbell et al. 2001, Halliday and McCann 2008). Truncation fragments of α-syn also have a higher aggregation propensity than the full length form, both in vitro and in vivo (Crowther et al. 1998, Tofaris et al. 2003, Tofaris et al. 2006, Bellucci et al. 2011).

As well as the association of α-syn with Lewy body pathology, there is a strong genetic association of α-syn with PD (Polymeropoulos et al. 1997) which has further piqued interest into the role of this protein in disease. Parkinson’s disease may be either early onset (before 40 years of age) or late onset (after 40 years of age). Although most commonly presenting as a late onset, sporadic disease of unknown aetiology, a familial form with an autosomal dominant pattern of inheritance among early onset patients was initially identified in two small kindreds (Golbe 1990, Golbe et al. 1990). This led to the identification of missense mutations in the α-syn gene which correlate to early-onset and aggressive PD: A53T, A30P, G51D, H50Q and E46K (Polymeropoulos et al. 1997, Kruger et al. 1998, Zarranz et al. 2004, Kiely et al. 2013, Proukakis et al. 2013). Multiplication of the α-syn gene has also been associated with the development of α-syn inclusions and PD (Baba et al. 1998, Campbell et al. 2001). Gene copy number is strongly correlated with the age of disease onset; duplication results in an average age of onset of 48.4 years while triplication results in an average age of onset of 33.4 years (Farrer 2006).
1.2.3 Aggregation of α-syn

Due to the presence of large amounts of aggregated α-syn in Lewy bodies and Lewy neurites, and the correlation between α-syn genetic abnormalities and early onset PD, the mechanism and kinetics by which α-syn aggregates to form amyloid fibrils \textit{in vitro} have been examined in detail (Figure 1.3). It has been generally accepted that α-syn exists as an unstructured monomeric protein in its native state in solution. However, when the N-terminus of monomeric α-syn interacts with lipids it attains an α-helical structure and recent evidence has suggested it may form a tetramer in some cell types (Eliezer et al. 2001, Bartels et al. 2011, Wang et al. 2011, Fauvet et al. 2012, Dettmer et al. 2013). Within inclusions, α-syn is assembled into highly-ordered, β-sheet rich amyloid fibrils (Polymeropoulos et al. 1997). Similarly, under conditions of physiological pH and temperature \textit{in vitro}, purified α-syn assembles into fibrils resembling those found in diseased brains (Rochet et al. 2000, Serpell et al. 2000, Uversky 2007). Amyloid fibril formation occurs via a nucleation-dependent mechanism in which the formation of oligomeric nuclei is the rate-limiting step, followed by rapid elongation and assembly into mature fibrils (Wood et al. 1999, Bhak et al. 2009, Li et al. 2009). All three α-syn mutations associated with PD have been found to influence the early stages of aggregation, either nuclei formation or fibril growth, when their aggregation propensities have been assessed \textit{in vitro} (Ono et al. 2011). Each α-syn mutant exhibits distinct fibrillation kinetics and/or aggregate morphologies: α-syn A53T and E46K form fibrils more rapidly than the wild-type (WT) protein, and α-syn A30P forms mature fibrils more slowly, although smaller oligomeric species are formed more rapidly by this mutant (Conway et al. 1998, Conway et al. 2000, Bruinsma et al. 2011). The difference in fibrillation kinetics is generally attributed to variations in the rate of nucleation, as opposed to the rate of elongation (Conway et al. 2000, Conway et al. 2000). As with gene multiplication, the aggregation propensity of each α-syn variant correlates well with disease onset and the
severity of familial PD, with the A53T variant aggregating the fastest and being associated with the earliest and most aggressive disease phenotype (Sellbach et al. 2006).

In addition to the inherent aggregation propensity of α-syn, a number of factors have been shown to influence the rate at which the protein forms fibrils. For example, aggregation is promoted by the presence of metals, pesticides, lipids, membranes, and under conditions of low pH or molecular crowding (Shtilerman et al. 2002, Uversky et al. 2002, Munishkina et al. 2004). In general, these factors increase the concentration of the prefibrillar intermediates crucial for the formation of nuclei during the rate-limiting step (Shtilerman et al. 2002, Uversky et al. 2002, Munishkina et al. 2004, Fink 2006). Conversely, aggregation is hindered by the presence of β- and γ-synucleins (Hashimoto et al. 2001, Uversky et al. 2002), as well as molecular chaperones such as sHsps (Bruinsma et al. 2011, Breydo et al. 2012). Changes in the rate at which aggregation occurs (e.g. through the influence of the above mentioned factors), may therefore contribute to the failure of proteostasis elements to prevent α-syn aggregation in a disease context.

**Figure 1.3: Amyloid fibrillar aggregation of α-syn.** Unfolded α-syn exists as a monomer that can interact with lipids, to form an α-helical structure (Eliezer et al. 2001). There is some evidence that a tetrameric α-helical form of α-syn also exists in cells (Dettmer et al. 2013) and monomeric unfolded forms of α-syn may be in equilibrium with this tetramer. Unfolded α-syn aggregates through a nucleation-dependent mechanism, in which aggregation-prone α-syn monomers associate to form soluble prefibrillar oligomeric nuclei. This is followed by the elongation of these nuclei into mature amyloid fibrils. Fibrillar aggregates may then be sequestered into Lewy bodies or Lewy neurites. Alternatively, fragmentation of mature fibrils can generate additional oligomeric nuclei which seed secondary aggregation events.
1.2.4 $\alpha$-Syn pathogenicity

Although the exact physiological function of $\alpha$-syn is yet to be defined, based on knowledge of its broad physiological role of modulating synaptic activity, some features of PD may be ascribed to a toxic loss of function encountered when $\alpha$-syn in the cell is sequestered and deposited following aggregation. The reduced availability of $\alpha$-syn would impact on its ability to interact with cellular membranes (Rajagopalan and Andersen 2001). Failure of $\alpha$-syn to complete its physiological role within the cell could potentially cause neuronal damage, particularly in the synaptic terminal (Stefanis 2012). However, it is considered unlikely that this is the primary pathological effect of $\alpha$-syn aggregation since $\alpha$-syn knockout mice do not display any overt neuropathological or behavioural phenotypes, in contrast to mice that overexpress $\alpha$-syn (Abeliovich et al. 2000, Welchko et al. 2012). Instead, it is now generally accepted that the accumulation of $\alpha$-syn into Lewy bodies and Lewy neurites leads to disease due to a toxic gain-of-function inherent in the protein when it exceeds a threshold concentration and adopts a fibrillar-type conformation (Stefanis 2012). The aggregation of $\alpha$-syn, exacerbated by a decrease in the ability of the cell to dispose of damaged proteins, results in the accumulation of non-functional (potentially toxic) $\alpha$-syn species, which may interfere with normal metabolic processes.

1.2.5 Identifying the toxic species in disease

For some time, the toxic $\alpha$-syn species responsible for neurodegeneration has been hotly debated. Initially, cytoplasmic inclusions were thought to be a characteristic feature of dead or dying neurons, with the deposition of mature amyloid fibrils into Lewy bodies identified as the neurotoxic event. This was primarily based on findings suggesting that inclusions may suppress organelle function, interfere with axonal transport, or induce energy failure via hyperubiquitination (Iwai 2000, Kaplan et al. 2003). However, Lewy bodies are commonly
found in living neurons, and are also present in up to 15% of healthy, aged individuals (Gibb and Lees 1988, Hindle 2010).

In cell-based models and in vivo, neurotoxicity correlates best with the appearance of soluble α-syn oligomers, as opposed to inclusions (Gosavi et al. 2002). Toxicity is usually observed in the absence of mature α-syn fibrils or detectable deposition into inclusions in cell models (Gosavi et al. 2002, Xu et al. 2002). Moreover, surviving dopaminergic neurons demonstrate equivalent viability irrespective of the presence or absence of Lewy bodies (Gertz et al. 1994). Furthermore, transgenic mice exhibit neurodegeneration outside the substantia nigra in the absence of fibrillary inclusions, and α-syn fibril-containing inclusions in Drosophila are observed in the absence of neurodegeneration (van der Putten et al. 2000, Auluck and Bonini 2002, Auluck et al. 2002).

A recent study utilised a rat lentivirus system to examine the cellular toxicity of α-syn E35K and E37K, which were specifically designed to form small oligomers, compared to an α-syn variant encompassing residues 30-110, which forms fibrils (Winner et al. 2011). Following injection into the substantia nigra, cell loss was assessed based on a reduction in tyrosine hydroxylase-positive neurons in this region. Higher toxicity was observed in dopaminergic neurons of animals exposed to the oligomer-forming variants of α-syn compared to that which formed fibrils (Winner et al. 2011). Finally, dopamine and its metabolites inhibit the conversion of protofibrils to mature amyloid both in vitro and in vivo (Conway et al. 2001, Mazzulli et al. 2006, Tsika et al. 2010), providing a potential rationalisation for the vulnerability of dopaminergic neurons (since they would therefore promote protofibril formation). Alternatively, dopamine may act as a source of reactive oxygen species (ROS) in a process potentiated by α-syn, thereby promoting apoptosis (Xu et al. 2002).
In light of this evidence, the predominant consensus is now that soluble prefibrillar oligomeric species of α-syn are the most toxic entity, and fibrils are the less toxic end-product of the aggregation process (Glabe 2008, Glabe 2009). Yet, mature fibrils can be a source of cytotoxic oligomeric species due to their fragmentation and potential to act as sites of secondary nucleation (Xue et al. 2009). Thus, it has been proposed that the formation of protein inclusions may represent an additional protective mechanism employed by the cell (Hashimoto and Masliah 1999, Goldberg and Lansbury Jr 2000, Glabe 2008). The formation of inclusion bodies within cells may therefore represent a protection strategy to actively sequester toxic oligomeric forms that may arise due to fibril fragmentation. In this way, potentially toxic species can be isolated in specific compartments in the cell as a protection mechanism (Tyedmers et al. 2010, Yerbury et al. 2016). However, the appearance of inclusions is an indication of the cell’s inability to maintain proteostasis and prevent aggregation. Thus, while it may provide short-term protection, the presence of inclusions is likely to signify that the long-term outcome will be cell death should the cell be unable to restore proteostasis.

The generic toxicity of prefibrillar (i.e. small soluble oligomeric) species is also evident in non-disease-associated proteins capable of forming amyloid. For example, using two unrelated and non-disease-associated protein domains, the SH3 domain from bovine phosphatidyl-inositol-3’-kinase and the amino terminal of HypF from E. coli, Bucciantini and colleagues (2002) demonstrated that oligomeric forms of these proteins, generated during the early stages of aggregation, were inherently cytotoxic when added to cells in culture. As well, early prefibrillar aggregates of apomyoglobin are toxic to cultured fibroblasts via their ability to alter membrane permeability (Sirangelo et al. 2004). Finally, soluble amyloidogenic oligomers of equine lysozyme are toxic to both primary and cultured neuronal cells; cytotoxicity is correlated with the size of the oligomers within the sample; larger species
being less cytotoxic (Malisauskas et al. 2005). Thus, it is concluded that prefibrillar aggregates are the most toxic entity formed during amyloid fibril formation.

The toxicity of aggregates formed from a variety of pathogenic and non-pathogenic proteins correlates with the level of exposed hydrophobicity at the aggregate surface (Bolognesi et al. 2010). Initially, there is an abundance of soluble oligomeric aggregates with a high surface-to-volume ratio and a high degree of exposed hydrophobicity (Cheon et al. 2007). As the aggregate increases in size over time, there is a decrease in the surface-to-volume ratio and amount of exposed hydrophobicity (Kremer et al. 2000, Cheon et al. 2007, Bolognesi et al. 2010). The higher proportion of hydrophobic residues exposed on the smaller oligomeric species enables them to participate in inappropriate interactions that can ultimately lead to cell death (Chiti and Dobson 2006). It is important to note, however, that although this mechanism appears generally applicable to pathogenic and non-pathogenic proteins alike, ultimately the amino acid sequence defines the kinetics by which a protein aggregates and the specific properties of any aggregate formed. Thus, the specific toxicity of a protein is influenced by its relative ability to form oligomers, the rate these oligomers are then converted to mature fibrils, and the amount of hydrophobicity exposed throughout this process (Bolognesi et al. 2010).

The relative hydrophobicity of prefibrillar aggregates appears to endow them with a large potential to cause cellular damage. Many mechanisms have been proposed for the toxicity of aggregated forms of α-syn (summarised in Figure 1.4). When considering the mechanism of toxicity of these species, it is important to also take into account their physical properties. For example, α-syn is capable of lipid-binding (Volles and Lansbury 2003, Zhu et al. 2003) and early oligomers formed from α-syn can be small, flexible spheroids, characteristics which favour their association with membranes and pore formation (Invernizzi et al. 2012). Pore
Figure 1.4: A schematic model for the potential mechanisms by which α-syn aggregation is toxic to neuronal cells. Aggregation-prone monomeric α-syn associates to form soluble oligomeric nuclei leading to the formation of mature fibrils. (1) Inclusion body formation: Fibrillar α-syn is sequestered into protein inclusions, which also contain various other cellular proteins, including sHsps, potentially depleting the cell of these essential components (2) Initiation of the heat shock response: An accumulation of toxic α-syn species within the cell activates the heat shock response pathway, initiating changes in transcription of stress response genes. (3) Blocking protein trafficking from the ER to Golgi: Aggregation of toxic forms of α-syn in the cell can block protein transport from the ER to Golgi, inducing Golgi fragmentation. (4) Oxidative stress: Aggregation of α-syn induces dopamine (DA)-dependent ROS production, resulting in oxidative stress. (5) Defects in axonal transport: Aggregated α-syn induces hyperphosphorylation of tau in the axon, which causes defects in axonal transport through restricting the ability of tau to modulate microtubule assembly. This impairs essential cellular transport as well as resulting in the accumulation of aggregated material in the cell body. (6) Altered synaptic terminal excitability and protein expression: The presence of aggregated α-syn in the synaptic compartment alters the distribution of synaptic terminal proteins, diminishing synaptic vesicle release and leading to changes in synaptic terminal protein expression and excitability. (7) Impaired autophagy: Binding of α-syn to lysosomal membranes impairs chaperone-mediated autophagic function, resulting in substrate accumulation and proteasomal impairment in the cell body. (8) Release of toxic α-syn species into extracellular space: Aggregated α-syn may be actively secreted (e.g. via exosomes) or passively released by dying neurons and be subsequently taken up by neighbouring neurons, resulting in seeded aggregation and altered synaptic terminal activity. Uptake by surrounding glia can induce proinflammatory activity including ROS production, which is toxic to surrounding neurons. (9) Impaired energy production and increased ROS production: Localisation of α-syn to mitochondrial membranes may impair energy production or increase ROS formation. (10) Membrane pore formation: Ring-like oligomeric α-syn species may infiltrate cellular membranes forming pores within the membrane and altering ion permeability. Figure compiled using information from (Gosavi et al. 2002, Volles and Lansbury 2002, Xu et al. 2002, Maries et al. 2003, Volles and Lansbury 2003, Cookson 2009, Bellucci et al. 2012, Chu et al. 2012, Lundblad et al. 2012, Marques and Outeiro 2012).

formation may occur on any of the cellular membranes, both inter- and intra-cellular, providing several targets for α-syn mediated toxicity (Cookson and van der Brug 2008). For instance, pore formation within the plasma membrane may allow the abnormal flow of ions, causing cellular dysfunction and leading to apoptosis (Volles and Lansbury 2002, Volles and Lansbury 2003). This is indirectly supported by work showing that cells expressing α-syn have increased cation permeability (Furukawa et al. 2006). Through interaction with lysosomal membranes, α-syn aggregates may inhibit chaperone-mediated autophagy, leading to an accumulation of substrates and proteasome inhibition (Cookson 2009). Alternatively, prefibrillar oligomers can impair axonal transport via hyperphosphorylation of Tau, a protein normally responsible for stabilising and regulating microtubule assembly and interacting with membranous cargo (Chu et al. 2012). By disrupting transport from the ER to Golgi, α-syn can cause ER stress and Golgi fragmentation (Gosavi et al. 2002, Cookson 2009). Energy
production can also be impaired due to effects of oligomeric α-syn on mitochondria (Maries et al. 2003). In addition, α-syn is implicated in reducing dopamine release and its subsequent reuptake in the synaptic terminal (Lundblad et al. 2012). The active secretion or passive release (following death) of aggregated forms of α-syn can also result in cell-to-cell transfer of toxic intermediates that alter synaptic protein expression and excitability (Bellucci et al. 2012). Moreover, these extracellular species of α-syn can activate surrounding astrocytes and glia, resulting in the production of ROS and proinflammatory cytokines, which in turn can be toxic to surrounding neurons (Marques and Outeiro 2012). Finally, although inclusion bodies may sequester potentially harmful aggregation intermediates, this process may also result in the depletion of proteins that become associated with the inclusions from the cytoplasm, leading to a loss in their biological activity (Maries et al. 2003). Together these factors may lead to compromised cell viability (and cell death), however, any one of these events (or combination thereof) may be sufficient given the delicate balance required to maintain proteostasis.

1.2.6 Current cell-based models of α-syn aggregation

Despite the lingering debate surrounding whether incorporation of proteins into inclusions is in fact protective or detrimental for a cell, it is clear that protein aggregation and the formation of these inclusions is closely associated with neurodegeneration and disease (Yerbury et al. 2016). It is therefore critical to examine these processes, and the potential impact of proteostasis elements, such as chaperones, in the cellular context. To date, this has been achieved through the development of a range of cellular models targeting the aggregation of disease-related target proteins. Most cell-based models of the toxicity associated with α-syn aggregation are based on the exogenous application of aggregated α-syn to cells in culture. Whilst exogenous application of α-syn fibrils may have relevance in
cell-to-cell propagation of aggregated α-syn (Desplats et al. 2009, Volpicelli-Daley et al. 2011, Freundt et al. 2012, Luk et al. 2012), the intracellular aggregation of α-syn into Lewy body-like deposits most likely recapitulates the earliest events in disease and therefore is considered especially relevant when considering the implications of proteostasis components on aggregation.

Overexpression of α-syn (via transfection or viral induction) has been examined in both primary and immortalised cell lines. Overexpression of both α-syn WT and A53T in primary mesencephalic neuronal cultures not only results in significant cell death, which is specific to dopaminergic neurons and does not impact the viability of the other cells in the culture, but also renders surviving dopaminergic neurons more susceptible to neurotoxic insults (Zhou et al. 2000, Zhou et al. 2002). Overexpression of α-syn WT, A53T and A30P in human neuroblastoma cells (SH-SY5Y) is not sufficient to cause extensive inclusion formation. However, co-treatment with various agents that generate ROS (e.g. rotenone, papaNONOate and FeCl₂) induces cytoplasmic protein inclusions in those cells which contain both α-syn and ubiquitin, and are therefore typical of Lewy bodies isolated from diseased tissue (Ostrerova-Golts et al. 2000, Matsuzaki et al. 2004). In these studies, the extent of α-syn aggregation was dependent on both the level and isoform of α-syn expressed: α-synA53T had an increased tendency to form inclusions, consistent with its increased propensity to form fibrils in vitro and its genetic association with early onset PD (Ostrerova-Golts et al. 2000). McLean et al (2001) expressed an α-syn isoform which was C-terminally tagged with EGFP in H4 neuroglioma cells and demonstrated that overexpression of this protein results in cytoplasmic inclusions in ~5% of transfected cells. Interestingly, co-transfection with synphilin-1, a protein which binds α-syn in vivo, increased the percentage of cells containing inclusions to ~55% (Engelender et al. 1999, McLean et al. 2001). Notably, although the α-syn was expressed with an EGFP fluorescent tag, inclusions were not fluorescent or reactive to
anti-GFP antibodies (McLean et al. 2001). This was found to be due to cleavage of part of the C-terminal region of EGFP from the α-syn-EGFP expressed in these cells, generating a truncated (and non-fluorescent) form of the protein which aggregated to form inclusions. Thus, these findings highlight the potential problems with tagging α-syn (and indeed other proteins) to monitor its aggregation in cells. Alternative methods for studying full length α-syn aggregation in cells have also been developed. For example, using a tetra-cysteine tagged α-syn, that was either microinjected or transfected into cells and detected by labelling with a fluorogenic biarsenical compound. Using this approach, Bertoncinni et al. (2007) demonstrated that the aggregation of α-syn into inclusions is significantly increased (up to 10%) by co-treating the cells with FeCl₂ to induce oxidative stress. More recently, α-syn aggregation in cells was modelled by the addition of a 16 amino acid peptide (termed CL1) to the C-terminus of α-syn, and this was used to demonstrate a correlation between increasing numbers of α-syn aggregates in cells and a reduction in cell viability (Wan and Chung 2012).

1.2.7 Lewy bodies and Lewy neurites: α-syn is not alone

Whilst α-syn is the main constituent of Lewy bodies and Lewy neurites, it is not the only protein found in these insoluble inclusions: a range of other proteins has been identified including synuclein-binding proteins, protein kinases, proteins implicated in the ubiquitin-proteasome system and proteins associated with the cellular stress response (e.g. molecular chaperones) (Wakabayashi et al. 2007). Immunostaining of post-mortem brain tissue from PD patients indicates that Hsp90, Hsp70, Hsp40 and the sHsps are associated with Lewy bodies and Lewy neurites (Auluck et al. 2002, McLean et al. 2002, Uryu et al. 2006). With regard to the sHsps, diffuse Hsp27 was identified throughout Lewy bodies and Lewy neurites in the substantia nigra (McLean et al. 2002, Outeiro et al. 2006) and αB-c also co-localises with α-syn in Lewy bodies and Lewy neurites (Lowe et al. 1990, Iwaki et al. 1992, Mizutani
et al. 1997, Outeiro et al. 2006). In addition, post-translationally modified forms of αB-c have been found to be a major component of oligodendral cytoplasmic inclusions isolated from clinically confirmed cases of multiple system atrophy (Pountney et al. 2005). Thus, it appears that although these chaperone proteins are available to inhibit α-syn aggregation in cells, under certain circumstances they are unable to prevent its deposition and instead become part of the inclusions that are the hallmarks of the α-synucleinopathies.

1.3 Small heat shock proteins (sHsps) as molecular chaperones

1.3.1 Structure and function of the sHsps

There are ten sHsps in the human genome (HSPB1 – HSPB10), and of these the most well characterised are Hsp27, αA-crystallin (αA-c), αB-c and Hsp22 (HSPB1, HSPB4, HSPB5 and HSPB8 respectively) (Kampinga et al. 2009, Carra et al. 2013). Whilst often described as ‘holdase’ chaperones, this term does not fully describe their chaperone activity (Kulig and Ecroyd 2012) and not all sHsps have been shown to be capable of suppressing protein aggregation (e.g. HSPB9 and HSPB10). Structural aspects of sHsps have been considered in detail elsewhere (Haslbeck et al. 2005, Sun and MacRae 2005, Basha et al. 2012, Hilton et al. 2013) and therefore the salient features are only summarised here. The sHsps are defined by their relatively small (compared to other Hsps) monomeric masses (12 – 43 kDa), and the presence of a conserved central region referred to as the α-crystallin domain. The α-crystallin domain is ~90 residues in length and contains eight anti-parallel β-strands organised into two β-sheets in an immunoglobulin-like fold (van Montfort et al. 2001, Stamler et al. 2005). It is flanked by N- and C-terminal regions of variable length and sequence that predominantly lack structure (Kim et al. 1998). In mammalian sHsps, the extreme C-terminus is a short, mobile and flexible extension which is typically polar in nature and contributes to stabilisation of the protein (and complexes it forms with target proteins) during chaperone
action (Carver et al. 1992, Lindner et al. 2000). The N-terminus contains regions of significant hydrophobicity and has been suggested to mediate the interaction between the chaperone and its target protein (Stromer et al. 2004, Giese et al. 2005, Aquilina and Watt 2007, Peschek et al. 2013), although recent work demonstrates that the N-terminus is not essential for chaperone action (Feil et al. 2001, Jehle et al. 2010, Hochberg et al. 2014). The formation of oligomeric assemblies is another defining feature of the sHsps. Whilst in some species sHsps form well-defined homogenous multimers (e.g. wheat Hsp16.5), many mammalian sHsps members (e.g. Hsp27, αB-c and αA-c (HSPB4)) form large polydisperse oligomers which undergo rapid subunit exchange (Bova et al. 1997, Bova et al. 2000, Van Montfort et al. 2002, Lelj-Garolla and Mauk 2006, Ahmad et al. 2008, Benesch et al. 2008, Jehle et al. 2010, Jehle et al. 2011). The popular model of sHsp chaperone action is that dissociated species (predominantly depicted as dimers) are the most chaperone-active form. In this model, the rate of subunit exchange of sHsps dictates how fast chaperone active subunits can be liberated from large oligomers, in order to interact with and prevent the aggregation of target proteins. Previous work has shown that αB-c is most effective (on a mole: mole basis) at preventing the aggregation of slowly aggregating target proteins compared to those aggregating more quickly (Lindner et al. 2001) presumably because of the requirement for active subunits to dissociate from larger oligomers as part of the chaperone action of sHsps. Thus, when protein aggregation occurs very fast it may exceed the rate at which active sHsp subunits can dissociate, therefore leading to a decrease in the chaperone efficacy of the sHsp.

1.3.2 Modulators of sHsp activity

As a dimer is the smallest unit observed in all X-ray structures of sHsps obtained to date, it is considered the smallest building block of sHsp oligomers and the primary species responsible
for the dynamic subunit exchange observed between oligomeric units (Sobott et al. 2002, Van Montfort et al. 2002). However, sHsps form large oligomeric assemblies with an odd number of subunits and therefore a dimer cannot be the sole building block for all assemblies (Aquilina et al. 2003, Aquilina et al. 2004). Dimers are generated through the formation of an inter-subunit composite β-sheet structure via domain swapping between two α-crystallin domains (Haslbeck et al. 2005, Baranova et al. 2011). The oligomeric state of a sHsp is highly dependent on solution conditions, including temperature and pH, which can also affect the rate of subunit exchange (Chernik et al. 2004, Lelj-Garolla and Mauk 2006). Although a degree of chaperone activity has been demonstrated for the larger oligomeric assembly (Augusteyn 2004, Franzmann et al. 2005), this remains contentious (Krushelnitsky et al. 2008). The consensus in most models of sHsp chaperone action is that dissociated species (predominantly depicted as dimers) are the chaperone active form (or at least the most chaperone active). This is based on the inference that they would have a higher degree of exposed hydrophobicity compared to the larger oligomeric form, thereby facilitating their interaction with partially-folded substrate proteins that also expose significant hydrophobicity to solution (Haslbeck et al. 1999, Van Montfort et al. 2002). It is thought that chaperone-bound substrate proteins then reassociate with the larger oligomers, to form a high molecular weight chaperone-substrate protein complex. This complex maintains the solubility of the partially-folded intermediates and, in doing so, sHsp oligomers acts as a reservoir of folding-competent intermediates (Leroux et al. 1997, Haslbeck 2002). This mechanism of action explains their classification as ‘holdase’ chaperones; however, the ‘holdase’ name is a misnomer since sHsps can prevent aggregation without ‘holding’ onto the substrate protein. sHsps may instead transiently interact with the substrates to stabilise them and prevent their aggregation (Ecroyd and Carver 2009, Kulig and Ecroyd 2012). As such, sHsps may be better classified as ‘stabilisers’ rather than holdases as this would more fully describe their
mechanism of action. Regardless of the mechanism of chaperone action used by sHsp to interact with substrate proteins (weak, transient interactions versus the formation of a stable complex), both are thought to be dependent on the dissociation of chaperone active species from the larger oligomers, a process which occurs at a constant rate in a given environment, but varies with temperature and solution conditions (Bova et al. 1997). Importantly, however, the exchange rate is independent of the chaperone concentration for αB-c (Bova et al. 2000) and thus, whilst cellular stress may act to up-regulate the rate of subunit exchange of sHsps in the cell, this rate of exchange may be insufficient to cope with the concomitant increase in the rate at which protein aggregation occurs. This may therefore be a significant factor that leads to sHsp activity being overwhelmed in the context of protein aggregation diseases.

Post-translational modifications also modulate mammalian sHsp structure and function. sHsps have been shown to undergo a range of post-translational modifications including methylglyoxal modification (Sakamoto et al. 2002), S-thiolation (Eaton et al. 2002) and truncation. However, the major modification identified to occur to sHsps is phosphorylation. Some mammalian sHsps have serine residues within the N-terminal domain that can be phosphorylated by various kinases (Vos et al. 2008). For example, Hsp27 is phosphorylated by MAPKAP kinase-2 and MAPKAP kinase-3 at serine residues 15, 78 and 82 (Rogalla et al. 1999), and αB-c undergoes phosphorylation at residues 45 and 59 by p44/p42 MAPK and MAPKAP kinase-2 respectively, and at serine 19 by an as yet unknown kinase (Ito et al. 1997, Kato et al. 1998). Whilst phosphorylation of αB-c and Hsp27 has been studied in detail, dephosphorylation and how this process is regulated has received much less attention. Dephosphorylation of Hsp27 can occur in vitro via the action of the phosphatases PP2A, PP1 and PP2B (Cairns et al. 1994), however, it remains to be ascertained which phosphatases fulfil this role in vivo.
Phosphorylation of some sHsps occurs rapidly in response to cellular stress and acts to modulate the oligomerisation of the sHsps (van den Ijssel et al. 1998). For example, phosphorylation of Hsp27 results in its dissociation into smaller species, such that the form phosphorylated on all three serine residues forms a monodisperse dimer in vitro (Kato et al. 1994, Lambert et al. 1999, Rogalla et al. 1999, Hayes et al. 2009, Jovcevski et al. 2015). Similarly, phosphorylation of αB-c has been shown to disrupt the interaction between dimer-forming αB-c subunits and is associated with an increase in oligomer polydispersity in vitro and decrease in oligomer size (Ito et al. 2001, Aquilina et al. 2004, Ecroyd et al. 2007). There remains some contention about the overall effect phosphorylation has on the chaperone activity of sHsps; whether it is beneficial or deleterious is thought to depend on the context and extent of phosphorylation (Bakthisaran et al. 2016). The effect of phosphorylation on Hsp27 and αB-c chaperone activity and structure have been widely investigated using phosphomimicking mutants, whereby the appropriate serine residues are replaced with either aspartate or glutamate residues in order to mimic the negative charge of the phosphate group (Panasenko et al. 2002, Ecroyd et al. 2007, Hayes et al. 2009). However, these mimics do have their limitations: the substitution of aspartate or glutamate at the serine residues introduces a -1 charge (as opposed to -2 that is introduced when a phosphate group is added by a kinase) and the carboxylic acid group is smaller than the phosphate group introduced by phosphorylation. The main advantage of using phosphomimics is that they enable a homogenous isoform of the sHsp to be studied as opposed to the mixed isoform populations that can occur when the protein is phosphorylated in vitro. Moreover, Hsp27 S15/78/82D has been shown to have similar, albeit not identical, size and chaperone activity to the triply phosphorylated form (Hayes et al. 2009).

Using a panel of related αB-c phosphomimics, Ecroyd and colleagues (2007) demonstrated that the size and relative chaperone activity of αB-c is highly dependent on the solution
conditions and substrate protein(s) used to assess the activity (Nicholl and Quinlan 1994, Wang et al. 1995, Ito et al. 2001, Panasenko et al. 2002, Ecroyd et al. 2007). Moreover, differences have been noted between recombinant forms of sHsps and those purified from other sources (e.g. lenses), although those from the lens most likely contain other post-translational modifications (Panasenko et al. 2002). In addition, it has also been postulated that phosphorylation may modulate the proteins targeted by the sHsps, such that under quiescent conditions the sHsps function with minimal phosphorylation and sufficient activity to maintain proteostasis. Exposure to stress results in the rapid phosphorylation of the sHsps, modifying their oligomeric structure and facilitating their interaction with destabilised proteins in danger of aggregation (Treweek et al. 2015). Thus, the activity of the sHsps upon phosphorylation may appear to increase or decrease according to the substrate tested. All these factors no doubt help explain the variation between workers on the overall effect phosphorylation has on sHsp chaperone activity and argue for a set of standard assays to be used in the field in order to enable comparison between studies, and overall consensus to be reached. In any case, intuitively it would be expected that phosphorylation of sHsps would act to boost chaperone activity against aggregating proteins (rather than decrease it), since the cell uses energy in an often energy poor environment to phosphorylate sHsps under stress conditions. When overall energy levels in the cell are low, and cell viability is compromised, the chaperone action of sHsps is crucial with regards to preventing the misfolding and aggregation of cellular proteins destabilised by the stress.

### 1.3.3 Expression of the sHsps in the brain

Of the eleven identified human sHsps, some are ubiquitously expressed (e.g. Hsp27 and αB-c), while others are found only within specific tissues (Stetler et al. 2010, Garrido et al. 2012). For example, αA-c (HSPB4) is only present at appreciable levels within the eye lens,
where, together with $\alpha$B-c, it forms $\alpha$-crystallin, the hetero-oligomeric lens protein which is responsible for maintaining lens transparency via its chaperone action and ordered arrangement (Klemenz et al. 1991, Haley et al. 1998). Given the important role sHsps have in proteostasis, it is surprising that there has not been a systematic study of sHsp expression and localisation in the human brain. In relation to neurodegenerative conditions such as the $\alpha$-synucleinopathies, five sHsps are expressed within the central nervous system. Myotonic dystrophy protein kinase binding protein (HSPB2) is expressed in smooth muscle of vessel walls of the brain (Wilhelmus et al. 2006), and compartment-specific expression of Hsp20 (HSPB6), Hsp22, Hsp27 and $\alpha$B-c has been demonstrated within other brain tissues of the mouse (Quraishe et al. 2008). Hsp27 is expressed in motor and sensory neurons in the brainstem and cranial nerve nuclei (Plumier et al. 1997), and is also constitutively expressed, along with $\alpha$B-c, in glial cells (Wilhelmus et al. 2006).

Although there is some evidence for the neuroprotective capabilities of Hsp20 and Hsp22 (Stetler et al. 2010), Hsp27 and $\alpha$B-c have attracted the most attention in relation to neurodegenerative disease. Both $\alpha$B-c and Hsp27 expression are highly induced in response to neurological stress (Iwaki et al. 1992, Kato et al. 1994). Hsp27 and $\alpha$B-c are expressed in reactive astrocytes adjacent to senile plaques in both normal aged brains and in neurodegenerative conditions, such as Alzheimer’s disease (Wilhelmus et al. 2006), and their expression is increased in reactive astrocytes in the hippocampus of PD patients with dementia (Renkawek et al. 1999). Moreover, Hsp27 is one of the most strongly induced proteins across several brain regions in PD patients (Zhang et al. 2005) and its levels are 2.5-fold higher in pathologically confirmed cases of Dementia with Lewy bodies than age-matched controls. Chen and Brown (2007) compared constitutive and inducible Hsp27 expression in several neuronal subtypes associated with neurodegenerative diseases, including PD and amyotrophic lateral sclerosis (ALS). Constitutive Hsp27 expression was
found within motor neurons of the spinal cord (the degeneration of which is associated with ALS), but Hsp27 was not detected within dopaminergic neurons of the *substantia nigra* (the degeneration of which is associated with PD). They speculated that one reason ALS is approximately 33 times less frequent than PD is that motor neurons in the spinal cord are better equipped to manage misfolded proteins than dopaminergic neurons due to the protection provided by the levels of Hsp27 (Chen and Brown 2007). Thus, the low basal expression of Hsp27 in dopaminergic neurons (Chen and Brown 2007, Carra et al. 2013) may facilitate the onset of α-syn aggregation in these cells. The increased expression of sHsps in the context of the α-synucleinopathies may be a consequence of the cellular stress conditions that accompanies the onset and progression of these diseases.

**1.3.4 sHsps interact with multiple species to inhibit aggregation**

The design of most experiments testing the chaperone function of sHsps (and indeed chaperones in general) to date has involved addition of the chaperone to a sample before aggregation has commenced (Horwitz 1992, Rekas et al. 2004, Ecroyd et al. 2007, Bruinsma et al. 2011). As such, these studies have focused on the interaction of the chaperones with species formed early on during the aggregation process. More recently, some studies have investigated the interaction of sHsps with preformed amyloid fibrils and shown that αB-c binds along the length of amyloid fibrils, including those formed by α-syn (Waudby et al. 2010, Shammas et al. 2011). In doing so, it acts to inhibit further fibril growth. αB-c binds to preformed amyloid-β (Aβ) and apolipoproteinC-II (apoC-II) fibrils with moderate (µM) affinity (Shammas et al. 2011, Binger et al. 2013). By binding to fibrils, sHsps may act to occlude the fibril ends, preventing the addition of further monomeric species and thus fibril growth. They may also stabilise fibrils against fragmentation or conceal hydrophobic surfaces that would otherwise be toxic. Fibril fragmentation (i.e. breaking) contributes to secondary
nucleation events, as the fragments act as nuclei for further fibril growth and are a source of toxic oligomers (Knowles et al. 2009). Likewise, sites of exposed hydrophobicity along the face of the fibril have also been identified as possible locations for secondary fibril nucleation, again leading to rapid fibril growth and dominating the overall kinetics of fibril growth (Ruschak and Miranker 2007, Knowles et al. 2009, Cohen et al. 2011). More recent work has also shown that by binding to fibrils, sHsps can promote their clustering into larger aggregates (Ojha et al. 2011, Mannini et al. 2012, Binger et al. 2013). Finally, using the amyloidogenic protein β2-macroglobulin, αB-cr has also been shown to reversibly dissociate soluble oligomers to produce monomeric β2-macroglobulin, potentially providing another protective mechanism against oligomer-induced toxicity (Esposito et al. 2013). Thus, it is now apparent that rather than having a mechanism of action that is based solely on binding to partially-folded monomeric states of proteins, sHsps can interact with multiple species formed during protein aggregation, including the mature fibrils. In each case, it appears that sHsps act to minimise the amount of toxic protein aggregates formed in the cell and thereby its exposure to these harmful species. However, this is yet to be demonstrated experimentally and is a key concept addressed in this work.

In addition, whilst the presence of sHsps within protein inclusions associated with disease has been considered a by-product of their failed attempt to mitigate aggregation, the recent work demonstrating the ability of sHsps to stabilise fibrils and mediate their tangling into larger aggregates provides another rationale for their localisation in these deposits. However, whether this is a beneficial effect in the cell remains to be established, since sequestering chaperones within these deposits may in fact have a deleterious effect on the cell as it reduces the ability of chaperones to participate in other physiological functions (Waudby et al. 2010, Ebrahim-Fakhari et al. 2012).
1.4 sHsps interact with α-syn

The presence of the sHsps within Lewy bodies and their upregulation in the surrounding neuronal tissues associated with a number of α-synucleinopathies has led to an examination of potential interactions between α-syn and various sHsps. These findings are summarised in Table 1.1 and are based on studies using a number of model systems, which can be grouped into three distinct categories, *in vitro* α-syn aggregation assays, cell-based models and animal models of α-syn aggregation.

Bruinsma et al (2011) assessed a panel of sHsps, including Hsp27, Hsp20, Hsp22, HspB2B3 and αB-c, for their ability to inhibit α-syn aggregation using both *in vitro* ThT assays and atomic force microscopy. They reported that Hsp27 is the most efficacious of these sHsps with regards to preventing fibril formation of α-synWT and it does so by inhibiting both the lag and elongation phases of aggregation (Bruinsma et al. 2011). The interaction of Hsp27 with α-syn leads to an overall reduction in the number and size of α-syn fibrils. Comparable effects were observed when Hsp27 was incubated with α-syn E46K and A30P, however, the presence of Hsp27 increased the aggregation of α-synA53T compared to when no chaperone was present (Bruinsma et al. 2011). αB-c also inhibits the aggregation of α-syn (and its disease-related mutant forms) *in vitro* and does so at sub-stoichiometric levels (Rekas et al. 2004). In addition, the *in vitro* fibrillar aggregation of α-synA53T isolated from brain tissue extracts of transgenic mice is significantly reduced by the presence of αB-c (Wang et al. 2008). As with Hsp27, the addition of αB-c not only increases the lag phase of α-syn aggregation, slowing the formation of prefibrillar intermediates, but also inhibits the elongation phase, indicating that it acts to stabilize monomeric and prefibrillar α-syn species (Rekas et al. 2004, Rekas et al. 2007). Notably, the efficiency with which αB-c inhibits α-syn fibril formation correlates with the aggregation-propensity of the α-syn isoform (Rekas et al.
Thus, at a given molar ratio, αB-c is more effective at inhibiting the aggregation of α-synWT (which aggregates the slowest) and is less effective against α-synA53T (which aggregates the fastest).

Much of what is known about the interaction between sHsps and α-syn in cells comes from cell-based models of α-syn which rely on overexpression of α-syn alongside cofactors such as synphillin-1, or treatment with inducers of cytotoxic stress (Engelender et al. 1999, Ostrerova-Golts et al. 2000, McLean et al. 2001, Matsuzaki et al. 2004). However, in general these cell models have only been used qualitatively to assess the effects of sHsps on α-syn aggregation. For example, using an α-syn overexpression system coupled with viral-mediated Hsp expression in ND7 cells, Zourlidou et al (2004) reported a reduction in α-syn-induced cellular toxicity following Hsp27 expression. Although this study focused on the inherent susceptibility of α-syn-expressing cells to external cell death stimuli, such as serum removal, without directly considering α-syn aggregation, it was concluded that Hsp27 is neuroprotective. Outeiro et al (2006) used overexpression of the truncated EGFP-tagged α-synWT in H4 cells to examine the effect of sHsps on α-syn aggregate formation, and reported that co-transfection with Hsp27 significantly reduced the percentage of cells containing α-syn inclusions. Both Hsp27 and αB-c reduced the inherent toxicity of α-syn expression to levels similar to that seen when Hsp70 is expressed in these cells (Outeiro et al. 2006). This reduction was reproduced when α-synA53T was overexpressed in primary midbrain cultures (Klucken et al. 2004, Outeiro et al. 2006). Furthermore, a reduction in the endogenous levels of Hsp27 (by siRNA knockdown) resulted in a concentration-dependent increase in α-syn toxicity in the transfected H4 model (Outeiro et al. 2006). Together, cell culture studies such as these have provided promising glimpses of the role of sHsps in preventing α-syn aggregation in cells. However, they also serve to highlight the failure of sHsps to prevent this aggregation in the context of the α-synucleinopathies.
Table 1.1: A summary of studies that have investigated the interaction between sHsps and α-syn. Key findings regarding this interaction are categorised according to the method used.

<table>
<thead>
<tr>
<th>Method used</th>
<th>α-Syn Isoform</th>
<th>sHsp</th>
<th>Key Findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>In vitro</em> aggregation assays, AFM</td>
<td>WT</td>
<td>Hsp27</td>
<td>sHsps bind α-syn variants in a weak, transient but specific manner</td>
<td>(Bruinsma et al. 2011)</td>
</tr>
<tr>
<td></td>
<td>A53T</td>
<td>αB-c</td>
<td></td>
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<tr>
<td></td>
<td>A30P</td>
<td>Hsp20</td>
<td>sHsps reduce the amount of fibrillar aggregation resulting in fibrils that are shorter, and have a clustered morphology.</td>
<td></td>
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<tr>
<td></td>
<td>E46K</td>
<td>Hsp22</td>
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<td></td>
<td></td>
<td>HspB2B3</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>In vitro</em> aggregation assays, EM</td>
<td>WT</td>
<td>αB-c</td>
<td>αB-c inhibits α-syn fibril formation at substoichiometric ratios.</td>
<td>(Rekas et al. 2004)</td>
</tr>
<tr>
<td></td>
<td>A53T</td>
<td></td>
<td>The number of fibrils and generation of amorphous-like aggregates are reduced.</td>
<td></td>
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<tr>
<td></td>
<td>A30P</td>
<td></td>
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<tr>
<td><em>In vitro</em> aggregation assays</td>
<td>WT</td>
<td>αB-c</td>
<td>The ability of αB-c to suppress α-syn aggregation increases with temperature.</td>
<td>(Rekas et al. 2007)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>αB-c inhibits further aggregation when introduced during the elongation phase of fibril growth.</td>
<td></td>
</tr>
<tr>
<td><em>In vitro</em> assay, EM</td>
<td>A53T</td>
<td>αB-c</td>
<td>The interaction of αB-c with α-syn monomers is weak and transient. αB-c binds along the face and ends of mature α-syn fibrils. Fibril-bound αB-c inhibits further elongation.</td>
<td>(Waudby et al. 2010)</td>
</tr>
<tr>
<td><em>In vitro</em> aggregation assays</td>
<td>A53T</td>
<td>αB-c</td>
<td>αB-c significantly reduces the <em>in vitro</em> aggregation of α-syn extracted and purified from brain tissue of transgenic mice.</td>
<td>(Wang et al. 2008)</td>
</tr>
<tr>
<td>Cell-based model</td>
<td>WT</td>
<td>Hsp27</td>
<td>Hsp27 expression protects stably transfected ND7 α-syn-expressing cells from cell death stimuli including serum withdrawal.</td>
<td>(Zourlidou et al. 2004)</td>
</tr>
<tr>
<td></td>
<td>A53T</td>
<td>αB-c</td>
<td></td>
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<tr>
<td></td>
<td>A30P</td>
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<tr>
<td>Cell-based model</td>
<td>WT</td>
<td>Hsp27</td>
<td>Both Hsp27 and αB-c co-localise with α-syn inclusions in co-transfected H4 neuroglioma cells. Hsp27 reduces inclusion formation, although both αB-c and Hsp27 reduce α-syn toxicity. Hsp27 protects primary dopaminergic neurons from α-syn toxicity.</td>
<td>(Outeiro et al. 2006)</td>
</tr>
<tr>
<td></td>
<td>A53T</td>
<td>αB-c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell-based model</td>
<td>WT</td>
<td>Hsp27</td>
<td>Hsp27 does not co-localise with α-syn inclusions</td>
<td>(McLean et al. 2002)</td>
</tr>
<tr>
<td>In vivo murine</td>
<td>A53T</td>
<td>Hsp25*</td>
<td>Hsp25 levels are significantly upregulated in both the soluble and insoluble fractions of spinal cord tissue of α-syn A53T over-expressing transgenic mice. αB-c levels are increased in the insoluble fraction of spinal cord tissue from these transgenic mice.</td>
<td>(Wang et al. 2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>αB-c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>In vivo Drosophila</td>
<td>WT</td>
<td>αB-c</td>
<td>αB-c reduces the ‘rough eye’ phenotype induced by α-syn expression and aggregation in <em>Drosophila</em></td>
<td>(Tue et al. 2012)</td>
</tr>
</tbody>
</table>

*Hsp25 is the murine ortholog of human Hsp27
Despite these investigations, little is known about the molecular mechanism(s) by which the sHsps, αB-c and Hsp27, interact with α-syn. As such, elucidating these details formed the basis of this work.

### 1.5 Summary and aims

The aggregation of α-syn into protein inclusions underlies the onset and progression of the α-synucleinopathies and represents a failure of the proteostasis network in maintaining the protein in a biologically active, non-toxic form. Key components of the proteostasis network are molecular chaperones and the sHsps are the cell’s first line of defence against protein aggregation; we need to better understand why they fail in the context of the α-synucleinopathies.

Whilst it has been generally considered that the role of sHsps in the chaperone network is to stabilise partially-folded intermediate states of protein to prevent their aggregation, recent work has demonstrated that their mechanism of chaperone action is multi-faceted. In the context of α-syn aggregation, sHsps interact with multiple species formed during the aggregation process, from monomeric partially-folded intermediate states through to the mature fibrils themselves. However, the precise mechanism(s) of this interaction is yet to be fully understood. Furthermore, the failure of sHsps to prevent α-syn aggregation in a disease context is often attributed to their protective capacity being ‘overwhelmed’. However, specific factors leading to this failure are yet to be determined.

In addition, whilst many studies have primarily considered the ability of the sHsps to inhibit or prevent α-syn aggregation in vitro, there is limited evidence of the physiological relevance of this interaction due to the limited number of cellular studies performed to date.
As such, the specific aims of this work were to:

i. Establish the mechanism by which the sHsps, αB-c and Hsp27, inhibit the initial stages of monomeric α-syn aggregation and determine whether an increase in the rate at which α-syn aggregates affects the ability of sHsps to prevent this process.

ii. Characterise the ability of Hsp27 and αB-c to bind α-syn fibrils, and determine whether this provides an alternate protective mechanism by which these chaperones act to prevent the toxicity associated with α-syn fibrils.

iii. Establish a robust cellular model of α-syn aggregation and use it to determine whether sHsps can prevent the aggregation of α-syn into inclusions in cells.
Chapter 2: Materials and Methods
Common methods used in this work are outlined in this chapter. Methods specifically pertaining to work presented in Chapters 3 – 5 are given in the relevant chapters.

2.1 Materials

All materials used in this work were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Amresco (Solon, OH, U.S.A.) unless otherwise indicated. Recombinant stocks of core domain variants of the sHsps, consisting of residues 68 – 153 of αB-c (αB-c<sub>core</sub>) and residues 84 – 176 of Hsp27 (Hsp27<sub>core</sub>), were kind gifts received from Prof. J. Benesch (University of Oxford, United Kingdom), Prof. A. Laganoswsky (Texas A&M Health Science Center, USA) and Assoc. Prof. Heath Ecroyd (University of Wollongong, Australia). Initial stocks of recombinant α-synA90C, produced to enable site-specific labelling of the cysteine using fluorescent dyes, were a kind gift from Dr. Laura Tosatto (University of Cambridge, UK).

Chemically competent DH5α or BL21(DE3) E. coli strains were kind gifts of Dr. Tracey Berg (University of Wollongong, Australia). Mouse neuroblastoma (Neuro-2a; N2a) were obtained from the American Type Culture Collection (Manassas, VA, U.S.A.). Human embryonic kidney (HEK293) cells were gifts from Assoc. Prof. Ron Sluyter (University of Wollongong, Australia). All cell lines were routinely tested for mycoplasma contamination every 6 months, and the identity of the human-derived cell line was verified via short tandem repeat (STR) profiling (Garvan Institute of Medical Research, Sydney, Australia).

Mouse monoclonal anti-αB-c (clone 1B6.1-3G4) and anti-Hsp27 (clone G3.1), rabbit polyclonal anti-αB-c (ab13497) and anti-Hsp27 (ab5579), and anti-mouse (ab37355) and anti-rabbit (ab171870) IgG control antibodies were all purchased from Abcam (Cambridge, UK). Goat anti-mouse secondary antibodies conjugated to DyLight488 (ab96879) and DyLight650 (ab96874), goat anti-rabbit secondary antibodies conjugated to DyLight488
(ab96895), DyLight550 (ab96900) and DyLight650 (ab96902), and a rabbit anti-mouse secondary conjugated to DyLight550 (ab98786) were also obtained from Abcam (Cambridge, UK). Both the mouse monoclonal anti-α-syn antibody (clone Syn211) and the peroxidase conjugated anti-mouse IgG secondary antibody were obtained from Sigma-Aldrich (St. Louis, USA).

2.2 Bacterial and mammalian expression constructs

2.2.1 Plasmids

The pET24d(+) or pET24a(+) bacterial expression vectors, containing the human HSPB5 (αB-c), HSPB1 (Hsp27) and SNCA (α-syn) genes, were kind gifts of Assoc. Prof. Heath Ecroyd, and were used for expression of recombinant WT proteins. Disease-related mutants of α-syn (A30P, A53T and E46K) and sHsp variants used in this work were produced via site-directed mutagenesis of the WT gene by GenScript (Piscataway, USA). In particular, variants of the sHsps designed to mimic phosphorylation were generated by mutation of the phosphorylatable serine residues to aspartic acid (Ser15, Ser45 and Ser59 to produce αB-c3D, and Ser15, Ser78 and Ser82 to produce Hsp273D). Plasmids for the expression of the core domains of the sHsps, i.e. residues 68 – 153 of αB-c (αB-c_core) and residues 84 – 176 of Hsp27 (Hsp27_core), were kind gifts from Prof A. Laganowsky (Texas A&M Health Science Center, USA). The pT7-7 vector harbouring the SCNA gene containing an A90C mutation was a kind gift from Dr. Laura Tosatto (University of Cambridge, UK) and was used for the expression of α-syn suitable for site-specific fluorescent labelling (α-synA90C).

The pEGFP-N3 mammalian expression vector, containing the human SNCA (α-syn) gene with or without the disease-related mutation (A53T), was a gift from Dr. Dean Poutney (Griffith University, Australia) and was used to induce cellular expression of α-syn variants
Chapter 2: Materials and Methods

C-terminally tagged with EGFP. The pCMV6-AC-GFP mammalian expression vector, containing M337V TDP-43 cDNA C-terminally tagged with TurboGFP (tGFP), was a kind gift from Dr. Daniel Whiten (University of Wollongong, Australia). Constructs encoding pT-Rex-Htt46Q-Tc1-mCherry and pT-Rex-Htt25Q-Tc1-mCherry, were gifts from Assoc. Prof. Danny Hatters (University of Melbourne, Australia). The bicistronic pIRES2-EGFP constructs containing WT αB-cr, Hsp27, Hsp40, Hsp70, Hsp90, or the non-chaperone controls LacZ or invisibile EGFP (EGFPinv; i.e. a non-fluorescent derivative of EGFP, Y66L EGFP (Olshina et al. 2010, Ramdzan et al. 2012)), were gifts from Dr. Tracey Berg (University of Wollongong, Australia).

2.2.2 Transformation of chemically competent E. coli

One aliquot (200 µL) of chemically competent cells (DH5α for plasmid storage or BL21(DE3) for recombinant protein expression) were thawed on ice before the addition of 100 ng of plasmid DNA (or 30 µL of ligation reaction) with gentle mixing. Cells were incubated on ice for 30 min, then heat shocked at 42°C for 30 s using an Accublock digital dry bath (Labnet International, Edison, USA). Cells were immediately transferred to ice, and diluted 1:4 into Luria-Bertani broth (LB; 1% (w/v) tryptone, 1% (v/v) NaCl, 0.5% (w/v) yeast extract, pH 7.4) for plasmid transformations or Super optimal broth with catabolite repression (SOC; 2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄ and 20 mM glucose) media for ligation transformations. Cells were then incubated at 37°C for 1 h with agitation. Transformation cultures were plated onto pre-warmed selective LB–agar plates (LB containing 15% (w/v) agar and the appropriate antibiotic) and incubated overnight at 37°C.

Successfully transformed colonies were screened for the plasmid of interest by polymerase chain reaction (PCR) using insert-specific primers. Plasmid DNA was extracted from the
clone using the Wizard® Plus SV Miniprep DNA Purification System (Promega, Madison, U.S.A.) according to the manufacturer’s instructions. Colonies identified to contain the correct plasmid DNA were then used to inoculate LB containing the appropriate antibiotic, which was incubated overnight at 37°C. This culture was then combined 1:1 with a sterile 30% (v/v) glycerol solution, and stored in sterile cryogenic tubes at -80°C.

2.2.3 Sequence verification

Extracted DNA constructs were prepared for sequencing using the BigDye Terminator v3.1 cycle Sequencing Kit (Applied Biosystems, Foster City, U.S.A.) according to the manufacturer’s instructions. The reaction mix contained 1X BigDye buffer, 1.6 µM of the appropriate primer, 1X BigDye Ready Reaction Premix and an appropriate concentration of plasmid DNA in a total of 10 µL. PCR was performed using a Mastercycler ProS (Eppendorf, Hamburg, Germany) and consisted of 35 cycles, with each cycle consisting of denaturation at 96°C for 10 s, annealing at 50°C for 5 s, and extension at 60°C for 4 min. The resulting DNA was then precipitated using 100% ethanol, 125 mM ethylenediaminetetraacetic acid (EDTA; pH8.0) and 3 M sodium acetate (pH 5.2) for 2 h at room temperature. The reaction was then centrifuged at 20000 x g for 20 min at 4°C and the supernatant discarded. The DNA was then washed with ice cold ethanol (70% (v/v)) and centrifuged as above. The supernatant was again discarded and any remaining ethanol evaporated at room temperature prior to sequencing. Sequencing was performed by Ms. Margaret Phillips (University of Wollongong, Australia) using a Hitachi 3130xl Genetic Analyser (Applied Biosystems, Mulgrave, Australia).
2.3 Recombinant protein production

2.3.1 Expression and purification

Recombinant proteins were expressed in BL21(DE3) *E. coli* cells transformed with each plasmid, and purified as described previously (Horwitz et al. 1998, Narhi et al. 1999, Laganowsky et al. 2010). Briefly, 100 mL of LB containing the appropriate antibiotic (100 µg/mL ampicillin or 50 µg/mL kanamycin) was inoculated with BL21(DE3) *E. coli* harbouring the plasmid of interest and incubated overnight at 37°C with constant agitation. Overnight cultures were then diluted 1:20 into fresh LB containing the appropriate antibiotic and incubated at 37°C with constant agitation. Once the optical density at 600 nm reached 0.6-0.8, protein expression was induced via the addition of 500 µM isopropyl β-D-1-thiogalactopyranoside (IPTG) and cultures were incubated for a further 4 h at 37°C with constant agitation. Bacteria were harvested at 5000 x g for 10 min at 4°C using an RC6 Sorval centrifuge (ThermoFischer Scientific, Waltham, USA).

Bacterial pellets were resuspended in lysis buffer (50 mM Tris, 100 mM NaCl, pH 8.0) supplemented with 0.5% (v/v) Halt™ Protease and Phosphatase Inhibitor Cocktail (ThermoFischer Scientific, Waltham, USA) at 3 mL/g. The solution was briefly vortexed before the addition of 0.25 mg/mL lysozyme, and was then incubated on ice for 20 min. Deoxycholic acid was added to a final concentration of 1.3 mg/mL and the solution was incubated at 37°C for 30 min. DNAse I was then added to a final concentration of 3.0 µg/mL prior to incubation at room temperature for 30 min with gentle rocking. Cellular debris were then pelleted at 5000 x g for 10 min at room temperature using an Heraeus Megafuge (ThermoFischer Scientific, Waltham, USA), before the supernatant was collected and further clarified by ultracentrifugation at 100000 x g for 30 min at 4°C in an MTX150 centrifuge (ThermoFischer Scientific, Waltham, USA). The supernatant was then collected and
dithiothreitol (DTT; 10 mM), polyethylenimine (0.3% v/v) and EDTA (1 mM) were added. The solution was stirred at room temperature for 30 min, before ultracentrifugation as above to remove any precipitate. The supernatant was again collected, and at this stage recombinant sHsp samples were stored at -20°C for purification.

Alternatively, for recombinant α-syn samples, additional proteins were precipitated via the dropwise addition of HCl such that the pH of the solution reached 4.0. The solution was again stirred at room temperature for 30 min, and then the precipitate was cleared via ultracentrifugation as above. The pH of the clarified supernatant was then raised to 8.0 via the dropwise addition of NaOH, and the solution was stored at -20°C for purification.

Extracted proteins were purified via anion exchange and subsequent size exclusion chromatography (SEC) using an AKTA Prime Plus FPLC system (GE Healthcare, Uppsala, Sweden) with an in-line UV detector to monitor the absorbance of eluted proteins at 280 nm ($A_{280nm}$). Thawed protein extracts were buffer exchanged into 20 mM Tris containing 1 mM EDTA and 0.02% (w/v) Na Azide (pH 8.0). Protein samples were then passed over a HiPrep DEAE FF 16/10 column (GE Healthcare, Uppsala, Sweden) and eluted by the addition of 20 mM Tris containing 1 mM EDTA, 0.02% (w/v) Na Azide and 2 M NaCl (pH 8.0) using a stepwise gradient (5% and 10%; each for 2 column volumes) for αB-c, or continuous gradient of 0 – 10% over 8 column volumes, or 0 – 25% over 4 column volumes, for Hsp27 and α-syn, respectively. Fractions containing the protein of interest were identified via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in combination with the in-line $A_{280nm}$ profile. Fractions were pooled and concentrated using an Amicon stirred cell model 8200 (Merck Millipore, Billerica, U.S.A.) according to the manufacturer’s instructions, with a 10 000 MWCO membrane. Protein samples were then passed over a HiPrep 26/60 Sephacryl S-300 High Resolution column (GE Healthcare, Uppsala, Sweden)
equilibrated with 50 mM sodium phosphate buffer (PB) containing 0.02% (w/v) Na Azide (pH 7.4). Fractions containing the protein of interest were identified, pooled and concentrated as above.

2.3.2 Quantification and storage

The purity of the resulting recombinant protein was confirmed via SDS-PAGE, and found to be > 90% in all instances. The concentration was routinely determined using a NanoDrop 2000c spectrophotometer (ThermoFischer Scientific, Waltham, USA) and the appropriate molar absorptivity value for the protein of interest. In particular, a molar absorptivity value of 0.44 M\(^{-1}\).cm\(^{-1}\) for α-syn (Narhi et al. 1999), 0.83 M\(^{-1}\).cm\(^{-1}\) for αB-c (Horwitz et al. 1998) and 1.65 M\(^{-1}\).cm\(^{-1}\) for Hsp27 (Hayes et al. 2009) were used. Protein samples were then aliquoted and stored at -20°C.

2.4 SDS-PAGE

Proteins were routinely assessed for purity and molecular weight using SDS-PAGE as previously reported (Laemmli 1970). Briefly, a resolving gel (15% (w/v) acrylamide/bis, 375 mM Tris (pH 8.8), 0.1% (w/v) SDS, 0.25% (v/v) tetramethylethylenediamine, 0.02% (w/v) ammonium persulfate) was overlaid with a stacking gel (4% (w/v) acrylamide/bis, 330 mM Tris (pH 6.8), 0.1% (w/v) SDS, 0.4% (v/v) tetramethylethylenediamine, 0.04% (w/v) ammonium persulfate). Samples were diluted into an appropriate volume of 3-times or 6-times loading buffer to give final concentrations of 0.5 M Tris-HCl (pH 8.8), 2% (w/v) SDS, 25% (w/v) glycerol, 0.01% (w/v) and bromophenol blue, 5% (v/v) β-mercaptoethanol. Prior to loading, samples were heated at 95°C for 5 min before being spun at 20000 x g for 30 s at room temperature in a benchtop centrifuge 5254 (Eppendorf, Hamburg, Germany). Samples were electrophoresed at 150 V using a Mini-Protean 2 Cell system (Bio-Rad, Hercules, CA) alongside Precision Plus Protein™ dual colour molecular weight standards (10-250 kDa)
(Bio-Rad, Hercules, CA) until the dye front reached the bottom of the resolving gel. Gels were then stained with Coomassie blue stain (0.1% (w/v) brilliant blue, 40% (v/v) methanol, 10% (v/v) glacial acetic acid) at room temperature overnight and destained using destaining solution (40% (v/v) methanol, 10% (v/v) glacial acetic acid). Gels were imaged using a Gel Logic 2200 Pro Imaging System (Carestream Health, Rochester, USA).

2.5 Immunoblotting

Following SDS-PAGE, proteins of interest were transferred onto Amersham HyBond-ECL nitrocellulose membrane (GE Healthcare, Uppsala, Sweden) at 100 V for 1 h in ice-cold transfer buffer (0.192 M glycine, 25 mM Tris, 20% (v/v) methanol, pH 8.6). The membrane was blocked for 1 h at room temperature in Tris-buffered saline (TBS; 50 mM Tris and 150 mM NaCl, pH 7.5) containing 5% (w/v) skim milk powder (Woolworths, Bella Vista, Australia), then incubated overnight at 4°C in TBS-Tween-20 (TBST; TBS containing 0.05% (v/v) Tween-20) containing 5% (w/v) skim milk powder and the appropriate primary antibody (diluted 1:5000). Blots were washed four times in TBST for 10 min with constant agitation, then incubated at room temperature for 1 h in TBST containing 5% (w/v) skim milk powder and the appropriate secondary antibody (diluted 1:5000). Blots were washed as above, and then labelled proteins were detected using SuperSignal West Pico Chemiluminescent Substrate or SuperSignal West Dura Extended Duration Chemiluminescent Substrate according to the manufacturer’s instructions (ThermoFischer Scientific, Waltham, USA). The membrane was exposed to Amersham Hyperfilm ECL chemiluminescence film (GE Healthcare, Uppsala, Sweden), or directly imaged using a Gel Logic 2200 Pro Imaging System (Carestream Health, Rochester, NY, USA) or Amersham Imager 600 (GE Healthcare, Uppsala, Sweden).
2.6 Fluorescent labelling of recombinant proteins

Recombinant proteins of interest were fluorescently labelled using succinimidyl ester or maleimide variants of CF488A or CF647 (Biotium, Hayward, USA), or AlexaFluor488 (AF488) or AlexaFluor647 (AF647; ThermoFischer Scientific, Waltham, USA) dyes according to the manufacturer’s instructions. Briefly, samples intended for maleimide labelling were pre-incubated with a 10-fold molar excess of tris(2-carboxyethyl)phosphine (TCEP) for 30 min at room temperature. Samples were then loaded onto a pre-equilibrated PD10 column (GE Healthcare, Uppsala, Sweden) and eluted with degassed phosphate buffered saline (PBS; 2.7 mM KCl, 1.75 mM K2HPO4, 135 mM NaCl, 10 mM NaH2PO4, pH 7.4). Fractions were collected (500 µL) and protein elution was monitored by determining the absorbance of each fraction at 280 nm using a NanoDrop 2000c spectrophotometer (ThermoFischer Scientific, Waltham, USA). Fractions containing protein were pooled and dye conjugates were added in a 1.5 molar (succinimidyl ester) or 10 molar (maleimide) excess relative to the protein concentration for labelling. Labelling reactions were incubated overnight at 4°C with agitation. Labelled protein was then separated from unreacted dye using a pre-equilibrated PD10 column as described above, using 50 mM phosphate buffer (pH 7.4). Labelled proteins were stored at -20°C, or flash frozen in liquid N₂ and stored at -80°C. The concentration and degree of labelling of each protein was calculated as per the manufacturer’s instructions and found to be greater than 60% in all cases. In some instances, protein labelling with the fluorophore was also confirmed via electrospray ionisation mass spectrometry by Mr. Blajoice Jovchevski (University of Wollongong, Australia).
2.7 Mammalian tissue culture

2.7.1 Passaging and plating

Mammalian cell lines (N2a and HEK293) were cultured in Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F-12 (DMEM/F12; ThermoFischer Scientific, Waltham, USA) supplemented with 10% (v/v) foetal bovine serum (FBS; Bovagen Biologicals, East Keilor, Australia) and 2.5 mM L-glutamine (ThermoFischer Scientific, Waltham, USA). All media was sterile filtered and warmed to 37°C for use. All tissue culture was performed within a laminar flow biosafety cabinet, and cells were incubated in a Heracell 150i CO2 incubator (ThermoFischer Scientific, Waltham, USA) under 5% CO2/95% air at 37°C.

Cells were passaged once at 80% confluency or after a period of 72 h, and reseeded into fresh CELLSTAR flasks or culture plates (both from Greiner Bio-one, Frickenhausen, Germany) where required. Briefly, culture media was removed from the cells and replaced with a sufficient volume of trypsin containing 0.05% (w/v) EDTA (ThermoFischer Scientific, Waltham, USA) to cover the cells. After incubation at 37°C for 5 min, the flask was gently tapped, if necessary, to encourage cells to dislodge. The flask was then washed with DMEM/F12 containing 1% (v/v) FBS. For passaging purposes, 10% of the cells were collected and harvested via centrifugation at 300 x g for 5 min at room temperature. The supernatant was discarded and cells resuspended in the appropriate volume of culture medium before being transferred to a fresh culture flask, such that the flask was seeded at 10-15% of the original density. For plating purposes, the remaining lifted cells were collected and harvested via centrifugation at 300 x g for 5 min at room temperature before being resuspended in culture medium. A sample of the cell solution was combined 1:1 with trypan-blue and the cell density counted using a Neubauer-improved counting chamber (Marienfeld
Superior, Lauda-Königshofen, Germany). Cells were then diluted with culture medium and seeded in the appropriate vessel at the desired cellular density.

### 2.7.2 Storage

Cell lines are maintained in liquid nitrogen for long term storage. To generate stocks for storage, culture media was removed from cells at 80% confluency and replaced with a sufficient volume of trypsin containing 0.05% (w/v) EDTA (ThermoFischer Scientific, Waltham, USA) to cover the cells. After incubation at 37°C for 5 min, if necessary the flask was gently tapped to encourage cells to dislodge, and the flask was then washed with DMEM/F12 containing 1% (v/v) FBS. The cells were collected and harvested via centrifugation at 300 x g for 5 min at room temperature before being resuspended in DMEM/F12 supplemented with 50% (v/v) FBS and 10% (v/v) dimethyl sulfoxide (DMSO). The solution was then aliquotted (1 mL) into sterile cryovials and placed in a pre-cooled Nalgene Cryo 1°C “Mr. Frosty” Freezing Container (ThermoFischer Scientific, Waltham, USA) containing isopropanol. Cells were stored at -80°C overnight before being transferred to liquid nitrogen.

When removing stocks from liquid nitrogen for use, cells were thawed and immediately added to a 10 fold excess of DMEM/F12 containing 10% (v/v) FBS. Cells were harvested via centrifugation at 300 x g for 5 min at room temperature before being resuspended in culture medium and transferred to a flask. After 24 h, the culture medium was discarded and replaced with fresh media and the cells were allowed to reach 80% confluency before being passaged.

### 2.8 Data Analysis

All statistical analyses (unless otherwise stated) were performed using GraphPad Prism v 5 (GraphPad Software Inc., San Diego, USA).
Chapter 3:

sHsp inhibit the aggregation of monomeric α-synuclein

Portions of this chapter have been previously published in the following work:


Author Contributions: DC designed, performed and analysed all experiments. HE conceived the aggregation and SEC assays and assisted with data analysis, ES and MG assisted with collection and analysis of AUC experiments. DC wrote the manuscript and generated figures; and all authors edited the manuscript for submission.
Chapter 3: sHsps inhibit the aggregation of monomeric α-synuclein

3.1 Introduction

The ability of sHsps such as αB-c and Hsp27 to prevent the fibrillar formation of α-syn in vitro is well established (Rekas et al. 2004, Waudby et al. 2010, Bruinsma et al. 2011). However, the mechanism by which sHsps achieve this inhibition and the fate of α-syn as a result of this interaction remains to be definitively established. Inhibition may involve one or more of the following mechanisms: (i) weak transient interactions with α-syn which prevent it from associating into oligomeric nuclei, (ii) the formation of a stable complex between α-syn and the sHsps which, when cellular conditions permit, enable monomeric α-syn to be released, or (iii) α-syn being induced to form amorphous aggregates rather than fibrils. Importantly, given the α-synucleinopathies (and other diseases) are associated with protein aggregation, it is clear that, under certain circumstances, aggregation-prone proteins can evade the chaperone action of the sHsps. This failure of the sHsps to prevent aggregation is often attributed to them being “overwhelmed” in the context of disease (Healy et al. 2013, Leak 2014, Bakthisaran et al. 2015). However, specific factors that lead to sHsp chaperone activity being overwhelmed are yet to be determined.

3.1.1 Experimental rationale

The ability of dyes such as ThT to bind to β-sheet structures (such as those found in α-syn fibrils) has been used to monitor the kinetics of α-syn aggregation and provide a quantitative measure of fibril formation. Assays incorporating ThT have long been used to assess the activity of aggregation modifiers such as molecular chaperones, including the sHsps (Ecroyd et al. 2007, Bruinsma et al. 2011, Jovcevski et al. 2015, Cox et al. 2016). Therefore, ThT fluorescence was used as a way to evaluate the effect of sHsps on α-syn aggregation in plate-based assays. In addition, the nature of the interaction between the archetypal sHsps, αB-c and Hsp27, and monomeric α-syn was addressed in this chapter using analytical SEC and
analytical ultracentrifugation (AUC). Analytical SEC is specialised for detecting stable, high molecular mass complexes which would be expected from a holdase-type interaction between the sHsps and α-syn. In contrast, AUC can distinguish between the formation of high molecular mass complexes and transient interactions between two proteins.

Finally, the parameter(s) of α-syn aggregation that act to overwhelm the sHsps were characterised. When evaluating sHsp chaperone activity, maintaining consistent buffer conditions is crucial given the confounding effects changes in temperature and pH may have on their oligomeric size and subunit exchange rate (Bova et al. 2000, Fu and Chang 2004, Sun and MacRae 2005, Lelj-Garolla and Mauk 2006). Therefore, the kinetics of aggregation was altered by two methods (concentration and mutation) which did not require the buffer conditions to be altered.
Chapter 3: sHsps inhibit the aggregation of monomeric α-synuclein

3.2 Methods

3.2.1 In vitro α-syn aggregation assays

Fibrillar aggregation of α-syn, in the absence or presence of sHsps, was monitored using a ThT fluorescence assay via a previously described method (Ecroyd et al. 2007) with adaptations. Briefly, α-syn was incubated at 300 µM in 50 mM phosphate buffer containing 100 mM NaCl (pH 7.4) and 0.01% sodium azide, unless otherwise indicated. Assays were conducted in triplicate using clear 384 microwell plates (Greiner Bio-One, Frickenhausen, Germany) with each well containing 30 µL of sample. Plates were incubated in a POLARstar OPTIMA plate reader (BMG Labtechnologies, Melbourne, Australia) at 37°C, with the plate sealed to prevent evaporation. The ThT fluorescence was measured using excitation and emission filters of 440 nm and 490 nm, respectively. Readings were taken every 625 s for a period of up to 60 h. Plates were subjected to linear shaking at 600 rpm for 540 s after each reading.

At the end of each assay, data for the change in ThT over time were fitted with Boltzmann sigmoidal curves. Data were only used when the R² coefficient of determination was > 0.8. Parameters from these fits were used to derive the length of the lag phase and rate of α-syn aggregation using equations previously derived (Nielsen et al. 2001), with modification (Figure 3.1). The equation of the Boltzmann curve is given as:

\[ F = F_i + \frac{(F_f - F_i)}{1 + e^{\frac{(t_{50} - t)}{k}}} \]
Chapter 3: sHsps inhibit the aggregation of monomeric α-synuclein

Figure 3.1: Schematic illustration of the Boltzmann sigmoidal curve used to describe the increase in ThT fluorescence upon α-syn fibril formation. \( F_i \) corresponds to the initial fluorescence value, \( F_f \) corresponds to the final fluorescence value, \( t_{50} \) refers to the time taken to reach half the maximal fluorescence (\( F_{t_{50}} \)), \( t_{lag} \) is the lag time and \( k \) denotes the steepness of the curve.

The slope of the line at any point is:

\[
\frac{dF}{dt} = \frac{(F_f - F_i)}{k} \times \frac{e^{(t_{50}-t)k}}{1 + e^{(t_{50}-t)k}}^2
\]

When \( t = t_{50} \) (i.e. the inflexion point, when the elongation rate is maximal):

\[
\frac{dF}{dt} = \frac{(F_f - F_i)}{k} \times e^{0} \frac{1}{(1 + e^{0})^2}
\]

Therefore the rate of α-syn aggregation at \( t_{50} \) was calculated according to the equation:

\[
Rate_{t_{50}} = \frac{(F_f - F_i)}{4 \times k}
\]

where \( F_i \) corresponds to the initial fluorescence value, \( F_f \) corresponds to the final fluorescence value, \( t_{50} \) refers to the time taken to reach half the maximal fluorescence, and \( k \) describes the gradient of the curve.
The lag phase ($t_{lag}$) is when

$$\left\{ \frac{(F_f - F_i)}{4 \times k} \right\} \times t_{50} + b = F_i$$

(2)

To solve for $b$, at $t_{50}$ (i.e. the mid-point between $F_f$ and $F_i$):

$$F_{t_{50}} = \frac{(F_f - F_i)}{2}$$

$$b = \left\{ \frac{(F_f - F_i)}{2} \right\} - \left\{ \frac{(F_f - F_i)}{4 \times k} \right\} \times t_{50}$$

So substituting $b$ into equation (2):

$$F_i = \left\{ \frac{(F_f - F_i)}{4 \times k} \right\} \times t_{lag} + \left\{ \frac{(F_f - F_i)}{2} \right\} - \left\{ \frac{(F_f - F_i)}{4 \times k} \right\} \times t_{50}$$

Solving for $t_{lag}$:

$$4 \times k \times F_i = \{(F_f - F_i) \times t_{lag}\} + \{2 \times k \times (F_f - F_i)\} - \{(F_f - F_i) \times t_{50}\}$$

$$(F_f - F_i) \times t_{lag} = 4 \times k \times F_i + \left\{ (F_f - F_i) \times t_{50} \right\} - \{2 \times k \times (F_f - F_i)\}$$

Therefore, $t_{lag}$ is determined using the equation:

$$t_{lag} = \frac{4 \times k \times F_i}{(F_f - F_i)} + t_{50} - \{2 \times k \}$$

(3)
The relative efficacy of the sHsps to inhibit α-syn fibril formation was determined by calculating the protection provided by each sHsp at the conclusion of the assay, according to the difference in maximum ThT fluorescence in the absence and presence of the chaperone using the equation:

\[
\% \text{ Protection} = \frac{\Delta I - \Delta I_{\text{chaperone}}}{\Delta I} \times 100
\]  

(4)

where \(\Delta I\) and \(\Delta I_{\text{chaperone}}\) correspond to the change in ThT fluorescence of α-syn in the absence and presence of the sHsp respectively. In each case, samples were assayed in triplicate and the percent protection is reported as a mean ± S.E.M of at least 3 independent (biological) replicates.

3.2.2 Analytical size exclusion chromatography (SEC)

The nature of the interaction between the sHsps and α-syn was analysed via SEC of the samples at the end of the aggregation assays. Samples containing 300 μM α-syn, in the absence or presence of 300 μM αB-c or Hsp27, were collected immediately following incubation and centrifuged at 14000 x g for 10 min at 4°C to remove any insoluble protein. Supernatants were collected and loaded onto a Superose-6 size exclusion column (GE Healthcare, Uppsala, Sweden), pre-equilibrated with 50 mM phosphate buffer containing 100 mM NaCl (pH 7.4) and 0.01% sodium azide, at a flow rate of 0.5 mL/min. Protein elution was monitored using an in-line UV detector, and concentrations were determined using the peak integration function of PrimeView v5.0 (GE Healthcare, Uppsala, Sweden), to monitor any loss of protein following centrifugation. Eluate fractions (1 mL) were collected and analysed via SDS-PAGE and immunoblotting. Representative results are presented from two independent experiments from two separate aggregation assays.
3.2.3 Analytical ultracentrifugation (AUC)

UV absorbance-detected AUC was performed as previously described (Binger et al. 2013). Briefly, the maximum concentration was calculated for each protein such that the absorbance at 230 or 280 nm was within the optimal detection range of the instrument. Samples (380 µL) were prepared at room temperature and incubated for 1 h before being loaded into a 12 mm double-sector epon-filled centrepiece, alongside the relevant reference solution (400 µL). Samples were centrifuged in an XL-I analytical ultracentrifuge (Beckman Coulter, CA, USA) at 50000 rpm, using either a Ti60 or Ti50 rotor, at 20°C. Radial absorbance scans were collected at 230 nm or 280 nm at 6 min intervals with a radial step size of 0.003 cm for a total of 10 h. Sedimentation velocity (SV) profiles were analysed with SEDFIT software using a continuous c(s) distribution model and regularisation by maximum entropy. Using a regularisation parameter of $p=0.95$ and 200 sedimentation coefficient increments, the data were fitted to produce c(s) distributions. Weight-average sedimentation coefficients ($S_{av}$) of each sample were then calculated by integration of the sedimentation distributions over the given range. The buffer density ($\rho$), buffer viscosity ($\eta$) and partial specific volume ($\bar{\nu}$) used for analysis were estimated using SEDNTERP software (http://sednterp.unh.edu).

Fluorescence-detected AUC was performed as previously described (Binger et al. 2013), using Hsp27-WT labelled with CF488A and unlabelled, monomeric α-syn. Briefly, samples (350 µL) were prepared at room temperature and incubated for 1 h before being loaded into a 12 mm double-sector epon-filled centrepiece, and covered with 50 µL of FC-43 perfluorotributylamine (Scientific Instrument Services Inc., NJ, USA). Samples were centrifuged in an XL-A analytical ultracentrifuge fitted with a fluorescence detection system (FDS; Aviv Biomedical, NJ, USA) at 50 000 rpm using either a Ti60 or Ti50 rotor at 20°C. Excitation was at 488 nm and fluorescence above 505 nm was measured. Radial fluorescence
scans were collected at 3 min intervals with a radial step size of 0.002 cm for a total of 5 h. Sedimentation velocity profiles were analysed as described above, fitting time-independent noise and according to a fixed sample meniscus position.

3.2.4 Bulk Förster Resonance Energy Transfer (FRET) analysis of sHsp subunit exchange rate

Subunit exchange rates of the sHsps were determined by monitoring changes in Förster Resonance Energy Transfer (FRET) between fluorescently labelled sHsp oligomers. Aliquots of fluorescently labelled sHsp, composed of an equimolar mixture of CF488A- and CF647-labelled protein, were prepared in PBS (pH 7.4) at concentrations ranging from 3 – 90 µM. Samples were incubated at 37°C with shaking at 60 rpm using a VorTemp incubator (Labnet, Edison, USA) for 1 h, to allow complete mixing of the two labelled populations (confirmed by monitoring the quenching of donor fluorescence at 495 nm). The labelled sHsps were then diluted 10-fold into a sample of unlabelled sHsp at an equivalent concentration so as to maintain the overall concentration of the sHsp. The loss of FRET due to subunit exchange of labelled sHsps with unlabelled sHsps was determined by the decrease in acceptor fluorescence at 670 nm and monitored using a POLARstar Omega platereader (BMG Labtechnologies, Melbourne, Australia). Experiments were performed in the absence or presence of a 10-fold molar excess of α-synWT, in black polystyrene clear-bottom 384-well plates (Greiner Bio-One, Frickenhausen, Germany). Readings were taken every 120 s over a period of 4 h, with linear shaking at 600 rpm for 90 s after each cycle. The rate constant was determined by fitting the data to a one-phase exponential decay curve. The rate of subunit exchange was derived from the equation:

\[ Y = (Y_0 - Y_t) \times e^{-kt} + Y_t \]
where $k$ is defined as the rate constant of the reaction, and $Y_0$ and $Y_t$ correspond to the fluorescence intensity at time $= 0$ and $t$ respectively.
3.3 Results

3.3.1 sHsp inhibition of α-syn aggregation is concentration dependent

The amyloid fibrillar aggregation of α-synWT has been well characterised in vitro (Bruinsma et al. 2011). As expected, there was no increase in ThT fluorescence when Hsp27, bovine serum albumin (BSA) or buffer were incubated alone. However, incubation of α-syn under these experimental conditions resulted in an increase in ThT fluorescence over time indicative of fibril formation (Figure 3.2A). This increase in ThT fluorescence was inhibited by the addition of Hsp27 at a 1:1 (α-syn:Hsp27) molar ratio, but was not inhibited by the presence of the non-chaperone control protein BSA. The change in ThT associated with α-syn aggregation can be fitted by a Boltzman sigmoidal curve. This reveals that when α-syn was incubated alone there was a lag phase of 15 ± 3 h, and the maximum ThT fluorescence occurred after 36 ± 3 h (Figure 3.2B). Addition of the sHsps αB-c and Hsp27 prevented the fibrillar aggregation of α-syn in a concentration-dependent manner (Figure 3.2B). At a 1:10 molar ratio (sHsp:α-syn), αB-c and Hsp27 inhibited the change in ThT fluorescence associated with α-syn fibril formation by 65 ± 4% and 74 ± 5% respectively (Figure 3.2C).

3.3.2 In preventing the fibrillar aggregation of α-syn, the sHsps do not form stable, high molecular mass sHsp-target protein complexes

To investigate whether the sHsps inhibit the fibrillar aggregation of α-syn via the formation of high molecular mass complexes, as has been reported for the interaction with amorphously aggregating proteins (Lee et al. 1997, Haslbeck et al. 1999, Kulig and Ecroyd 2012), the samples containing a 1:1 molar ratio of the sHsp and α-syn (i.e. a concentration corresponding to near complete inhibition of fibril formation of α-syn by the sHsps, see Figure 3.2B) were collected after the aggregation assay for subsequent analysis by size
Chapter 3: sHsps inhibit the aggregation of monomeric α-synuclein

Figure 3.2: sHsps inhibit α-syn aggregation in a concentration-dependent manner. Recombinant α-syn was incubated at 300 μM in 50 mM sodium phosphate buffer with 100 mM NaCl and 0.01% NaN₃ (pH 7.4), in the absence or presence of sHsps or the non-chaperone control protein BSA. Fibril formation was monitored by the change in ThT fluorescence at 490 nm over time. (A) A representative trace of 2 independent experiments is shown for α-syn, in the absence or presence of Hsp27 or BSA at a 1:1 molar ratio (Hsp27/BSA:α-syn). Hsp27, BSA and buffer alone samples are also included for comparison, and the data for these overlay one another along the x-axis due to them showing no change in fluorescence over the course of the assay. (B) A representative trace of 4 independent experiments is shown for α-syn in the presence of various ratios of αB-cWT. Data were fitted with a Boltzmann sigmoidal curve. (C) Values obtained for the maximum change in ThT fluorescence from these fits was used to determine the percent protection afforded by WT αB-c or Hsp27 at a range of molar ratios. Results are presented as mean ± S.E.M (n ≥ 4).

exclusion chromatography (Figure 3.3A). Prior to incubation, in the absence of sHsp, α-syn eluted from the column in a peak centred at 17 mL. Following incubation of α-syn in the absence of sHsp, there was very little soluble α-syn present in the sample, consistent with its aggregation into fibrils. When present alone in solution, αB-c eluted as a well-resolved peak at 13 mL, consistent with it being a large polydisperse oligomer of average mass ~650 kDa under these solution conditions (Haley et al. 1998, Horwitz 2005). In the sample containing both αB-c and α-syn, there was no detectable shift in the elution volume or size of the individual peaks corresponding to monomeric α-syn and oligomeric αB-c, or the appearance of any additional peaks. Thus, there was no significant difference in the amount of soluble oligomeric αB-c and monomeric α-syn in this sample compared to when each of these (non-aggregated) proteins were analysed alone. The presence of only αB-c in the peak eluting at 13 mL and only α-syn in the peak eluting at 17 mL in this sample was confirmed by immunoblotting (Figure 3.3C) (the detectable limits of the immunoblotting procedure used in this work was ~30 nM for each protein). Even when a crosslinker, bis[sulfo succinimidy] (with a spacer arm length of 8 atoms or 11.4 Å), was added to the sample following
incubation and prior to SEC, there was no evidence of co-elution of αB-c and α-syn from the column (data not shown). Thus, under the conditions used in this work, there was no evidence of a stable high molecular mass complex formed between αB-c and aggregation-prone α-syn. Similar results were obtained for samples containing Hsp27 and α-syn (Figure 3.3B, D).

**Figure 3.3:** sHsps prevent α-syn aggregation but, in doing so, do not form a stable high molecular mass sHsp-target protein complex. (A, B) Size-exclusion chromatograms of soluble α-syn (300 μM), αB-c (300 μM), Hsp27 (300 μM) or post-aggregation samples of soluble α-syn (300 μM) in the absence and presence of 1:1 molar ratio of αB-c or Hsp27. (C, D) Immunoblot analysis of the eluate fractions collected from the size-exclusion column after loading with the sample from A or C containing α-syn and αB-c or Hsp27. Aliquots from every fraction (1 mL) collected between 8–20 mL were loaded on to a SDS/PAGE gel, transferred to nitrocellulose membrane and blotted with an anti-αB-c, anti-Hsp27 or anti-α-syn antibody. Results shown are representative of two independent experiments.

To further investigate the interaction between these sHsps and aggregation-prone α-syn, AUC was employed. Due to the propensity of the WT sHsps to form large, high molecular mass, polydisperse oligomers in solution (see Figure 3.3A), variant forms of these sHsps that either mimic phosphorylation (Hsp27_{3D}) or consist of only the core α-crystallin domain (αB-c_{core}) were selected for use in these experiments. This is because these variants exist predominately as either monomers or dimers (Hochberg et al. 2014, Jovcevski et al. 2015) and therefore their molecular masses more closely match that of α-syn (an important factor in these absorbance-based AUC experiments as it ensures that both species sediment at similar rates).
The ability of these variant forms to inhibit the fibrillar aggregation of α-syn was first established (Figure 3.4A). The protective capacity of these variants, as well as the αB-c3D and Hsp27\textsubscript{core} variants (see Appendix I), was seen to be similar to that of the WT proteins. Notably, the ability of the core domains (i.e. αB-c\textsubscript{core} and Hsp27\textsubscript{core}) to inhibit α-syn aggregation indicates that the ability of the sHsps to prevent α-syn fibril formation is inherent to the α-crystallin domain, and does not require the N- or C-terminal regions.

Sedimentation velocity profiles for α-syn alone, and in the presence of either Hsp27\textsubscript{3D} or αB-c\textsubscript{core} are shown in Figure 3.4. These profiles were fitted using a c(s) model which considers both sedimentation and diffusion to determine sedimentation coefficients for the species in solution (Schuck 2000). Single peaks calculated for α-syn (Figure 3.4B), αB-c\textsubscript{core} (Figure 3.4C) and Hsp27\textsubscript{3D} (Figure 3.4D) correspond to sedimentation coefficients of approximately 1.1, 1.7 and 2.7 S, respectively. Fitting the SV profile resulting from co-incubation of α-syn with αB-c\textsubscript{core} (Figure 3.4E) results in a bimodal distribution, with peak positions of approximately 1.2 and 1.9 S (Figure 3.4F). The small shift of the peak positions may suggest that these proteins interact transiently. Comparison of the predicted $S_{av}$ for the two components with the $S_{av}$ calculated for the mixture shows a small shift from 1.37 S to 1.40 S. Similarly, co-incubation of α-syn with Hsp27\textsubscript{3D} (Figure 3.4G) results in a bimodal distribution, with peaks corresponding to sedimentation coefficients of approximately 1.1 and 2.7 S (Figure 3.4H). In contrast to the mixture of α-syn with αB-c\textsubscript{core}, the peak positions for α-syn and Hsp27\textsubscript{3D} do not change significantly. The absence of additional peaks confirms the lack of a detectable stable complex between α-syn and Hsp27\textsubscript{3D}. 

Chapter 3: sHsps inhibit the aggregation of monomeric α-synuclein

Figure 3.4: Analysis of the interaction between sHsps and aggregation-prone α-syn by absorbance-based AUC. (A) Recombinant α-syn was incubated at 300 μM in 50 mM sodium phosphate buffer with 100 mM NaCl and 0.01% NaN₃ (pH 7.4), in the absence or presence of sHsps variants (30 μM). Fibril formation was monitored by the change in ThT fluorescence at 490 nm over time, and data were fitted with a Boltzmann sigmoidal curve. Sedimentation velocity analysis of 5 μM (B) α-syn, (C) αB-core or (D) Hsp27_3D. Data collected at 60 min intervals is presented, overlaid with theoretical fits to the c(s) model generated by SEDFIT. Sedimentation velocity profiles of α-syn co-incubated with (E) αB-core or (G) Hsp27_3D are also shown, along with c(s) distributions for these profiles (F and H respectively).
Chapter 3: sHsps inhibit the aggregation of monomeric α-synuclein

The use of fluorescently labelled protein allows specific detection of one component of a complex mixture using fluorescence-detected AUC. As such, CF488A-labelled Hsp27WT was used in fluorescence-based AUC experiments to obtain SV profiles with a range of α-syn concentrations (Figure 3.5). As above, these profiles were fitted using the c(s) model and the resulting distribution had three maxima (Figure 3.5B). The shoulder centred at 0.9 S results from non-sedimenting fluorescence, and is attributed to residual free CF488A dye in solution from the labelling process. The other two main peaks, centred at approximately 2.4 and 10.5 S, reflect the equilibrium characteristic of Hsp27WT in which smaller species (most likely predominately dimers) dissociate from polydisperse oligomers in order to provide chaperone-active subunits (Van Montfort et al. 2002). Importantly, with increasing unlabelled α-syn concentration, there is no concentration-dependent increase in the $S_{av}$ of Hsp27 that would otherwise be indicative of a stable, high molecular mass complex being formed between Hsp27 and α-syn. Rather, the $S_{av}$ decreases as the concentration of α-syn increases (Figure 3.5C), indicating that the average size of Hsp27 species in solution decreases with increasing amounts of α-syn. This trend is supported by a reduction in the signal at 10.5 S and an increase in the signal at 2.4 S with increasing (unlabelled) α-syn concentration. Together, these data suggest that the presence of aggregation-prone α-syn causes the dissociation of Hsp27 polydisperse oligomers in a concentration-dependent manner.

**Figure 3.5:** Aggregation-prone α-syn induces the dissociation of oligomeric Hsp27. Sedimentation velocity analysis of 0.5 µM CF488-labelled Hsp27 in the absence or presence of increasing concentrations of α-syn (0 – 50 µM). (A) Radial scans are displayed for the highest α-syn concentration overlaid with theoretical fits to the c(s) model generated by SEDFIT. Data are collected at 60 min intervals is presented. (B) c(s) distributions for each profile are shown, and (C) the $S_{av}$ was calculated via integration from 1 - 25 S for each concentration of α-syn. Data were then fitted with a linear regression model.
3.3.3 The ability of sHsps to prevent the aggregation of α-syn is dependent on the kinetics of the aggregation process

Despite the proven ability of the sHsps to prevent α-syn aggregation in vitro, the mechanism(s) by which aggregation-prone α-syn may ‘overwhelm’ sHsps, and result in the formation of disease-associated amyloid fibrils, remain unknown. To determine whether the rate at which α-syn aggregates affects the ability of the sHsps to prevent fibril formation, two methods were used to alter the kinetics of α-syn’s aggregation, both of which avoid the confounding effect a change in solution conditions (e.g. pH, temperature) may have on the chaperone activity of the sHsps (Bova et al. 2000, Fu and Chang 2004, Sun and MacRae 2005, Lelj-Garolla and Mauk 2006). First, we exploited the nucleation-dependent mechanism of amyloid fibril formation of α-syn, whereby an increase in the concentration of aggregation-prone monomeric protein increases the rate of aggregation (Wood et al. 1999). Since the dissociation of small chaperone-active species from larger polydisperse oligomers is proposed to be a key component of the chaperone activity of the sHsps (Aquilina et al. 2004, Ecroyd et al. 2007, Benesch et al. 2008), we first determined the rate of sHsp subunit exchange over the sHsp concentration range to be used in these experiments (i.e. 3 – 90 µM) using FRET. During subunit exchange, mixing of labelled and unlabelled sHsp subunits resulted in an exponential decrease in the emission fluorescence intensity of the acceptor, which can be used to calculate the rate of subunit exchange (Figure 3.6A). Over the concentration range used in this work, the rate of subunit exchange of αB-c reached a maximum at ~30 µM (Figure 3.6B). Importantly, this rate was not significantly affected by the presence of α-syn (Figure 3.6B). Thus, in the samples containing 300 – 750 µM α-syn, the rate of subunit exchange of αB-c is constant. In contrast to αB-c, the oligomeric state of Hsp27 (Jovcevski et al. 2015) and its subunit exchange rate (Figure 3.6C) are both significantly affected by concentration. Therefore, Hsp27 was unsuitable for use in these
Figure 3.6: The ability of αB-c to inhibit the fibrillar aggregation of α-syn is dependent on the kinetics of aggregation. (A) Fluorescently labelled αB-c was incubated for 1 h at 37°C in PBS (pH 7.4) at concentrations ranging from 3 – 90 µM, consisting of an equimolar mixture of fluorescently labelled protein capable of FRET. Samples were diluted 10-fold into unlabelled αB-c, in the absence or presence of α-syn at a 1:10 (αB-c:α-syn) molar ratio, and the loss of fluorescence in the acceptor fluorescence channel was used to calculate (B) the rate of subunit exchange in the absence and presence of α-syn. (C) The rate of subunit exchange was similarly calculated for Hsp27, in the absence and presence of α-syn. (D) Recombinant A53T α-syn was incubated at concentrations ranging from 150 – 750 µM in 50 mM phosphate buffer containing 100 mM NaCl and 0.01% NaN3 (pH 7.4), in the presence or absence of a 1:10 molar ratio of αB-c. Samples were incubated at 37°C for 60 h and aggregation was monitored via the change in ThT fluorescence at 490nm. (A) A representative plot is shown for α-syn in the absence of αB-c with Boltzmann-sigmoidal curves fitted to the data. Values obtained from A were used to calculate the lag phase, elongation rate and plateau phase for each α-syn concentration. The percent protection afforded by αB-c when present in the sample was calculated and correlated with the (B) duration of the lag phase and (C) rate of elongation. Symbols represent the calculated parameters from each of three independent repeats, with each point corresponding to values calculated from a fit of triplicate samples and shaded according to the concentration of α-syn as indicated in panel A. Data in B, E and F were fitted with non-linear regression analysis, while data in C were fitted with linear regression analysis, and the R² coefficients of determination are shown.

experiments due to these confounding effects. Thus, the disease-associated A53T variant of α-syn was incubated at concentrations from 150 – 750 µM in the absence and presence of αB-c (Figure 3.6D). In the absence of the chaperone, increasing the concentration of α-syn led to an increase in the rate and maximum ThT fluorescence associated with α-synA53T fibril formation, and a decrease in the lag phase (from 9 ± 2 h to 3 ± 1 h over this concentration range). When αB-c was present, such that the molar ratio of α-syn:αB-c remained constant (i.e. 1:10 αB-c:α-syn), the ability of αB-c to inhibit α-syn fibril formation was dependent on the kinetics of the aggregation process (Figure 3.6E-F). Thus, when the
kinetics of α-syn aggregation were relatively slow (i.e. longer lag phase and slower rate of aggregation), αB-c was a more effective inhibitor of aggregation. When the kinetics of aggregation increased (i.e. shorter lag phase and faster rate of aggregation), αB-c was a less effective chaperone, only decreasing the amount of aggregation of α-syn by ~20%.

The second method employed to alter the aggregation kinetics of α-syn aggregation was the use of disease-related mutants which aggregate at different rates (Bruinsma et al. 2011). Recombinant α-syn mutant proteins were incubated at 300 µM in the absence or presence of either αB-c or Hsp27 (1:10 molar ratio, sHsp:α-syn). As expected, each of the mutant proteins displayed different aggregation kinetics, with α-synA30P having a similar lag phase, elongation rate and maximal fibril formation to that of α-synWT (Figure 3.7A). In contrast, α-synA53T had the shortest lag phase (3 ± 1 h), slowest elongation rate and lowest maximum increase in ThT fluorescence. The α-synE46K variant was characterised by a slow rate of elongation, high maximal ThT fluorescence, and longest lag phase (42 ± 9 h) of any of the α-syn variants tested. Whilst both αB-c and Hsp27 were able to inhibit the aggregation of the α-syn proteins (Figure 3.7A), their ability to do so was dependent upon the isoform of α-syn. Both chaperones were least effective at inhibiting the aggregation of α-synA53T, which aggregated the fastest (i.e. shortest lag phase), whereas they were most effective at inhibiting α-synE46K, which aggregated the slowest (i.e. longest lag phase) (Figure 3.7B and C).
Chapter 3: sHsps inhibit the aggregation of monomeric α-synuclein

Figure 3.7: The ability of the sHsps αB-c and Hsp27 to prevent the aggregation of disease-associated mutant forms of α-syn. Recombinant WT or disease-related mutant forms of α-syn (A30P, A53T or E46K) were incubated at 300 μM in 50 mM phosphate buffer containing 100 mM NaCl and 0.01% NaN₃ (pH 7.4), in the presence or absence of 30 μM αB-c or Hsp27. Samples were incubated at 37°C for 60 h and aggregation was monitored via the change in ThT fluorescence at 490 nm. (A) Representative plots of the four independent repeats, each consisting of triplicate samples, are shown for each of the α-syn proteins, including Boltzmann-sigmoidal curves fitted to the data. Values obtained from these fits were used to calculate the lag phase for each protein, which was then correlated with the percent protection when (B) αB-c or (C) Hsp27 was present in the sample. Data shown are the results of four independent repeats, with each point corresponding to values calculated from a fit of triplicate samples. Non-linear regression analysis of the correlation between the lag phase and the percent protection was then performed.
Chapter 3: sHsps inhibit the aggregation of monomeric α-synuclein

3.4 Discussion

The sHsps play a critical role in maintaining cellular proteostasis by preventing protein aggregation associated with disease. Here, the sHsps αB-c and Hsp27 are confirmed as potent inhibitors of α-syn fibril formation in vitro (Rekas et al. 2004, Wang et al. 2008, Aquilina et al. 2013). The phosphomimicking variants of αB-c and Hsp27 (i.e. αB-c3D and Hsp273D) were also effective chaperones at inhibiting α-syn fibril formation, adding to the growing debate surrounding the effect of phosphorylation on chaperone activity (Bakthisaran et al. 2016). Notably, the αB-c core and Hsp27 core isoforms inhibited the aggregation of α-syn with similar efficacy to the WT (full length) protein, demonstrating that the sites required to inhibit α-syn aggregation are present in the core domain of these sHsps. The finding that the core domain is sufficient to prevent α-syn aggregation is consistent with previous studies showing that this region is capable of preventing other target proteins from forming amorphous or fibrillar aggregates (Hochberg et al. 2014, Mainz 2015).

Although the ability of the sHsps to inhibit the amyloid fibrillar aggregation of a range of target proteins in vitro is well established, the mechanism by which they do so is not clearly defined and may be dependent on the target protein (Hatters et al. 2001, Raman et al. 2005, Wilhelmus et al. 2006, Kulig and Ecroyd 2012). The molecular mechanism by which αB-c and Hsp27 interact with α-syn to prevent its aggregation was therefore characterised by several techniques. The results presented here demonstrate that both Hsp27 and αB-c increase the lag phase of α-syn aggregation and inhibit the elongation phase, suggesting that they primarily act through stabilising aggregation-prone monomeric α-syn to prevent it forming fibrils. However, a high molecular mass complex between α-syn and αB-c or Hsp27 was unable to be detected under the experimental conditions used in this work. Thus, in the
absence of any evidence of a stable interaction, it is inferred that these proteins interact transiently and this acts to prevent α-syn aggregation.

Transient interactions between the sHsps and α-syn are difficult to detect. Small shifts in the c(s) distributions and calculated $S_{av}$ for the α-syn/αB-c$_{core}$ mixture may suggest a transiently interacting, rapidly exchanging system. That these shifts were not observed for Hsp27$_{3D}$ does not rule out that a transient interaction occurs between α-syn and Hsp27$_{3D}$. Rather, it indicates that this interaction was below the detection limit for these experimental conditions. This may be due to differences in the binding kinetics or the amount of α-syn interacting with Hsp27$_{3D}$ compared to αB-c$_{core}$. If the fraction of species in this interacting population is too small, or if the interaction occurs too fast, the interaction may not be resolved in the sedimentation experiment (Lebowitz et al. 2002, Balbo and Schuck 2005, Howlett et al. 2006). Transient interactions between αB-c and α-syn (and also of αB-c with other fibril-forming target proteins) are also apparent from NMR spectra acquired on mixtures of these proteins due to general broadening of spectra of the target protein with no indication of specific binding site(s) (Rekas et al. 2004, Rekas et al. 2007, Robertson et al. 2010, Esposito et al. 2013). It is therefore concluded that these sHsps prevent α-syn aggregation via transient interactions in a similar way as has been previously described for apoC-II (Hatters et al. 2001) and reduced and carboxymethylated α-lactalbumin (α-lac) (Kulig and Ecroyd 2012). In doing so, the manner by which these sHsps act as chaperones to prevent α-syn aggregation is not through a ‘holdase’ mechanism, as has been well characterised for amorphously aggregating target proteins under stress conditions (Lee et al. 1997, Haslbeck et al. 1999). Instead, these data support a model in which sHsps can act as protein stabilisers, interacting transiently with relatively ordered protein intermediates that have entered off-folding pathways, a process that facilitates them re-entering the on-folding pathway (Jakob et al. 1993). Considering the cellular implications of this type of interaction, a transient interaction mechanism is
favourable as it does not deplete the pool of sHsp available to interact with aggregation-prone proteins in the cells. In contrast, a holdase-type chaperone mechanism, in which stable high molecular mass complexes are formed, likely occurs with more disordered intermediates. These intermediates commonly expose higher degrees of hydrophobicity on their surface prior to undergoing aggregation, as occurs under significant stress conditions with the formation of amorphous aggregates (Kulig and Ecroyd 2012).

Importantly, it is unclear at this stage whether the interaction between the sHsp and α-syn impacts the conformation of aggregation-prone monomeric α-syn. However, previous studies have demonstrated that depletion of αB-c from aggregation-inducing conditions containing amyloidogenic substrate can allow aggregation to proceed (Kulig and Ecroyd 2012). This implies that transient interactions between aggregation-prone species and sHsp may not significantly alter the conformation or aggregation propensity of the target protein.

Previous work has extensively investigated the interaction of αB-c with a variety of amorphously aggregating target proteins under conditions of elevated temperature, reductive stress or chemical stress. As a result of these studies, it is apparent that there are similarities between the mechanisms of sHsp chaperone interaction with amorphous and fibrillar aggregating target proteins. As observed here with fibril-forming α-syn, sHsp are more efficient chaperones when interacting with slowly (amorphously) aggregating target proteins (Lindner et al. 2001, Carver et al. 2002). They also interact with target proteins early along their aggregation pathway, i.e. monomeric forms that are in a disordered, intermediate state (Lindner et al. 2001, Carver et al. 2002). Dynamic, transient interactions between the target protein and the sHsp are also crucial factors in determining chaperone efficacy with target proteins (Devlin et al. 2003).
Given that both αB-c and Hsp27 are highly effective molecular chaperones at inhibiting α-syn fibril formation in vitro, the question remains as to how α-syn intermediates escape the sHsps to form fibrils and plaques in the context of the α-synucleinopathies? A key factor explored in this chapter was whether the rate of aggregation has a significant effect on the ability of these chaperones to prevent aggregation. Similar to the reported effect of metals (Uversky et al. 2002), pesticides, membrane lipids (Lee et al. 2002) and molecular crowding (Uversky et al. 2002, Munishkina et al. 2004), increases in the initial monomer concentration of α-syn promotes nucleation during the rate-limiting step, increasing the kinetics of aggregation (Shtilerman et al. 2002, Uversky 2002, Munishkina et al. 2004, Uversky 2007). Furthermore, the three most studied disease-related mutations in α-syn results in markedly different aggregation kinetics (Bruinsma et al. 2011), which was also observed in this study. Increases in α-syn monomer concentration and disease-related mutants of α-syn were therefore exploited in order to alter the kinetics of α-syn’s fibrillar aggregation. In both cases, increasing the rate of α-syn aggregation led to a decrease in the chaperone ability of αB-c and Hsp27 to prevent fibril formation. In particular, there was a marked correlation between the efficacy of the chaperone and the lag phase of aggregation, i.e. the longer the time taken to form nuclei, the more efficacious the chaperones were in preventing aggregation. These results also support previous work in which the rate of α-syn fibril formation was increased by adding the inert crowding agent dextran (Rekas et al. 2004). Under these conditions αB-c was a much poorer chaperone (Rekas et al. 2004). However, the presence of a crowding agent also reduces the rate of subunit exchange of αA-c (HSPB4) (Ghahghaei et al. 2007), which may have contributed to the decrease in αB-c’s chaperone efficacy in this case.

The effect of aggregation kinetics on the ability of sHsps to prevent fibril formation is significant given the association of mutations and duplication or triplication of the SCNA gene with early onset PD (Polymeropoulos et al. 1997, Baba et al. 1998, Kruger et al. 1998,
Chapter 3: sHsps inhibit the aggregation of monomeric α-synuclein

Campbell et al. (2001, Zarranz et al. 2004). Moreover, the link between increased aggregation rate and a decrease in chaperone efficacy provides a potential mechanism for aggregation-prone α-syn overwhelming the chaperone capability of the sHsps. While fluorescence-based AUC suggested that the presence of aggregation-prone α-syn causes the dissociation of Hsp27 oligomers, this technique provides a measure of the oligomeric distribution at equilibrium. The real-time availability of chaperone-active subunits, through their dissociation from large polydisperse oligomers that are able to interact with aggregation-prone proteins, is governed by the subunit exchange rate (Vos et al. 2008). The rate of subunit exchange for αB-c increased with concentration up to ~30 µM, after which it remained constant, which supports the notion that subunit exchange is a result of dissociation from sHsp oligomers, as opposed to oligomeric collisions, similar to that demonstrated for αA-c (HSPB4) (Bova et al. 2000). Moreover, factors that increase the rate of α-syn aggregation in cells (such as mutation, gene multiplication or macromolecular crowding) are likely to overwhelm the protective capacity of the sHsps due to an insufficient supply of chaperone-active subunits capable of interacting with the aggregation-prone α-syn monomer. Such a mechanism is also consistent with the dissociated subunits of sHsps being the chaperone-active species in cells.

The results presented here contribute to a greater understanding of the molecular mechanisms by which sHsps interact with monomeric disease-related target proteins to prevent their aggregation. Importantly, this provides further support for the reclassification of the sHsps as protein stabilisers rather than holdase chaperones since the latter does not fully describe the manner by which they can interact with aggregation-prone proteins (Kulig and Ecroyd 2012). Thus, although the ability to form complexes with destabilised, particularly amorphously aggregating, proteins is a key element of their chaperone activity (Arrigo et al. 2007), this work adds to a growing body of evidence highlighting that this is not the only mechanism by
which sHsps inhibit protein aggregation (Hatters et al. 2001, Rekas et al. 2004, Robertson et al. 2010, Bruinsma et al. 2011, Kulig and Ecroyd 2012). Furthermore, the rate of aggregation is highlighted as a significant factor that governs the relative ability of αB-c and Hsp27 to prevent α-syn fibrillar aggregation. This provides a potential rationale for how sHsp chaperone activity is ‘overwhelmed’ in the context of diseases associated with protein aggregation, i.e. factors that increase the rate at which aggregation occurs also compromise the ability of sHsps to prevent it.
Chapter 4:

sHsps interact with mature α-synuclein fibrils

Portions of this chapter are to be submitted for publication under the following title:


Author Contributions: DC designed, performed and analysed all experiments. HE conceived and assisted with data analysis for the sucrose and fluorescence pelleting assays, MH and DW assisted with smFRET, SAVE and sPAINT data collection and analysis, JB assisted with analysis of seeded aggregation assays, DW assisted with DHE data collection and analysis, ES and MG assisted with collection and analysis of AUC experiments. DC wrote the manuscript and generated figures. All authors edited the manuscript in preparation for submission.
Chapter 4: sHsps interact with mature α-synuclein fibrils

4.1 Introduction

4.1.1 sHsps interact with aggregation intermediates

Many investigations of sHsp chaperone activity have focussed on their interaction with aggregation-prone monomers. The work presented in Chapter 3 aimed to characterise the interaction of the sHsps, αB-c and Hsp27, with monomeric α-syn. This required the addition of chaperone prior to aggregation commencing, in a similar manner to previous studies examining the ability of sHsps to prevent aggregation (Horwitz 1992, Rekas et al. 2004, Ecroyd et al. 2007, Bruinsma et al. 2011). However, little is known about the potential for sHsps to interact with species formed during the aggregation of α-syn. Recently, the interaction of sHsps with preformed amyloid fibrils has been investigated; it has been reported that αB-c can bind along the length of amyloid fibrils formed by α-syn (Waudby et al. 2010), Aβ (Shammas et al. 2011) or apoC-II (Binger et al. 2013) with moderate (µM) affinity.

Addition of monomeric units to fibril ends occurs during the elongation phase of aggregation. In addition, sites of exposed hydrophobicity along the face of the fibril have been identified as possible locations for secondary nucleation (via surface-templated nucleation or fragmentation), leading to rapid fibril growth. These secondary nucleation processes can dominate the overall kinetics of aggregation once fibrils have formed (Ruschak and Miranker 2007, Knowles et al. 2009, Cohen et al. 2011). Interaction of the sHsps with amyloid oligomers or fibrils may contribute to the ability of sHsps to inhibit further aggregation, by competing with monomeric units for access to fibril ends or occluding sites of potential secondary nucleation (Waudby et al. 2010). These mechanisms may complement the interaction of the sHsps with monomeric α-syn. The association of small amyloid aggregates
with cytotoxicity highlights the importance of considering them as potential targets of the sHsps.

**4.1.2 Single molecule techniques**

Traditional techniques for the study of protein aggregation, such as the ThT assays used in Chapter 3, rely on triggering aggregation in a population of molecules and monitoring the ensemble average of the population. These techniques have great utility in evaluating the effect of aggregation modulators, such as chaperones, on the process of aggregation. However, a significant limitation of such bulk techniques is the inability of these methods to detect short-lived or rare species that may be present due to ensemble averaging over the population of molecules. Given intermediates of α-syn aggregation (oligomers) typically comprise a small fraction of the total protein concentration, traditional techniques are ill-suited to detect and characterise them (Horrocks et al. 2015). Single molecule analyses have thus been employed to study both oligomers and fibrils formed during protein aggregation, as these methods allow detection, characterisation and direct observation of rare and/or short-lived species, as well as their potential interactions with the sHsps, that are otherwise inaccessible to bulk analyses (Horrocks et al. 2016).

The two most common single molecule experimental setups are confocal microscopy and TIRF microscopy. Confocal microscopy is routinely used to detect molecules as they diffuse (or are flowed) one-by-one through the confocal volume, and thus, with regards to protein aggregation, is ideally suited to detecting and characterising rare oligomeric intermediates formed during aggregation (Horrocks et al. 2011, Cremades et al. 2012). Flowing molecules through the confocal volume greatly reduces the acquisition time required to detect rare species, and limits the heterogeneous paths taken by molecules through the confocal volume, such that experiments can be conducted on time-frames relevant to aggregation (min)
Chapter 4: sHsps interact with mature α-synuclein fibrils

(Horrocks et al. 2011). In addition, application of FRET in this context enables some structural characterisation of oligomers, with a resolution of approximately 8 nm (Cornish and Ha 2007). This technique was previously employed to demonstrate that α-syn oligomers undergo a structural reorganisation from amorphous oligomers possessing low FRET efficiencies to more ordered, β-sheet rich oligomers possessing high FRET efficiencies (Cremades et al. 2012).

In contrast to confocal microscopy, TIRF microscopy involves imaging entire fields of view simultaneously (Cornish and Ha 2007). Combining amyloid-specific dyes, such as ThT, with this technique (in a method known as SAVE imaging (Horrocks et al. 2016)), enables oligomeric and fibrillar structures to be distinguished from their monomeric counterparts without the need for covalent attachment of a fluorophore (Horrocks et al. 2016). In addition, the newly developed technique of sPAINT enables simultaneous characterisation of the spatial position and emission spectrum of single dye molecules (Bongiovanni et al. 2016).

Using nile red, a phenoxazone-based dye whose fluorescence emission wavelength is known to be sensitive to the hydrophobicity of its environment (Greenspan and Fowler 1985) (Bongiovanni et al. 2016), it is possible to characterise the hydrophobicity of regions along the surface of mature fibrils at superresolution (Bongiovanni et al. 2016).

4.1.3 Experimental rationale

Whilst the work described in Chapter 3 established that the sHsps, Hsp27 and αB-c, interact transiently with monomeric α-syn to prevent its aggregation, little is known about the interaction of sHsps with other species formed during the aggregation of α-syn. Although αB-c has been shown to bind fibrillar α-syn (Waudby et al. 2010), the mechanism of this interaction and whether it provides a protective effect by inhibiting the cytotoxicity of the fibrils themselves is yet to be established. Moreover, it remains to be determined whether
other sHsps, such as Hsp27, can also bind to amyloid fibrils, and thus whether fibril binding is likely to be a generic property of the sHsps. The following chapter specifically addresses these gaps in knowledge by examining the ability of Hsp27 to interact with oligomeric and fibrillar forms of α-syn. Single molecule techniques, such as those described above, are employed, along with traditional bulk techniques, in order to gain a comprehensive understanding of how Hsp27 interacts with aggregated forms of α-syn and the potential physiological relevance of these interactions.
Chapter 4: sHsps interact with mature a-synuclein fibrils

4.2 Methods

4.2.1 Single molecule confocal microscopy

4.2.1.1 Microscope setup

smFRET measurements were performed using a custom built confocal microscope assembled by Dr. Mathew Horrocks (University of Cambridge, UK). The components, filter spectra and layout of this microscope have been previously published (Horrocks et al. 2013). Prior to use, the alignment of the microscope optics was measured using dual labelled DNA duplexes. Synthetic DNA oligonucleotides synthesised by IBA GmbH (Gottingen, Germany) and labelled with AF488 (5’–TAGTGTAACCTTAGGATAAGGAGCAGT AATCGGTA–3’) or AF647 (5’–TACCGATTACTGGCTCTTATCCTAGGCTTAAGGT ACACTA–3’) were prepared to a final concentration of 2 µM (containing a 1:1 mixture of each oligonucleotide) in 10 mM Tris containing 1 mM EDTA and 100 mM NaCl (pH 7.5, 0.02 µm filtered). To form duplexes, the mixture was heated to 95°C then cooled to room temperature over a 7 h period. smTCCD measurements (Orte et al. 2008, Cremades et al. 2012) of the duplex were then performed and the association quotient (Q) was determined, where Q is defined as:

\[
Q = \frac{C - D}{A + B - (C - D)}
\]

In this instance, A and B refer to the event rates in the donor and acceptor channels respectively, C refers to the rate of coincident events (i.e. events above background in both channels), and D refers to the rate of desynchronised events (i.e. coincident events that occur after randomisation of one channel, acting as a measure of chance coincidence) (Clarke et al. 2007). This calculation was completed using custom software for Python 2.7 (available at:...
4.2.1.2 Preparation of microfluidic devices

Microfluidic devices were prepared as described by Horrocks et al (2011). Briefly, ‘master’ moulds used for the fabrication of these devices were generated by spin-coating approximately 1 mL SU-8 3025 photoresist (Microchem, Westborough, U.S.A.) onto a 76.2 mm silicon wafer (Compart Technology Ltd, Tamworth, UK) using a Spincoat G3P-8 (Specialty Coating Systems, Indianapolis, U.S.A.). The wafer was spun at 800 rpm for 5 s, then accelerated to 3000 rpm at a rate of 300 rpm/s for 60 s, resulting in a final thickness of 25 µm. The wafer was prebaked by heating at 96°C for 12 min using an SD300 digital hotplate (Stuart Equipment, Staffordshire, UK), before being exposed to UV light for 15 s using an OAI UV source (OAI, USA) through a UV-blocking mask. A single design containing 25 straight 100 x 10000 µM channels was used in this work, and the appropriate mask was provided by Dr. Mathew Horrocks. The master was then washed with propylene glycol monomethyl ether followed by isopropanol to remove any unexposed photoresist.

Microfluidic devices were produced by casting the master mould with SYLGARD 184 PDMS elastomer according to the manufacturer’s instructions. Approximately 25 mL of elastomer mixture (containing elastomer and curing agent at a 10:1 volumetric ratio) was vigorously mixed, poured over the master and degassed to remove any air pockets. The elastomer was hardened by incubation at 65°C overnight in a laboratory oven (CarboLite Gero, Hope Valley, UK). Individual devices were cut from the elastomer according to the design, and access holes for inlet tubes were introduced using Unicore biopsy punches of 1.0 mm or 0.75 mm diameter (ThermoFischer Scientific, Waltham, USA). Devices were then exposed to oxygen plasma for 7 s using a Plasma system FEMTO (Diener Electronic,
Ebhausen, Germany), sealed to borosilicate glass cover slides (thickness number 1; VWR International, Radnor, U.S.A.) and baked overnight at 65°C overnight in a laboratory oven (CarboLite Gero, Hope Valley, UK).

The straight channel design used in this work enables the detection of oligomeric proteins under conditions of flow, allowing the rate of detection to be increased and removing the bias for small, faster diffusing species (Horrocks et al. 2011). For use, the device was connected to a PhD 2000 Infusion or PhD 2000 Programmable syringe pump (Harvard Apparatus, USA) using FineBore polyethylene tubing (Scientific Laboratory Supplies, UK), and the sample drawn through the device from a sample reservoir at 200 µL/h (equivalent to 2 cm/s).

4.2.1.3 Single molecule Förster resonance energy transfer (smFRET)

smFRET was performed to measure the accumulation, growth and structural conversion of fluorescently labelled, oligomeric α-syn species in solution. To enable site specific conjugation of a fluorophore to α-syn, the α-synA90C variant was chosen, which allows the engineered cysteine to be targeted by succylimide fluorescent dyes (see Section 2.6 for labelling details). To monitor the aggregation of α-syn, an equimolar mix of AF488 and AF647 labelled α-synA90C was prepared to a total concentration of 70 µM in PBS (pH 7.4, 0.02 µm filtered), in the absence or presence of Hsp27 (7 µM). Samples were incubated for up to 48 h at 37°C with shaking at 200 rpm in an Innova43 Incubator Shaker (Eppendorf, Hamburg, Germany), and aliquots taken at the time points indicated in the corresponding figure legend. To monitor the disaggregation of α-syn fibrils, an equimolar mix of AF488 and AF647 labelled α-synA90C was prepared to a total concentration of 70 µM in PBS (pH 7.4, 0.02 µm filtered) and incubated as above for up to 7 days. The sample was centrifuged at 17000 x g for 20 min at 4°C to isolate mature fibrils, before the supernatant was discarded and gently replaced with fresh PBS. The fibrils were washed with PBS twice, before being
resuspended in fresh PBS in the absence or presence of αB-c or Hsp27, at a 1:5 molar ratio (chaperone:α-syn), according to the original α-syn monomer concentration. Samples were incubated for up to 21 days at 37°C without shaking, and aliquots taken at the time points indicated in the corresponding figure legends. All incubation steps were completed in DNA LoBind 1.5 mL tubes (Eppendorf, Hamburg, Germany) wrapped in aluminium foil to limit protein adsorption and photobleaching over the course of the assay.

Immediately prior to analysis, aliquots were centrifuged at 20000 x g for 20 min at room temperature to remove any large, insoluble aggregates. The supernatant was then collected and diluted to approximately 100 pM using fresh PBS. Samples were then loaded into the sample reservoir of a microfluidic channel and drawn through the confocal volume (see Section 4.2.1.2). Samples were excited at 488 nm and the emitted fluorescence from both the donor (AF488) and acceptor (AF647) fluorophores was then detected.

### 4.2.1.4 Data analysis

Data analysis was completed in Origin and IgorPro v 6.3.4.1 using custom scripts written by Dr. Mathew Horrocks (University of Cambridge, UK). Datasets were first thresholded to remove background photons and data points falling below this threshold were discarded. Thresholding was completed using the ‘and’ criterion, such that only those bursts which were above the threshold in both the acceptor and donor channels simultaneously were selected. To determine the appropriate thresholds, the Q value (see Section 4.2.1.1) was calculated at each possible pair of thresholds ranging from 0 – 100 in both the donor and acceptor channels (equating to over 10000 possible threshold pairs). The thresholds for the donor and acceptor channels were then selected as those which produced the maximum value of Q.

Once data has been thresholded, the size of oligomeric α-syn was then calculated according to the following equation:
\[ \text{Size} = 2 \left( \frac{I_D + I_A \left(\frac{1}{\gamma}\right)}{I_M} \right) \]

where \( I_D \) and \( I_A \) correspond to the intensity in the donor and acceptor channels respectively, \( I_M \) corresponds to the intensity of the monomer (calculated as the average intensity of non-coincident events in the donor channel prior to incubating the sample under aggregation-inducing conditions) and \( \gamma \) is an experimentally determined calibration factor, in this case 1.01, used to compensate for inter-instrumental differences (Ye et al. 2012).

The efficiency of FRET can also be used as a measure of proximity, as it is dependent on the distance between two fluorophores. This property was exploited as an indirect measure of the structurally compact nature of the detected oligomers, such that more compact, well ordered species exhibit a higher FRET efficiency (Cremades et al. 2012). The FRET efficiency of a given coincident burst was calculated according to the following equation:

\[ \text{Efficiency} = \frac{I_A}{I_A + \gamma \times I_D} \]

where \( I_D \) and \( I_A \) correspond to the intensity in the donor and acceptor channels respectively, and \( \gamma \) is an experimentally determined calibration factor used to compensate for inter-instrumental differences (Ye et al. 2012).

Populations of small (2 – 6 mers) and medium (7 – 300 mers) oligomers were then identified, and the FRET efficiency histograms for each were fitted by a Gaussian distribution. This distinguishes small oligomers with low FRET from medium oligomers with low or high FRET efficiencies, such that the change in these populations can be characterised over time as a measure of the structural conversion of oligomers from relatively disordered to more stable, compact proteinase-K-resistant oligomers (Cremades et al. 2012). Oligomers larger
than 300 monomeric units were excluded from the analysis as there were too few events in this range.

4.2.2 Seeded aggregation assays

In an attempt to differentiate an interaction of Hsp27 with monomeric or fibrillar α-syn, seeded aggregation assays were employed that were an adaption of a method previously described (Buell et al. 2014). Briefly, to produce seed fibrils, 1 mL aliquots of monomeric α-syn were prepared at concentrations ranging from 100 – 200 µM in 50 mM phosphate buffer (pH 7.4), in the absence of added salt. Samples were incubated at 45°C with maximal stirring with a Teflon flea on a WiseStir heat plate (Witeg, Wertheim, Germany) for 24 h, then sonicated (three cycles of 10 s at 30% power). Samples were then incubated for a further 24 h under the conditions described above, before being sonicated again as described. Finally, samples were distributed into aliquots, flash frozen in liquid N₂ and stored at -20°C. In the experiments described below, the concentration of fibril seeds is reported as the monomer-equivalent concentration.

Seeded aggregation of α-syn was monitored using a microplate assay. Assays were conducted in clear 384 microwell plates (Greiner Bio-One, Frickenhausen, Germany) with each well initially containing a total of 40 µL of sample (all samples were run in duplicate). In order to probe the interaction of Hsp27 with monomeric α-syn, α-syn was incubated at concentrations from 10 – 100 µM in the absence or presence of 50 µM Hsp27 (or the negative control protein BSA) in 50 mM phosphate buffer (pH 7.4). Samples were equilibrated to 37°C, then seed fibrils were spiked into each sample to a final concentration of 5% (w/w), resulting in a final volume of 50 µL for each sample. Alternatively, to probe the interaction of Hsp27 with fibrillar α-syn, 50 µM α-syn was incubated in the absence or presence of Hsp27 (or BSA) at concentrations ranging from 0.1 – 10 µM in 50 mM phosphate buffer (pH 7.4). Samples were
equilibrated to 37°C, then seed fibrils were spiked into each sample to a final concentration of 1 – 10% (w/w), resulting in a final volume of 50 µL for each sample. All wells contained a final concentration of 50 µM ThT, with the plate sealed to prevent evaporation. Plates were incubated in a FLUOstar Optima plate reader (BMG Labtechnologies, Melbourne, Australia) at 37°C with no shaking, and ThT fluorescence measured using excitation and emission filters of 440 nm and 490 nm, respectively. Readings were taken every 300 s for a period of up to 20 h.

The change in ThT fluorescence intensity was calculated by subtracting the value at t = 0 h from subsequent measurements. The elongation rate for each sample was then determined by fitting data from the linear elongation phase of the assay (consisting of the initial 1 – 3 h or 0 – 2.5 h where appropriate) with linear regression. The relationship between rate and concentration was then fit to a Michaelis-Menten kinetic model.

**4.2.3 Preparation of mature, unlabelled α-syn fibrils**

Mature α-syn amyloid fibrils were grown from recombinant monomeric α-syn by adding pre-formed seeds to the reaction. Briefly, monomeric α-syn, at concentrations ranging from 50 – 300 µM, was incubated in 50 mM phosphate buffer (pH 7.4) in the presence of 1% (w/w) pre-formed α-syn seeds (formed as described above). To monitor fibril growth, samples were incubated in the presence of 50 µM ThT using a black-walled, clear bottom 96-well microplate (Greiner Bio-One, Frickenhausen, Germany), with each well containing 100 µL of sample. Plates were incubated without agitation at 37°C in a FLUOStar Optima plate reader (BMG Labtechnologies, Melbourne, Australia), with the plate sealed to prevent evaporation. The ThT fluorescence was monitored via excitation and emission at 440 nm and 490 nm, respectively. Readings were taken every 300 s for a period of up to 48 h. Samples were considered to contain mature amyloid fibrils once the ThT fluorescence had reached a plateau.
Chapter 4: sHsps interact with mature α-synuclein fibrils

(typically after 24 h of incubation). Fibril samples were collected from the plate and stored at 4°C for use.

4.2.4 Analytical sucrose gradient centrifugation

Mature α-syn fibrils (formed as described above, Section 4.2.3) were prepared at a final concentration of 75 µM in 50 mM phosphate buffer containing 100 mM NaCl (pH 7.4). Fibrils were incubated at 37°C for 1 hr in a VorTemp shaking incubator (Labnet International, Edison, USA) with shaking at 60 rpm, in the absence or presence of Hsp27 or αB-c variants (15 µM). An aliquot was taken (load sample) prior to samples (100 µL) being layered on top of a 20% (w/v) sucrose solution (900 µL; Amresco, Solon, USA) prepared in 50 mM phosphate buffer containing 100 mM NaCl (pH 7.4). Following centrifugation at 200000 x g for 20 min at 4°C using an MTX150 ultracentrifuge (ThermoFischer Scientific, Waltham, USA), 100 µL fractions were collected and any pelleted material resuspended in the final 100 µL (top – fraction 1, bottom – fraction 10). SDS-PAGE analysis of the load, and sample fractions was performed using 15% polyacrylamide gels, which were visualised via staining with Coomassie Brilliant Blue R250.

4.2.5 Fluorescence fibril pelleting assay

In order to determine the binding affinity (Kd) of Hsp273D (i.e. a Hsp27 isoform containing mutations that mimic phosphorylation at residues S15, S78 and S82) for mature α-syn fibrils, a pelleting assay was developed. CF488-labelled Hsp273D (or the non-binding control protein α-lac) was incubated at concentrations ranging from 0 – 20 µM in the absence or presence of 25 µM α-syn fibrils. Samples were incubated in 50 mM phosphate buffer (pH 7.4) at 37°C for 1 h without shaking using an Accublock digital dry bath (Labnet International, Edison, USA), before being centrifuged at 20000 x g for 1 h at 4°C. The supernatant was collected, and the pellet washed with an equivalent volume of fresh 50 mM phosphate buffer. Samples were
again centrifuged at 20000 x g for 1 h at 4°C, and the supernatant was discarded. Pelleted material was then resuspended in an equivalent volume of fresh 50 mM phosphate buffer, and the absorbance at 495 nm ($A_{495\text{nm}}$) was determined in triplicate using a NanoDrop 2000c spectrophotometer (ThermoFischer Scientific, Waltham, USA). The concentration of bound labelled protein was then determined using a standard curve ranging from 0 – 20 µM. The concentration of bound Hsp27$_3D$ was correlated with the total amount of Hsp27$_3D$ added to the sample, and the data fitted with a one-site saturation model, such that the $K_d$ and $B_{max}$ could be determined.

4.2.6 Total internal reflection fluorescence (TIRF) microscopy

4.2.6.1 Microscope setup

TIRF microscopy, including super resolution imaging, of mature α-syn fibrils was performed using a custom designed microscope built by Dr. Mathew Horrocks. Briefly, an inverted microscope (Nikon Eclipse TI, Tokyo, Japan) was configured to operate in objective-type TIRF with three light sources, a 405 nm CW diode 200mW Obis laser (Coherent, Santa Clara, USA), a 514 nm solid-state 200 mW Sapphire laser (Coherent, Santa Clara, USA) and a 647 nm CW diode 200mW Obis laser (Coherent, Santa Clara, USA). The lasers were directed via dichroic mirrors through a high numerical aperture, 60X oil immersion CFI Apochromate objective lens (Nikon Eclipse TI, Tokyo, Japan) to the sample coverslip. The emitted fluorescence was collected and filtered through long-pass filters specific for each excitation (see Section 4.2.6.2) and finally projected onto an electron multiplied charged coupled device (EMCCD) camera (Evolve II 512, Photometrics, Tuson, AZ). For sPAINT imaging, a physical aperture (VA100/M, Thorlabs) and a transmission diffraction grating (300 Grooves/mm 8.6° Blaze Angle - GT13-03, Thorlabs) were mounted on the camera port path prior to the detector.
4.2.6.2 Sample preparation

To prepare slides for microscopy, glass coverslips were cleaned using an argon plasma cleaner (Diener Electronic, Ebhausen, Germany) for 1 h and frame-seal 99 mm slide chambers (Bio-Rad, Hercules, CA) were fixed to the cleaned surface. The glass surface within the chamber was coated with poly-L-lysine (0.01% w/v), and incubated for 30 min at room temperature. The chamber was then washed three times with filtered PBS, before the slide was transferred to the microscope stage and coupled to the lens using immersion oil (n=1.518, Olympus, Tokyo, Japan). Samples for microscopy were prepared in 50 mM phosphate buffer (pH 7.4) or PBS containing 50 µM α-syn and 1 µM fluorescently labelled Hsp27-WT or negative control proteins (α-lac or lysozyme). Samples were incubated for 5 min at room temperature, before being diluted 100-fold in the appropriate imaging buffer. Imaging buffer consisted of GLOX-mercaptoethylamine buffer (0.5 mg/mL glucose oxidase, 40 μg/mL catalase and 10% (v/v) glucose in 50 mM Tris-HCl (pH 8.0) with 10 mM NaCl and 10 mM β-mercaptoethylamine) supplemented with 5 µM ThT for SAVE imaging, or 100 nM nile red for sPAINT imaging (Bongiovanni et al. 2016). These buffers enable the detection of fibrillar α-syn (via ThT or nile red fluorescence), while also allowing the labelled Hsp27 (or control fluorescent proteins) to be imaged via the red fluorescence. Excitation of ThT at 405 nm allowed the detection of fluorescence using an ET488LP long pass filter (Semrock, Rochester, USA). Alternatively, nile red was excited at 514 nm and fluorescence collected using an ET590LP long-pass filter (Semrock, Rochester, USA). In both cases, CF647-labelled Hsp27 or control proteins were imaged via excitation at 647 nm and the emitted fluorescence was detected using an ET655LP long-pass filter (Semrock, Rochester, USA). Each channel was imaged sequentially such that there was no spectral overlap in detected fluorescence.
4.2.6.3 Image processing

Data were analysed to generate sPAINT images as described previously (Bongiovanni et al. 2016) using the Genome Damage and Stability Centre Single Molecule Localisation Microscopy (GDSC SMLM) ImageJ Plugin (University of Sussex, Brighton, UK), using a typical ‘signal strength’ threshold of ~30. Quantitative co-localisation analyses were performed using IgorPro v 6.3.4.1 using custom scripts (Appendix II) written by Dr. Daniel Whiten, and wavelength analyses were completed using custom scripts written for Python 2.7 (Appendix III).

4.2.7 Dihydroethidium (DHE) assay

A dihydroethidium (DHE) cell-based method, similar to that described previously (Cremades et al. 2012, Zhang and Soldati 2013), was used to investigate the toxicity of α-syn fibrils when added exogenously to cells. N2a cells were routinely cultured as described in Section 2.7.1. For use, cells were seeded into 8-well chamber slides (Ibidi, Martinsried, Germany) and cultured to 60% confluency. Treatments containing monomeric or fibrillar α-syn (to a final concentration of 20 µM) were prepared in PBS in the absence or presence of sHsp (or the control proteins BSA or α-lac) at a 1:100 molar ratio (sHsp:α-syn) and incubated at 37°C for 30 min. Immediately prior to use, DHE (to a final concentration of 2 µM) was also added to these treatments to exclude effects due to dilution of the DHE.

Prior to addition of the samples, cells were washed with PBS and then DHE (2 µM in PBS) was added. Images were taken every 30 s for 15 min using an epifluorescence microscope to quantify the amount of DHE (excitation 325 – 375 nm and emission 435 – 485 nm) and its oxidised product (i.e. ethidium, excitation 540 – 580 nm and emission 592 – 668 nm) as a measure of basal ROS production (Zhang and Soldati 2013). Treatments were then added to cells, and images were taken every 30 s for a further 15 min. Data analysis was completed.
using custom programs written for Python 2.7 (Appendix IV) and, where appropriate, data fitted with linear regression. Briefly, the change in fluorescence, corresponding to the conversion between DHE and its oxidised product ethidium, was monitored for 20 cells and the ratio of DHE fluorescence to ethidium fluorescence calculated for each of these cells. The difference in the rate of change of the DHE/ethidium fluorescence ratio before and following treatment was then determined, and normalised to the PBS control. Statistical analysis was performed using a one-way analysis of variance followed by a Bonferroni’s multiple comparison test.

4.2.8 Analytical ultracentrifugation (AUC) of fibrils

Absorbance-detected AUC was performed as previously described (Binger et al. 2013). Briefly, the maximum concentration of each protein in the experiment was calculated such that the absorbance at 260 or 280 nm was within the optimal detection range of the instrument. Samples (380 µL) containing α-syn fibrils (25 µM) in the absence or presence of αB-c or Hsp27 (5 µM) were prepared at room temperature and incubated for 1 h before being loaded into a 12 mm double-sector epon-filled centrepiece, alongside the relevant reference solution (400 µL). Samples were centrifuged in an XL-I analytical ultracentrifuge (Beckman Coulter, CA, USA) at 3000 rpm using either a Ti60 or Ti50 rotor at 20°C. Radial absorbance scans were collected at 280 nm at 6 min intervals with a radial step size of 0.003 cm for a total of 10 h. Sedimentation velocity (SV) profiles were analysed with SEDFIT software using the least-squares (ls) boundary model, ls-g*(s) (Schuck 2000, Schuck and Rossmanith 2000). Regularisation by second derivative was used to obtain the apparent (g*(s)) distribution (Schuck 2000, Schuck and Rossmanith 2000). The S_{av} of each sample was then determined by integration of the distributions. Samples were again analysed via AUC as described above, following incubation at room temperature for 48 h without shaking.
4.3 Results

4.3.1 Hsp27 inhibits the growth and structural conversion of α-syn oligomers during early aggregation

To investigate the interaction of Hsp27 with intermediates formed during the early stages of α-syn aggregation, smFRET was employed (Cremades et al. 2012). This enables fluorescently labelled oligomeric species, which typically represent <1% of the total α-syn species present in solution, to be detected and characterised with regards to their size and relative structure. Incubation of α-syn alone resulted in an increase in the total number of oligomeric species over time, which was inhibited by the presence of Hsp27 at a 1:10 molar ratio (Hsp27:α-syn) (Figure 4.1A). When considering the proportion of events corresponding to small (2-6 monomeric subunits) α-syn oligomers, Hsp27 inhibited the formation of these small oligomers but did not completely prevent their formation (Figure 4.1B). The growth of these small oligomers can be monitored by measuring the proportion of medium sized (7 – 300 monomeric equivalent) α-syn oligomers formed over time. In addition, the conversion of medium sized oligomers from a disordered to more ordered, compact species can also be monitored via the relative FRET efficiency of the medium sized oligomers (Cremades et al. 2012). Analysis of the medium sized α-syn oligomer population revealed that, in the absence of Hsp27, there was an accumulation of medium sized, low FRET oligomers over time followed by an increase in the proportion of medium sized high FRET population in solution (Figure 4.1C, D). This is consistent with previous observations, supporting the structural conversion of α-syn oligomers during aggregation to more structured, compact oligomers over time (Cremades et al. 2012). Hsp27 prevented the growth of small oligomers into medium sized oligomers, such that there was negligible appearance of medium oligomers of either low or high FRET efficiency when Hsp27 was present (Figure 4.1C, D). This may be
reflective of Hsp27 interacting either with monomeric subunits to prevent their addition to growing oligomeric nuclei, or directly with the small α-syn oligomers to prevent monomer addition.

**Figure 4.1: Hsp27 inhibits the growth of oligomers formed during α-syn aggregation.** An equimolar mix of AF488- and AF647-labelled α-synA90C was prepared to a total concentration of 70 µM in PBS (pH 7.4), in the absence (blue) or presence of Hsp27 (7 µM, orange). Samples were incubated for up to 32 h at 37°C. Aliquots were analysed via smFRET to determine (A) the proportion of events that corresponded to oligomers. The fraction of these events was normalised to time 0, and those events corresponding to (B) small α-syn oligomers (2 – 6 monomer equivalents) and (C and D) medium α-syn oligomers (7 – 300 monomer equivalents) were determined. For those events corresponding to medium α-syn oligomers (7 – 300 monomer equivalents), the population was separated into those exhibiting (C) low and (D) high FRET efficiencies, corresponding to lower and higher degrees of structural compactness respectively. Data is presented as mean ± S.E.M. of triplicate samples, and is representative of three biological repeats.

**4.3.2 Hsp27 inhibits the elongation of α-syn fibril fragments**

Seeded aggregation assays were employed to enable direct analysis of the elongation rate of small α-syn amyloid fibril fragments (seeds). Under these conditions, aggregation occurs primarily through monomer addition at fibril ends, while primary nucleation and secondary fibril amplification processes do not contribute significantly to the observed aggregation kinetics (Buell et al. 2014). For example, incubation of monomeric α-syn in the presence of 5% (w/w) fibril seeds does not produce an observable lag phase typical of non-seeded aggregation (in which primary nucleation occurs), and instead is best described by a one-phase association model (Figure 4.2A). Elongation of these α-syn fibril seeds is inhibited by the addition of Hsp27 (Figure 4.2A), and this effect is specific to the chaperone as there is no inhibition when a non-chaperone control protein (BSA) is used at the same concentration.
Figure 4.2: Hsp27 inhibits the elongation of α-syn fibril fragments. (A) Recombinant monomeric α-syn (50 µM) was incubated in 50 mM phosphate buffer and 0.01% NaN₃ (pH 7.4) in the absence or presence of 50 µM Hsp27 or the control protein BSA. After equilibration at 37°C, α-syn seeds (5% (w/w)) were added to each sample containing monomeric α-syn and the elongation of these seeds monitored via the change in ThT fluorescence at 490 nm over time. Data shown are representative of two independent repeats. (B-E) Recombinant monomeric α-syn was incubated in 50 mM phosphate buffer (pH 7.4) at concentrations ranging from 10 – 100 µM in the (B, C) absence or (D, E) presence of 50 µM Hsp27. After equilibration at 37°C, α-syn seeds (5% (w/w)) were added to each sample and the elongation of these seeds monitored via the change in ThT fluorescence at 490 nm over time. The data from 1 – 3 h (red dotted box) of incubation were (C, E) fit to a linear regression. (F) The rate of elongation of the α-syn seeds, in the absence and presence of Hsp27, was calculated using values from these fits and correlated with the monomeric α-syn concentration. Data for α-syn alone were fit to a Michaelis-Menten non-linear regression model. Data in B – E are representative of four independent repeats, and data in F is presented as mean ± S.E.M. of these four repeats.

Moreover, there is no increase in ThT fluorescence in the absence of α-syn (i.e. in samples that contain Hsp27, BSA or buffer alone) (Figure 4.2A). Importantly, when the concentration of monomeric α-syn is increased in the presence of a constant ratio of seeds (i.e. 5% (w/w)), the rate of elongation and maximal fibril formation increases (Figure 4.2B, D). By considering the linear elongation phase of the reaction (in this case the time between 1 – 3 h; Figure 4.2C, E), the relationship between elongation rate and the concentration of monomeric α-syn is best described by a Michaelis-Menten model. In this case, elongation is predicted to reach a half maximal rate at approximately 300 µM monomeric α-syn (Figure 4.2F). In the presence of Hsp27 (50 µM; Figure 4.2D, E), there is no significant increase in the rate of elongation at low concentrations of monomeric α-syn (up to 50 µM) (Figure 4.2D). However, when the concentration of Hsp27 is held constant (50 µM) and the concentration of
monomeric α-syn is increased (to 75 and 100 µM), such that the molar ratio of monomeric α-syn:Hsp27 exceeds 1:1, the rate of α-syn aggregation increases (Figure 4.2F). Importantly, the elongation rate in the presence of Hsp27 is no longer able to be fitted to a Michaelis-Menten model. Together, these data suggest that the interaction between Hsp27 and monomeric α-syn is elongation-rate dependent. These findings may also reflect an interaction between Hsp27 and the fibrillar seeds of α-syn, whereby Hsp27 is in direct competition with monomeric α-syn for access to fibril ends, thus inhibiting elongation of the fibril seeds. As the concentration of monomeric α-syn increases, it may out-compete Hsp27 leading to the observed increase in the rate of elongation.

To further explore these possibilities, the ratio of α-syn fibril seeds to monomeric α-syn was varied and Hsp27 added to the samples at a range of concentrations (Figure 4.3). As expected, incubation of monomeric α-syn in the presence of 2.5% (w/w) α-syn seeds resulted in a characteristic aggregation profile without an observable lag phase (Figure 4.3A). Addition of Hsp27 inhibited this aggregation, such that there was a concentration-dependent decrease in the rate and maximal amount of α-syn aggregation as the concentration of Hsp27 was increased (Figure 4.3A and 2B). By repeating this experiment using different concentrations of α-syn fibril seeds (from 1%-10% (w/w) of the monomeric α-syn concentration, Appendix V) the relationship between the elongation rate and concentration of Hsp27 was established (Figure 4.3C). This relationship is best described by a one-phase exponential decay curve (Figure 4.3C), whereby, the rate constant from this fit provides a measure of the ability of Hsp27 to prevent fibril elongation at each ratio of seed:monomeric α-syn (Figure 4.3D). There was a strong negative exponential correlation between the concentration of seed and the ability of Hsp27 to prevent fibril elongation, i.e. as the percentage of α-syn seed in a sample increased, Hsp27 was less effective at inhibiting fibril elongation. Together, these data therefore support a stable interaction between Hsp27 and
fibrillar α-syn. Importantly, binding of Hsp27 to fibrillar α-syn is in stark contrast to the transient interaction seen between Hsp27 and monomeric α-syn (Chapter 3).

Figure 4.3: Hsp27 interacts with α-syn fibril fragments. Recombinant monomeric α-syn was incubated in 50 mM phosphate buffer and 0.01% NaN₃ (pH 7.4) at 50 μM in the absence or presence of Hsp27 at concentrations ranging from 0.1 – 10 μM. After equilibration at 37°C, α-syn seeds were added at concentrations ranging from 0.5 – 5 μM (i.e. 1%-10% (w/w) when expressed as a percentage of the soluble protein concentration) and elongation monitored via the change in ThT fluorescence at 490 nm over time. (A) A representative trace is shown in the presence of 5% seed, and the linear portion (0 – 2.5 h, indicated by red dotted box) was (B) fit to a linear regression curve. (C) The rate of elongation was calculated using values from this fit, which was then normalised to the α-syn alone sample and correlated with Hsp27 concentration at each seed ratio. These data were then fit with one phase exponential decay. (D) Parameters of this fit were used to compare the chaperone efficacy of Hsp27 at each seed ratio, and the data fit to a one phase exponential decay. Data in A – C is representative of at least three independent experiments, and data in D is reported as mean ± S.E.M. of these independent repeats.

4.3.3 sHsps forms a stable complex with mature α-syn fibrils and this is mediated by the N- and/or C-termini

Although the interaction between fibrillar α-syn and Hsp27 significantly impacts the rate of α-syn aggregation in vitro, the mechanism by which Hsp27 interacts with fibrillar α-syn remained to be established. In addition, whilst αB-c has been shown to bind α-syn fibrils (Waudby et al. 2010), the regions responsible for this interaction had not been determined. Sucrose centrifugation assays were therefore employed to investigate the ability of both Hsp27 and αB-c to form stable complexes with α-syn fibrils (Figure 4.4). When non-fibrillar
(monomeric) α-syn was applied to the top of the sucrose gradient it was retained in the upper fractions (fraction 1-3). In contrast, fibrillar α-syn sedimented through the sucrose gradient and was detected in the bottom fraction (fraction 10) (Figure 4.4A). When Hsp27 or αB-c was incubated alone, they did not sediment, and were localised in fractions 1-5 from the sucrose gradient consistent with the large, polydisperse and oligomeric nature of these sHsps (Figure 4.4B, D) (Haley et al. 1998, Jovcevski et al. 2015). However, pre-incubation of Hsp27 and αB-c with mature α-syn fibrils resulted in these sHsps co-sedimenting with the fibrils and therefore being detected in the bottom fraction from the sucrose gradient (Figure 4.4C, E).

To gain further insight into the mechanism by which these sHsps form complexes with mature α-syn fibrils, core domain (i.e. sHsps forms which lack the flanking N- and C-terminal domains, Hsp27\textit{core} and αB-c\textit{core}) and a phosphomimicking variant (Hsp27\textit{3D}) of the sHsps were used in this assay. These variants were chosen as they only form dimers and monomers under these experimental conditions rather than large polydisperse oligomers such as those formed by WT αB-c and Hsp27 (Hochberg et al. 2014, Jovcevski et al. 2015). Thus, these sHsp variants enabled us to test whether only large oligomeric forms of sHsp are capable of binding to α-syn fibrils. Since αB-c\textit{3D} does not form small oligomeric species (Ecroyd et al. 2007) it was not used in these experiments. Importantly, the core domain and Hsp27\textit{3D} variants have all previously been shown to retain the ability to prevent monomeric α-syn aggregation and therefore are chaperone active (Chapter 3, Appendix I) (Hochberg et al. 2014, Jovcevski et al. 2015). Consistent with them existing as monomers and dimers under these experimental conditions, Hsp27\textit{3D} (Figure 4.4F), Hsp27\textit{core} (Figure 4.4H) and αB-c\textit{core} (Figure 4.4J) were found in fractions 1-4 from the sucrose gradient when incubated alone. Incubation of Hsp27\textit{3D} with α-syn fibrils resulted in the Hsp27\textit{3D} co-sedimenting with the fibrils, such that it was detected in the bottom fraction from the sucrose gradient (Figure
However, when Hsp27\textsubscript{core} and αB-c\textsubscript{core} were incubated with mature α-syn fibrils, they did not co-sediment with the fibrils (Figure 4.4I, K). Together these data demonstrate that the small oligomeric forms of sHsps can bind to α-syn fibrils, however, binding requires the N- and/or C-terminal regions of these sHsps.

\textbf{Figure 4.4: sHsps bind to α-syn fibrils and this is mediated by the N- and/or C-termini.} α-Syn fibrils (75 µM) were incubated in 50 mM phosphate buffer and 0.01% NaN\textsubscript{3} (pH 7.4) in the (A) absence or presence of (C, E) WT, (G) phosphomimicking and (I, K) core domain isoforms of Hsp27 (C, G and I) or αB-c (E and K). The sHsp isoforms were present at a final concentration of 15 µM. Hsp27 and αB-c (B, D) WT, (F) phosphomimicking and (H, J) core domain isoforms, incubated in the absence of α-syn fibrils, are also included for comparison. Samples were incubated for 1 h at 37°C before being layered on top of a 20% (w/v) sucrose cushion and centrifuged at 200000 \textit{x} g for 20 minutes. Sequential fractions were collected from the top of the cushion and analysed via SDS-PAGE.

In order to determine the binding affinity of Hsp27 for α-syn fibrils, a fluorescence binding assay was developed. In this instance, Hsp27\textsubscript{3D} was selected as a representative sHsp, as it exists primarily as a dimer and does not sediment independently under the conditions used. The presence of a cysteine at position 137 of Hsp27 enabled site-specific labelling of
Hsp27\textsubscript{3D}, such that a maximum of one fluorophore can be conjugated to each Hsp27\textsubscript{3D} monomer. Incubation of a range of concentrations of Hsp27\textsubscript{3D} with mature α-syn fibrils resulted in a saturable binding curve, which was not evident when the non-chaperone control protein α-lac was incubated with α-syn fibrils (Figure 4.5). Fitting of the data revealed a $K_d$ and $B_{\text{max}}$ for the binding of Hsp27\textsubscript{3D} to α-syn fibrils of $2.7 \pm 1.9$ µM and $5.4 \pm 1.1$ µM respectively. Given the concentration of α-syn utilised here (25 µM monomer equivalent), this $B_{\text{max}}$ corresponds to approximately 1 molecule of Hsp27\textsubscript{3D} for every 5 α-syn monomeric units at saturation.

**Figure 4.5: Hsp27 binds mature α-syn fibrils with µM affinity.** Mature α-syn fibrils (25 µM) were prepared in 50 mM phosphate buffer and 0.01% NaN\textsubscript{3} in the absence or presence of a range of concentrations of fluorescently labelled Hsp27\textsubscript{3D} or the non-binding control protein α-lac. After incubation at 37°C for 1 h, samples were centrifuged and washed to remove unbound protein. The concentration of bound Hsp27\textsubscript{3D} (or α-lac) was determined by correlating the absorbance at 495 nm with a known concentration of labelled protein. The concentration of bound Hsp27\textsubscript{3D} was correlated with the total concentration of Hsp27\textsubscript{3D} added to the sample, and fit with a one-site saturation model to enable the $K_d$ and $B_{\text{max}}$ to be calculated. Data is displayed as mean ± S.E.M., where n=3 and n=2 for Hsp27\textsubscript{3D} and α-lac respectively.

**4.3.4 Binding of Hsp27 to α-syn fibrils occurs along the surface, leading to a decrease in hydrophobicity**

Given the stable association of Hsp27 with α-syn fibrils, we sought to visualise this interaction directly using TIRF microscopy. In this instance, fibrils were detected using SAVE imaging, which works by staining the aggregates with the amyloid specific dye ThT.
(Figure 4.6A; α-syn) (Bongiovanni et al. 2016), while CF647-labelled Hsp27 was imaged via its red fluorescence (Figure 4.6A; Hsp27). The merged image (Figure 4.6A) demonstrates the association of the labelled Hsp27 with α-syn fibrils. Hsp27 was seen to be distributed along the fibril surface. There was a significant increase in the co-incidence between ThT-reactive and red-fluorescent localisations when fibrils were co-incubated with Hsp27 (65 ± 6%) compared to the control (non-chaperone) proteins α-lac (20 ± 7%) or lysozyme (25 ± 0.4%), providing further evidence that the binding of Hsp27 to α-syn fibrils is the result of a specific interaction.

**Figure 4.6: Hsp27 binds along the surface of α-syn fibrils.** α-Syn fibrils (50 µM) were incubated in the presence of 1 µM CF647-labelled Hsp27, α-lac or lysozyme for 30 min at room temperature. Samples were incubated in PBS containing 50 µM ThT to allow α-syn fibrils to be visualised and imaged via TIRF microscopy. (A) TIRF images are shown for α-syn fibrils stained with ThT (top left panel) in the presence of Hsp27 (bottom left panel), along with the corresponding merge of these two images (right panel). Scale bars represent 5 µm. (B) The percentage of red fluorescent pixels coincident with ThT-reactive pixels was quantified. Data are mean ± S.E.M. (n=9 images) and were analysed via a one-way ANOVA with a Bonferroni post-hoc test (*** denotes p<0.01). Data are representative of two separate experiments.

In an attempt to further characterise the nature of the interaction between Hsp27 and α-syn fibrils, the recently described TIRF-based imaging technique sPAINT (Bongiovanni et al. 2016) was used. This technique relies on the use of nile red in solution to image the fibrils in place of ThT. Importantly, when nile red interacts with amyloid, it undergoes a characteristic
shift in its emission wavelength according to the hydrophobicity of its surrounding
environment (Greenspan and Fowler 1985, Bongiovanni et al. 2016). Thus, sPAINT enables
regions of hydrophobicity along the face of fibrils to be mapped at super-resolution (Figure
4.7A). The addition of fluorescently labelled Hsp27, which was imaged below the
diffraction-limit using STORM (Rust et al. 2006), allows the association of the chaperone
with the fibrils to be mapped at the nanometer level (Figure 4.7B), and correlated with
regions of higher or lower hydrophobicity along the fibril surface (Figure 4.7C). The
emission wavelengths of the nile red bound to α-syn fibrils recorded for each localisation in
the absence or presence of Hsp27 were grouped into regions of low (550 - 600 nm), medium
(600 - 650 nm) or high (650 - 700 nm) hydrophobicity. Notably, binding of Hsp27 to α-syn
fibrils significantly affected the emission wavelength of the nile red associated with the fibrils
(F(5, 27) = 1255, p<0.0001). The presence of Hsp27 significantly decreased the proportion of
localisations corresponding to high levels of hydrophobicity (i.e. 650 – 700 nm; p<0.0001) and
significantly increased the proportion of localisations corresponding to medium levels of
hydrophobicity (i.e. 600 – 650 nm; p<0.0001) (Figure 4.7D).
Figure 4.7: Hsp27 decreases the relative hydrophobicity at the surface of α-syn fibrils. α-Syn fibrils (50 µM) were incubated in the absence or presence of CF647-labelled Hsp27 in GLOX buffer containing 100 nM nile red, to allow α-syn fibrils to be visualised and imaged via TIRF microscopy. (A) An example super-resolution image is shown for α-syn, with localisations coloured according to the wavelength of emission of the nile red. (B) The labelled Hsp27 is coloured according to pixel intensity. (C) In the corresponding merged image, the nile red localisations have been binned into low, medium and high levels of hydrophobicity according to their wavelength of emission (colour scale given at the bottom of the figure). (D) The percentage of localisations in each wavelength range in the absence and presence of Hsp27 was quantified and data are displayed as the mean ± S.E.M. (n=6). Scale bars represent 2 μm, or 1 μm in inset. Data were analysed via a student’s t-test, where *** denotes a significant (p<0.001) difference between group means. Data are representative of two separate experiments.

4.3.5 sHsps protect against the cellular toxicity of exogenous α-syn fibrils

To establish whether, by binding to α-syn fibrils, αB-c and Hsp27 decrease fibril-associated cytotoxicity, a DHE assay was developed. The dye DHE has been used extensively to monitor the intracellular production of ROS, specifically superoxide (Zhao et al. 2003, Wang et al. 2013, Zhang and Soldati 2013), as a measure of cellular toxicity. Therefore, this was chosen as a method to measure the cellular toxicity of α-syn fibrils when added exogenously to cells. Traces of the fluorescence ratio before and after treatment with monomeric or
fibrillar α-syn demonstrated that there is an increase in the ratio of reduced to oxidised DHE upon introduction of α-syn, indicative of ROS being produced by the cells (Figure 4.8A, B). The difference in the rate of change in the fluorescence ratio (reported as a fold change relative to the PBS control) is a measure of relative cytotoxicity, i.e. a faster rate of change in the fluorescence ratio is due to higher rates of ROS production associated with increased toxicity. There was a significant effect of treatment on the generation of ROS within cells (F(7,247) = 33.96, p<0.001). Post-hoc tests revealed mature α-syn fibrils induced significantly more ROS production than monomeric α-syn (p<0.0001) (Figure 4.8F). However, when the α-syn fibrils were pre-incubated with αB-c, ROS production was decreased by 50 ± 4% when compared to α-syn fibrils alone (Figure 4.8D). Similarly, when the α-syn fibrils were pre-incubated with Hsp27 there was a dramatic decrease (by 65 ± 7%) in ROS production compared to when fibrils alone were added to cells (Figure 4.8D). Importantly, pre-incubating α-syn fibrils with the non-chaperone control proteins BSA or α-lac did not significantly decrease the production of ROS by cells. Thus, the cytoprotective effect was specific to the sHsps binding to the α-syn fibrils. Also, incubation of the chaperone or control proteins in the absence of α-syn fibrils did not significantly alter basal levels of ROS production.
Figure 4.8: sHsps reduce the generation of ROS by N2a cells exposed to exogenous α-syn fibrils. N2a cells were incubated in PBS containing DHE (2 µM) before addition of monomeric (Mono) or fibrillar α-syn (20 µM) that had been pre-incubated in the absence or presence of αB-c or Hsp27 (or the control proteins BSA and α-lac; each at 2 µM). The ratio of oxidised to reduced DHE before (blue) and after (red) treatment was determined for 20 cells per treatment. Example traces are shown for an individual cell treated with (A) monomeric α-syn, (B) fibrillar α-syn, and fibrillar α-syn preincubated with (C) BSA, (D) αB-c or (E) Hsp27. The fluorescence ratio before and after treatment was fit by linear regression and the change in the rate of ROS production due to treatment was calculated as the difference in the gradient of the fitted lines. (F) The fold change in ROS production was then determined relative to a buffer only control (dashed line) and is reported as mean ± S.E.M. (n=3 biological repeats, with each repeat an average of 20 cells). Data were analysed via one-way ANOVA with a Bonferroni’s multiple comparison post-hoc test, where *** denotes a significant (p<0.001) difference compared to the fibril alone sample.
4.3.6 Binding of sHsps promotes dissociation of mature α-syn fibrils

In order to investigate how sHsps bound to α-syn fibrils may influence the size distribution of the fibrils, absorbance AUC was employed. Sedimentation velocity profiles are shown for α-syn in the absence and presence of αB-c or Hsp27 (Figure 4.9A-C). Fitting these profiles with the least-squares (ls) boundary model, ls-g*(s), allows the g*(s) distributions to be calculated (Figure 4.9D). In the absence of sHsps, the sedimentation coefficient distribution reveals a large range of species, reflective of fibrils with a broad size distribution (Figure 4.9D). In the presence of αB-c and Hsp27, the maximum sedimentation coefficients decrease by approximately 40% and 60% respectively (Figure 4.9D). Following integration of these distributions it is evident that the presence of chaperone increases the abundance of small (1 – 4000 S) species (Figure 4.9E), and this is accompanied by a corresponding decrease in the proportion of large (4000 – 100000 S) species in solution. This difference was noted in both the 0 h and 48 h distributions. Importantly, this apparent change in the size distribution of α-syn fibrils when bound by αB-c or Hsp27, is also reflected by the S_av values (Figure 4.9F). Thus, in the presence of αB-c, the S_av of α-syn fibrils decreased by 27% when compared to α-syn fibrils alone. Similarly, the presence of Hsp27 resulted in a 47% decrease in the S_av. Similar results are also evident at the 48 h time point (Figure 4.9F). Furthermore, incubation of α-syn fibrils alone resulted in an increase in the S_av over 48 h (Figure 4.9F), indicating the species present in solution increase in size over this time. In contrast, the presence of chaperone resulted in decreases in the S_av over this time frame, as the S_av values of α-syn fibrils decreased by 9% and 21% when incubated with αB-c or Hsp27 respectively (Figure 4.9F). This indicates that, when sHsps were present, the species in solution decreased in size over the 48 h of incubation. Therefore, the presence of αB-c and Hsp27 prevents any increase in the S_av of α-syn fibrils which would otherwise occur over this period. This may be reflective of sHsps preventing ‘clumping’ of fibrils together (e.g. by coating the fibril
surface), or influencing the monomer-oligomer-fibril equilibrium such that the size of fibrils themselves decreases in the presence of the sHsps.

Figure 4.9: sHsps decrease the apparent size of mature α-syn fibrils. Mature α-syn fibrils (µM) were prepared in 50 mM phosphate buffer (pH 7.4), in the absence or presence of αB-c or Hsp27 (µM). Samples were incubated for 48 h at room temperature, with aliquots being analysed via absorbance-based AUC after 0 h and 48 h. Sedimentation velocity profiles of (A) α-syn fibrils alone, or α-syn fibrils in the presence of (B) αB-c or (C) Hsp27 are shown, with radial scans collected at 6 min intervals. Data is presented for every 10th scan overlaid with theoretical fits to the c(S) model generated by SEDFIT. (D) Continuous g*(s) distributions for all samples were obtained from the raw data using SEDFIT. (E) The percentage of species having low (1 – 4000) and high (4000 – 100000) S values was determined by integration of D over the ranges indicated. (F) The S_{av} was determined by integration of D over the whole range. Representative data is shown in A-E following 48 h incubation.

To further investigate these possibilities, smFRET was employed. Given this technique is specialised to detect oligomers, it was utilised to monitor the dissociation of fibrils in the absence and presence of bound Hsp27 or αB-c during prolonged incubation (i.e. once residual monomeric and oligomeric α-syn had been removed by washing). When incubated alone, the liberation of oligomers declined for up to 13 days (Figure 4.10). The presence of αB-c or Hsp27 increased the proportion of oligomeric events detected, relative to α-syn incubated alone (Figure 4.10A, B). This suggests that, by binding fibrils, both αB-c and Hsp27 promote their dissociation into smaller oligomeric species.
Figure 4.10: sHsps promote the dissociation of α-syn fibrils into smaller oligomers. An equimolar mix of AF488- and AF647-labelled α-synA90C was prepared to a total concentration of 70 µM in PBS (pH 7.4), and incubated for up to 7 days at 37°C. Mature fibrils were then washed, and resuspended in PBS in the absence or presence of (A) αB-c or (B) Hsp27 (7 µM). Aliquots were analysed via smFRET to determine the fraction of events that corresponded to oligomers. Data is presented as mean ± S.E.M. of triplicate samples.
4.4 Discussion

Given small oligomeric species formed during amyloid fibril aggregation are considered the most toxic to neurons (Kayed et al. 2003, Glabe 2008, Winner et al. 2011), primary and secondary nucleation processes become a crucial element to understanding the pathogenesis of aggregation-prone α-syn. Although the soluble oligomer model of amyloid cytotoxicity suggests that mature fibrils are a relatively inert end product, it is important to note that fibrils themselves can be a significant source of these small cytotoxic species, through secondary nucleation and fibril fragmentation. In contrast to the transient interaction between the sHsps and monomeric α-syn (Chapter 3), the data presented in this chapter demonstrates that the sHsps are able to stably interact with oligomeric and fibrillar forms of α-syn to prevent further aggregation and mitigate fibril-associated cytotoxicity.

Hsp27 was able to inhibit, but not prevent, the formation of small soluble α-syn oligomers when present prior to aggregation being initiated in a population of soluble, monomeric α-syn. This suggests small oligomers, which were not detected via ThT fluorescence in Chapter 3, accumulate even in the presence of Hsp27 and represent a small proportion of α-syn species present. However, the results presented here demonstrate Hsp27 prevented the growth of these small oligomers into larger aggregates, and therefore prevents the accumulation of oligomers consisting primarily of β-sheet (i.e. high FRET oligomers). It remains unclear whether this effect is primarily mediated by an interaction with monomeric α-syn (to prevent its addition to growing oligomers), or with the small oligomers themselves (to occlude sites of monomer addition). However, it suggests a role for Hsp27 in protecting against soluble α-syn oligomers, given these small, β-sheet-rich oligomers are thought to exert intracellular toxicity (see Section 1.2.5) (Cremades et al. 2012).
Another source of small aggregates during α-syn aggregation is the fragmentation of fibrils. Incorporating these fragments in bulk aggregation assays allows their elongation to be considered, free from the confounding effects of primary nucleation due to the association of monomers. In this case, increases in the elongation rate associated with increasing monomer concentration are consistent with previous studies completed in the absence of chaperone (Buell et al. 2014). The results presented here support those of Chapter 3 by showing that the ability of Hsp27 to prevent the aggregation of monomeric α-syn is dependent on the rate of aggregation. The elongation of α-syn seeds is completely inhibited by Hsp27 at low concentrations of α-syn monomer, however, when the molar ratio of monomeric α-syn:Hsp27 exceeds 1:1, some aggregation occurs. The finding that Hsp27 inhibits the growth of the α-syn seed fibrils (significantly decreasing the rate of elongation) are comparable to those of earlier work showing that αB-c inhibits further aggregation of α-syn when introduced during the elongation phase of fibril growth (Rekas et al. 2007). The α-syn fibril seeds used in this case likely represent physiologically relevant products of fibril fragmentation that have a range of potentially toxic effects in the cell.

Fragmentation decreases the length of individual fibrils, which may increase their membrane permeability or cellular uptake (Jakhria et al. 2014). The surface area of fibrils is also increased by fragmentation as the fibril face is exposed when fibril-fibril associations are disrupted. Thus, fragmentation increases the potential for deleterious interactions with cellular components (Xue et al. 2009). Fragmentation also increases the number of fibrillar particles, resulting in an increase in the likelihood of particle-dependent interactions such as lipid-associated depolymerisation (Martins et al. 2008). Finally, fragmentation increases the total surface area of fibril ends available to solution, which can act as seeds for further fibril extension and thereby enhance the rate of amyloid deposition (Shvadchak et al. 2015), or alternatively drive the dissociation of cytotoxic species that exist in a dynamic equilibrium.
with exposed fibril ends (Carulla et al. 2005). In this way, not only does fragmentation have the potential to drive amyloid production, but also the ability to alter the physical properties of the fibrils themselves. Therefore, fragmentation has the capacity to enhance the cytotoxicity of so-called inert fibrillar material. Given this, the ability of these sHsps to prevent the elongation of fibril fragments suggests a potential protective role for them in cells when fibrils have formed and are prone to fragmentation.

The rate of elongation of α-syn fibrils was also dependent on the amount of seeds added, such that increases in seed concentration resulted in increases in the apparent elongation rate (Buell et al. 2014). At each concentration of monomeric α-syn, when the concentration of α-syn fibril seed added was increased, more Hsp27 was also required to completely inhibit elongation. Thus, the efficacy of Hsp27 to inhibit elongation (as measured by the rate constant of the elongation process) was inversely correlated to the seed concentration. This suggests that there is a strong interaction between Hsp27 and the α-syn fibril seeds. Together, this implies that the ability of Hsp27 to inhibit fibril elongation may result from a combination of transient interactions with monomeric α-syn (to prevent association and addition to fibril ends) and interactions with fibrillar material (essentially outcompeting monomeric α-syn for access to the fibril ends). The overall outcome is the inhibition of elongation of α-syn fibrils, and suggests that Hsp27 (and possibly other sHsps) can prevent further fibril formation of α-syn even after aggregation has commenced. This highlights the potential to target sHsps levels and/or activity in cells as a means of halting both the onset and progression of α-syn aggregation associated with the α-synucleinopathies.

The mechanism by which sHsps interact with α-syn fibrils was also characterised. The results presented here demonstrate that both αB-c and Hsp27 form a stable complex with α-syn fibrils. Moreover, an Hsp27 phosphomimicking variant (Hsp27_{3D}) that forms dimers and
monomers in solution (not large oligomers) was shown to be capable of binding to α-syn fibrils, however, the core-domain variants of αB-c or Hsp27 do not. Thus, the sHsps do not need to be large oligomers in order to bind to α-syn fibrils. However, the inability of αB-c\textsubscript{core} and Hsp27\textsubscript{core} to bind to α-syn fibrils demonstrates that binding is mediated by the N- and/or C-terminal regions of the protein. Given that these core domains are still chaperone active (Hochberg et al. 2014, Cox et al. 2016) (Appendix I), these data suggest that the sHsp domains play distinct functional roles with regards to interactions with aggregation-prone proteins. The central α-crystallin domain interacts with monomeric proteins to prevent them entering off-folding pathways leading to aggregation (Hochberg et al. 2014, Cox et al. 2016), whilst the flexible N- and/or C-terminal regions mediate stable interactions with aggregated (fibrillar) protein (Mainz 2015).

The affinity of the binding interaction between sHsps and α-syn fibrils was probed using Hsp27\textsubscript{3D}, and found to be in the micromolar range, consistent with previous estimates of the affinity of αB-c for apoC-II and Aβ fibrils (5µM and 2µM respectively) (Shammas et al. 2011, Binger et al. 2013). In addition, the binding interaction was saturated at substoichiometric molar ratios of Hsp27, which is also consistent with previous investigations of αB-c binding to apoC-II fibrils (Shammas et al. 2011, Binger et al. 2013). Together, the data presented here suggests that the ability to bind to fibrils is a generic property of the sHsps, independent of the protein from which the fibrils are initially formed.

Using TIRF microscopy, it was possible to directly visualize the interaction between Hsp27 and α-syn fibrils. It was observed that Hsp27 binds along the surface of fibrils in a similar manner as described for αB-c (Waudby et al. 2010). By coating the fibril surface, Hsp27 may act to inhibit surface-dependent templated secondary nucleation and/or fibril fragmentation, both of which lead to increases in the abundance of small oligomeric species and total fibril
load (Xue et al. 2009, Jakhria et al. 2014, Shvadchak et al. 2015). Importantly, it was noted that the distribution of Hsp27 bound to the fibril was not uniform, suggesting that it may bind preferentially to specific regions along the surface of the fibril. To investigate this further, a novel imaging technique, known as sPAINT, was employed. This TIRF microscopy-based technique utilises nile red (a fluorescent dye exhibiting a characteristic emission wavelength shift according to the hydrophobic nature of the surrounding environment) to provide a quantitative measure of the relative hydrophobicity at any given region along the fibril surface. The results presented here demonstrate that, by binding to α-syn fibrils, Hsp27 significantly decreases hydrophobicity at the fibril surface. This is significant given that the toxicity of aggregates formed from both pathogenic and non-pathogenic proteins correlates with the level of exposed hydrophobicity at the aggregate surface (Bolognesi et al. 2010), such that the more hydrophobic the aggregate the higher the toxicity. The observed reduction in the relative hydrophobicity of Hsp27-bound α-syn fibrils suggests that, by binding to regions of high hydrophobicity, Hsp27 may decrease the toxicity of aggregates.

In light of this, to assess whether binding of the sHsps to α-syn fibrils acts to decrease their cytotoxicity, the production of ROS by cells was monitored upon exposure to preformed α-syn fibrils. The production of ROS associated with protein aggregates is thought to be a significant mediator of neuronal toxicity in neurodegenerative disorders, including PD (Tabner et al. 2001). ROS can damage DNA, induce modification of macromolecules, inhibit protein function and promote apoptotic cell death (Simon et al. 2000, Circu and Aw 2010). As anticipated, exposure of cells to exogenous α-syn fibrils resulted in a specific, rapid and significant increase in cellular ROS production. However, pre-incubation of the α-syn fibrils with Hsp27 or αB-c prevented this toxicity, indicating that the binding of these sHsps to fibrillar α-syn represents a previously uncharacterised protective function of these
chaperones, i.e. by binding to fibrils, Hsp27 and αB-c (and most likely other sHsps) prevent the toxicity associated with extracellular amyloid fibrils.

Although α-syn has traditionally been considered an intracellular protein, recent advancements in understanding the transmission of pathology between neuronal cells has highlighted the role of extracellular α-syn in disease progression (Lee et al. 2005, Kordower et al. 2008, Lee et al. 2008, Li et al. 2008). Aggregates may be released via exocytosis of calcium-dependent exosomes or direct penetration of the cell membrane, entering the extracellular space and gaining access to neighbouring cells (Lee et al. 2008, Emmanouilidou et al. 2010). Aggregates have been shown to enter neighbouring cells, through endocytosis, membrane-receptor mediated access or direct penetration of the cellular membrane (Lee et al. 2008, Tang et al. 2012), where they can then seed the aggregation of endogenous α-syn (Desplats et al. 2009, Luk et al. 2009, Volpicelli-Daley et al. 2011). In addition to inducing intracellular ROS production, exogenous α-syn fibrils have been associated with active adaptation of the proteome, whereby cells upregulate defence mechanisms against dysfunction and death (Pieri et al. 2016). These effects have been demonstrated in cultured cells as well as in mouse models recapitulating disease-related inclusion body formation (Luk et al. 2012). It remains to be determined whether Hsp27 can impact these other cytotoxic effects resulting from exposure to exogenous fibrils, e.g. by binding to fibrils shSps may prevent the uptake of fibrils by cells or may inhibit other downstream, intracellular effects. However, due to their well-described anti-apoptotic activity, it is likely that the shSps play multiple roles in preventing the toxicity associated with fibrils, in addition to those involving direct interactions with the aggregated protein.

Whilst the relevance of extracellular aggregates is becoming apparent, the accumulation of intracellular aggregates remains a defining characteristic of α-syn aggregation. Currently,
these deposits are thought to be storage spaces for the cell in which they may sequester potentially toxic intermediates (Yerbury et al. 2013). In fact, chaperones have been shown to promote the accumulation of cytotoxic oligomers into larger, non-toxic aggregates (Ojha et al. 2011, Mannini et al. 2012). Cells may go on to live with these ‘storage’ inclusions over extended periods of time, as they continually attempt to re-establish proteostasis. The association of sHsps with these deposits may therefore have implications for their clearance, as dismantling or degradation of these structures would expose the cell to potentially toxic aggregates. Previous work using apoC-II fibrils and AUC demonstrated that the binding of αB-c to fibrils both prevents their fragmentation and causes the dissociation of monomeric species (Binger et al. 2013). The results presented here support these findings and extend them to a disease-relevant model of protein aggregation: by binding to α-syn fibrils, Hsp27 and αB-c decrease the apparent size of the fibrils and promote their dissociation over a period of up to 21 days. Whilst the production of monomeric α-syn from sHsp-bound fibrils was not directly assessed here, the ability of the sHsps to dissociate α-syn fibrils into oligomers suggests that the sHsps play an important role in assisting the clearance of aggregates should cellular proteostasis be restored. In addition, by coating the dissociating species, the sHsps could mitigate any cytotoxic effects. The potential protective effects of the sHsps being bound to aggregated α-syn represent a significant area for future investigation.

Given that both αB-c and Hsp27 bind α-syn fibrils, it appears fibril binding may represent a generic aspect of sHsp chaperone action. The work presented here provides evidence that the fibril-binding capacity of the sHsps provides a second protective mechanism by which these chaperones may prevent the toxicity associated with protein aggregation. Thus, rather than being ‘caught up’ in inclusions as a by-product of their failed attempt to prevent aggregation, the work presented in this chapter indicates that the localisation of sHsps to protein deposits is likely due to their specific association with the aggregated protein as a protective measure.
in cells. Investigating the molecular mechanisms by which sHsps are able to interact with aggregating proteins, including monomeric, oligomeric and fibrillar species, is essential to understanding the potential protective capabilities of sHsps in cells.
Chapter 5: sHsp chaperone activity in cellular models of aggregation
Chapter 5: sHsp chaperone activity in cellular models of aggregation

5.1 Introduction

The work in Chapters 3 and 4 of this thesis demonstrated that the sHsps αB-c and Hsp27 interact with various α-syn species formed during its aggregation into fibrils. This work involved characterising the mechanisms by which sHsps interact with α-syn in simple buffers in vitro, however, it is essential to consider the relevance of this interaction in a cellular context. Much of what is known about the interaction between sHsps and α-syn in cells comes from qualitative studies using cell-based models of α-syn aggregation (as summarised in Section 1.4). One issue with these cell culture models of α-syn aggregation, which has hampered their use in quantitative studies, is that typically only a small proportion of the cells in culture develop inclusions (typically less than 5% in studies where this is quantified) (McLean et al. 2001). This severely limits the types of biochemical analyses that can be undertaken with these models. Therefore, there is a need to develop a robust and reliable cell-based model of α-syn aggregation so that the effect the sHsps have on this process can be quantified in cells.

5.1.1 Modelling cellular aggregation

Detecting and quantifying aggregates in cell models most commonly relies on biochemical or fluorescence microscopy techniques (Shiber et al. 2014). The formation of aggregates in a population of cells is often assessed via techniques such as differential centrifugation, detergent insolubility and/or size exclusion gel filtration, however, these methods are not suited to resolving the time-dependent changes that occur during the aggregation process (Shiber et al. 2014). In addition, exposure to detergents can dissociate aggregates producing false-negative results (Muchowski 2002), while lysis and centrifugation techniques may increase the non-specific adhesion of proteins resulting in false-positive results (Shiber et al. 2014). Fluorescence microscopy can provide qualitative co-localisation analyses and is
amenable to real-time investigation of protein aggregation, however, the subjective nature of this technique can result in bias and random error (Shiber et al. 2014). Obtaining quantitative information from microscopy analyses is possible, although it generally requires manually-assisted counting as image analysis algorithms can be highly dependent on background thresholding and restricted in their capacity for feature extraction and segmentation of individual cells (Ramdzan et al. 2012). As such, these techniques are inherently incompatible with high throughput analyses.

In addition to these challenges, perhaps the most significant limitation of these techniques is the inability to recover distinct populations for further analysis (Ramdzan et al. 2012). The emergence of flow cytometry-based methods for detecting aggregate-associated fluorescence overcomes this limitation and is amenable to high throughput analyses. Mitsui and colleagues (2006) describe a method for purification of fluorescently labelled Htt aggregates from N2a cell lysates, in which fluorescence activated cell sorting (FACS) selectively collects aggregates of the labelled protein of interest. This technique is powerful as it enables further biochemical analysis of the aggregates, e.g. identification of proteins within the aggregate via mass spectrometry (Mitsui et al. 2006). More recently, a method using flow cytometric analysis of intact cells was described by Ramdzan and colleagues (2012). The technique, termed pulse-shape analysis (PulSA), exploits the reduction in pulse width and increase in pulse height associated with an intracellular (fluorescently tagged) protein when it is in an aggregate compared to when it is diffuse in the cytoplasm. PulSA also enables cells with aggregates to be sorted via FACS, thereby allowing a population of live, intact cells which are enriched with aggregates to be isolated. However, whilst this technique works well when large inclusions are formed by an aggregation–prone protein in cells (e.g. the exon 1 fragment of the Htt protein containing an extended poly-glutamine repeat), it is limited in its sensitivity to detect the smaller aggregates formed in cells by other disease-relevant proteins (e.g. TDP-
43 or SOD1). This limitation is a result of PulISA not being capable of distinguishing shifts in the pulse height and pulse width when small aggregates are present in a cell, despite inclusions being visible via microscopy (Ramdzan et al. 2012, Shiber et al. 2014, Whiten et al. 2016).

There currently exists a plethora of cellular models which recapitulate the cellular aggregation of disease-associated proteins, each displaying characteristics specific to the protein of interest. Ideally, when developing model systems to investigate the cellular aggregation of a protein, compatibility with high throughput assays that provide the capability to recover live cells is advantageous. This enables aggregation to be related to specific biological outcomes, or the utility of targeting specific aggregation events with therapeutic agents to be established (Ramdzan et al. 2012). However, the characteristics of a given cell-based model of aggregation will determine which techniques are suitable to analyse the aggregation process. In any case, such cell-based models have provided crucial insight into protein-specific or generic aggregation properties associated with disease, and represent an essential link between in vitro and in vivo studies.

5.1.2 Monitoring chaperone action in cells

As described in Section 1.1.3, tagging a protein expressed in cells with a fluorescent protein or fluorophore is a common way of tracking its intracellular localisation. However, Hsps often form dynamic oligomers which interact with various cofactors and substrate proteins. In particular, the sHsps αB-c and Hsp27 form large polydisperse oligomers which undergo dynamic subunit exchange (Bova et al. 2000, Van Montfort et al. 2002), a process that is thought to be a critical mediator of their chaperone activity. As such, these functions can be significantly affected by the addition of bulky fluorescent tags. Recent work has demonstrated that the addition of a fluorescent protein tag to the N- or C-terminus of αB-c or
Hsp27 significantly impacts the ability of these sHsps to form oligomeric assemblies and act as chaperones, compared to the non-labelled protein (Datskevich et al. 2012, Datskevich et al. 2012, Datskevich and Gusev 2014). Thus, the addition of bulky fluorescent tags to sHsps is not a suitable option when attempting to study their chaperone activity in cells. However, the expression of untagged sHsps in cells does not allow differences in transfection efficiency and expression levels to be taken into account when comparing between individual cells and samples in an experiment, which can confound analyses.

There is therefore a need to develop an alternative method to evaluate the effect of sHsps in cells that does not involve tagging them with a fluorescent protein, but does take into account potential differences in transfection efficiencies and protein expression levels. One such method is the use of bicistronic expression constructs which enable the expression of a gene of interest (in this case a sHsp) and a fluorescent (reporter) protein (in this case EGFP) (Assoc. Prof. Heath Ecroyd, personal communication). In these bicistronic constructs, the mRNA contains an internal ribosome entry site (IRES) such that the expression of the sHsp of interest and fluorescent protein is correlated. In this way the levels of the fluorescent protein acts as a reporter of the expression of the sHsp. Use of these IRES constructs therefore enables transfected cells to be detected, and the relative levels of chaperone to be quantified (based on the expression levels of the fluorescent reporter) without the addition of a fluorescent tag directly to the protein.

5.1.3 Experimental rationale

Despite their many limitations, cell-based models of α-syn aggregation have revealed some evidence for sHsps inhibiting α-syn aggregation or toxicity in cells (Klucken et al. 2004, Zourlidou et al. 2004, Outeiro et al. 2006). In order to advance our understanding of the ability of sHsps to prevent the aggregation of α-syn in cells, a suitably robust and reliable
cell-based model of α-syn aggregation was sought. Once this cell-based assay was developed, the ability of sHsps to prevent the aggregation of α-syn in cells was assessed. Since the addition of a fluorescent tag potentially impacts the function of sHsps in cells (Datskevich et al. 2012, Datskevich et al. 2012, Datskevich and Gusev 2014), bicistronic IRES vectors were used to evaluate and compare the ability of αB-c and Hsp27 to prevent intracellular aggregation of α-syn. In these assays, an IRES construct encoding EGFP and a non-chaperone control protein EGFPinv was utilised to ensure all cells received equal DNA concentrations and an equivalent number of proteins to express. Finally, in order to assess whether the sHsps are able to prevent the aggregation of other disease-relevant proteins in cells, the IRES constructs encoding EGFP and a range of other Hsps (or the non-chaperone controls EGFPinv or LacZ) were used in the well characterised cell model of Htt exon 1 aggregation and evaluated via PulSA.
5.2 Methods

5.2.1 Cloning strategy to produce truncated variant of α-synA53T-EGFP

Two variants of the α-syn expression construct (see Section 2.2.1) were employed in an attempt to generate a robust and reliable cellular model of α-syn aggregation. First, constructs encoding EGFP-tagged α-synWT and α-synA53T were utilised without modification. The cloning strategy for the second construct is summarised in Figure 5.1, and was designed according to findings published by McLean and colleagues (2001) in which an aggregation-prone cleavage product of α-syn was identified within cells initially transfected to express α-syn with a C-terminal EGFP tag (i.e. α-syn-EGFP). Specifically, this product was approximately 27 kDa, and corresponded to a cleaved form of α-syn-EGFP in which the last 155 residues from the C-terminal region of EGFP were removed. This resulted in α-syn fused to a non-fluorescent EGFP fragment. The region corresponding to α-synA53T and the first 85 residues of EGFP (i.e. lacking the last 155 residues, designated α-synA53T*) was amplified from the original α-synA53T-EGFP construct by PCR using MyTaq DNA polymerase, the CMV-F primer (5’ – CGCAAATGGGCGGTAGGCGTG – 3’) and a custom designed primer (5’ – CTTGCAGCCGCCTAGAAGAAGTCGTGCTGC – 3’) containing the NotI restriction site (bold text). PCR was performed using a Mastercycler ProS (Eppendorf) and consisted of 35 cycles, with each cycle consisting of denaturation at 95°C for 15 s, annealing at 55°C for 15 s, and extension at 72°C for 10 s. The amplified product was combined with agarose loading buffer (final concentrations: 30% (v/v) glycerol, 0.25% (w/v) bromophenol blue in dH2O) and resolved via electrophoresis using a 1% (w/v) Tris-acetate-EDTA (TAE; 40 mM Tris-base, 20 mM glacial acetic acid, 1 mM EDTA, pH 8.0) agarose gel alongside the Hyperladder I (200 – 10037 bp) and Hyperladder II (50 – 2000 bp) molecular weight markers. Gels were submerged in 1 × TAE buffer in a MiniSub Cell GT electrophoresis tank.
(Bio-Rad, Hercules, USA) and electrophoresis was performed at 100 V until the dye front reached the end of the gel. DNA bands were briefly visualised under UV light using a Gel Logic 2200 Pro Imaging System (Carestream Health, Rochester, USA) following staining with 0.005% (w/v) ethidium bromide solution for 15 min and destaining with dH2O for 5 min.

The PCR product was excised from the gel and purified using the Isolate II PCR and Gel kit (Bioline, Toronto, Canada) according to the manufacturer’s instructions. The purified PCR product (insert) and the pEGFP-N3-α-synA53T-EGFP construct were double digested using Acc65I and NotI, according to the manufacturer’s instructions, before heat inactivation at 80°C for 20 min. The desired products were separated from the digestion fragments via agarose gel electrophoresis, and purified using the Isolate II PCR and Gel kit (Bioline, Toronto, Canada) as described above. Ligation of the insert and digested plasmid was performed at 16°C for 16 h using T4 DNA ligase (5 μg/mL) in a 20 μL reaction mixture containing 1 × ligase buffer and 1 × ATP solution. The ligation mixture was then used to transform chemically competent DH5α E. coli as per Section 2.2.2. Successfully transformed colonies were screened for the α-synA53T* insert by PCR using the CMV-F and SV40 (5’ – CTCTACAAATGTGGTGTTAATGGC – 3’) primers. PCR was performed using a Mastercycler ProS (Eppendorf) and consisted of 30 cycles, with each cycle consisting of denaturation at 95°C for 15 s, annealing at 52°C for 15 s, and extension at 72°C for 15 s. A colony positive for the α-synA53T* insert was selected and plasmid DNA was extracted using the Wizard® Plus SV Miniprep DNA Purification System (Promega, Fitchburg, U.S.A.) according to the manufacturer’s instructions. The construct was then verified via restriction digest with the preparative enzymes as described above to confirm the correct size of the liberated insert, and sequencing (see Section 2.2.1).
Figure 5.1: Cloning strategy used to generate α-synA53T* construct for mammalian expression. From the original α-synA53T-EGFP construct, PCR amplification and restriction enzyme digestion generated a linear construct and insert fragment, which was then ligated using T4 DNA ligase. Construct maps are annotated with solid, coloured arrows to indicate genes of interest, primer sites (red text) and enzyme digestion sites (purple text). Orange line in the original construct indicates the original PCR amplification product.
Chapter 5: sHsp chaperone activity in cellular models of aggregation

5.2.2 Transfection and treatment with inducers of cellular stress

For transfection, cells were seeded at 100 000 cells/mL in 6-well, 12-well or 24-well plates (Greiner Bio-One, Frickenhausen, Germany), or 8-well chamber slides (Ibidi, Martinsried, Germany) and grown overnight. Cells were then transfected using Lipofectamine® LTX (Invitrogen, Waltham, U.S.A.) according to the manufacturer’s instructions. Briefly, DNA:liposome complexes were generated by incubation of 1 μg of plasmid DNA in serum free DMEM/F12 with 3 μL of Lipofectamine® LTX and 1 μL PLUS™ reagent per ~3.5 cm² of cells to be transfected, at room temperature for 30 min. In the case of dual transfections, DNA complexes for each plasmid were prepared separately then combined after the 30 min incubation period to allow mixing. In this way, each plasmid is incorporated into separate liposomes, which can then be independently taken up by cells. Dual transfections (unless stated) were completed at a 1:4 (IRES:α-syn or Htt) (w/w) ratio of DNA. Complexes were then applied to cells in fresh culture medium and incubated for 48 h at 37°C under 5% CO2/95% air.

Where necessary, cells were treated with various inhibitors of proteostasis pathway components to induce cellular stress. Treatments included a proteasome inhibitor (MG132; 10 μM), an autophagy inhibitor (3-methyladenine (3-MA); 10 mM), an inducer of ER stress (thapsigargin; 3 μM), or an inducer of oxidative stress (FeCl₂; 10 mM), all of which were solubilised in DMSO as 1000-times stocks and subsequently diluted into serum free DMEM/F12 for addition to cultured cells. Mock treated cells were exposed to the appropriate concentration of DMSO in serum free DMEM/F12.

5.2.3 Immunocytochemistry

Proteins of interest were routinely visualised in cells via immunocytochemistry. Cells were seeded at 100 000 cells/mL onto sterilised 19 mm glass coverslips (ProSciTech, Thuringowa,
Chapter 5: sHsp chaperone activity in cellular models of aggregation

Australia) or in 8-well chamber slides (Ibidi, Martinsried, Germany) and transfected as described above. After 48 h, or following treatment for the times indicated, culture medium was removed from the cells and the cells washed twice with PBS. Cells were fixed via incubation with 4% (w/v) paraformaldehyde (PFA, pH 7.4) for 20 min at room temperature and washed twice with PBS. Cells were then permeabilised using 0.5% (v/v) Triton X-100 in PBS for 20 min at room temperature, before being washed twice with 1% (w/v) BSA in PBS. Cells were then blocked using 5% (w/v) BSA in PBS, and washed twice in 1% (w/v) BSA in PBS-Tween 20 (PBS-T; PBS containing 0.05% (v/v) Tween-20).

Intracellular proteins were immunolabelled using the appropriate primary antibody diluted 1:500 with 1% (w/v) BSA in PBS-T. Cells were incubated at 37°C for 1 h in a humidity chamber, before being washed three times with 1% (w/v) BSA in PBS-T. The appropriate fluorophore-conjugated secondary antibody was diluted 1:500 with 1% (w/v) BSA in PBS-T, and applied to the cells. Following incubation at 37°C for 1 h in a humidity chamber, cells were then washed four times with 1% (w/v) BSA in PBS-T.

5.2.4 Confocal microscopy imaging

Coverslips with cells containing fluorescent or immunolabelled proteins were mounted onto 26 × 76 mm glass slides (ThermoFischer Scientific, Waltham, USA) using Citifluor™ Anti-Fadent Mounting Solutions (9:1 (v/v) ratio of Citifluor™ CFPVOH: Citifluor™ AF100 anti-fadent; ProSciTech, Thuringowa, Australia) and allowed to set at room temperature for 1 h prior to imaging. Alternatively, cells in chamber slides were overlayed with PBS containing 10% (v/v) Citifluor™ AF100 anti-fadent (ProSciTech, Thuringowa, Australia) and imaged immediately. Cells were observed using a Leica TCS SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany). Fluorescent conjugates were excited at 488 nm, 561 nm, or 633 nm by argon, DPS 561, and HeNe lasers, respectively. Fluorescent emissions were
acquired by sequential scanning using the Leica Application Suite – Advanced Fluorescence (LAS-AF) software v3 (Leica Microsystems, Wetzlar, Germany). Either photomultiplier tube (PMT) or hybrid (HyD) detectors were selected and custom filter windows set according to the intensity and emission spectrum of the fluorophore of interest. Gains and filter windows were set according to the untransfected and isotype (mouse or rabbit IgG) stained controls using the overflow colour map, such that samples were below the saturation point, background fluorescence was not visible and bleed through was eliminated.

5.2.5 Inclusion quantification

5.2.5.1 Flow cytometry

A recently described flow cytometry method of distinguishing inclusions in whole cells, known as PulSA, was used (Ramdzan et al. 2012). Briefly, transfected cells were harvested by replacing culture media with a sufficient volume of trypsin containing 0.05% (w/v) EDTA to cover the cells. After incubation at 37°C for 5 min the plate was gently tapped to dislodge cells, if necessary. The plate was then washed with DMEM/F12 containing 1% (v/v) FCS, and cells were collected. Cells were harvested via centrifugation at 300 x g for 5 min at room temperature, and washed twice in PBS. Cells were then resuspended in PBS and analysed via flow cytometry using an LSR II or LSRFortessa X-20 Flow Cytometer (BD Biosciences, San Jose, U.S.A). In addition to forward and side scatter, area, height and width parameters of GFP fluorescence (excited at 488 nm) and mCherry fluorescence (excited at 561 nm) were collected at 525/50 nm and 586/15 nm respectively, using FACS Diva™ software v6.1.3 (BD Biosciences, San Jose, U.S.A.). Gating and data analysis was performed using FlowJo software v10 (Tree Star, Ashland, U.S.A.). Using the PulSA gating strategy (Ramdzan et al. 2012), live transfected cells of interest were selected, then a scatterplot of the GFP fluorescence width versus height parameters was generated for cells that did not contain
inclusions ($n_{\text{pop}}$) but instead contain diffuse, cytosolic fluorescence. It was then possible to distinguish cells containing compact, punctate fluorescence (indicating protein inclusions), according to a shift in both GFP fluorescence height and width which results in the appearance of a population of cells with inclusions ($i_{\text{pop}}$) in the upper left quadrant (i.e. narrower pulse width and higher pulse height than cells without inclusions). The percentage of cells in this population was then compared across treatment groups relative to the EGFP$^{\text{inv}}$ control.

5.2.5.2 Manual quantification using ImageJ

Images collected via confocal microscopy were processed manually with ImageJ (National Institutes of Health, Bethesda, U.S.A.) using custom written macro scripts (Appendix VI). These methods were developed to enable non-subjective quantification of the number of cells with aggregates between samples. Briefly, cell boundaries of individual cells were defined using the brightfield image, and the transfected cells determined automatically via thresholding of the fluorescent image. Cells containing inclusions (visually defined as fluorescent puncta) were manually selected according to the fluorescent image, and the percentage of transfected cells (or co-transfected cells in experiments involving the IRES constructs) with and without inclusions for each treatment was calculated using a custom written Python script (Appendix VII). This was completed with at least 50 cells per treatment over four biological repeats.

5.2.6 Cell lysate analysis via filter trap assay

In order to confirm the presence of insoluble aggregates in cellular $\alpha$-syn inclusions, a filter trap assay was performed. Cells were transfected, treated with inhibitors where appropriate and harvested as described in Section 2.7.1. Cell pellets were washed twice in PBS, and then resuspended in lysis buffer (PBS containing 0.5% (v/v) Triton-X 100, pH 7.4, supplemented
with 0.5% (v/v) Halt™ Protease and Phosphatase Inhibitor Cocktail). The total protein concentration for each sample was then determined using a Pierce BCA assay (ThermoFischer Scientific, Waltham, USA) according to the manufacturer’s instructions. Samples were diluted using lysis buffer to generate 200 µL aliquots containing 200 µg total protein, and kept on ice until use. In addition, aliquots containing known concentrations of recombinant monomeric or fibrillar α-syn were similarly prepared in PBS or spiked into lysis buffer and kept on ice.

In order to determine the concentration of α-syn in samples produced following transfection with the α-synA53T* construct, a dot blot was first performed using these samples. Samples were spotted (to a total volume of 5 µL) onto nitrocellulose membrane, and left to dry at room temperature for 1 h. The membrane was then blocked at room temperature for 1 h in 5% (w/v) skim milk powder in TBS, before being analysed via the immunoblotting technique described in Section 2.5. The intensity of spots of interest were analysed using the gel quantification function in ImageJ, and a standard curve of concentration of recombinant α-syn versus spot intensity was generated. The concentration of α-syn in the transfected cell extracts was then determined via interpolation from the standard curve and was found to be less than 1 µM (see Appendix VIII).

The Bio-Dot microfiltration apparatus (Bio-Rad, Hercules, CA) was prepared according to the manufacturer’s instructions. Briefly, 0.22 µm cellulose acetate membrane (Whatman, Maidstone, UK) and three sheets of Bio-DOT SF filter paper (Bio-Rad, Hercules, CA) were equilibrated in PBS containing 1% (w/v) SDS (pH 7.4) for 10 min at room temperature. The Bio-Dot apparatus was then assembled containing the membrane and filter paper and tightened under vacuum. Each slot for use was rehydrated with 200 µL PBS, which was then filtered under vacuum immediately prior to the addition of 200 µL of the prepared cell lysate.
or appropriate control samples. Any unused slots were sealed with clear sealing film, and samples filtered under gentle vacuum. Once all samples had been filtered, 200 µL of PBS was added to the sample well and filtered under gentle vacuum. The membrane was then removed and blocked in 5% (w/v) skim milk powder in TBS, before being analysed via the immunoblotting technique described in Section 2.5.
5.3 Results

5.3.1 Developing a cell model of α-syn aggregation

5.3.1.1 Transfection with fluorescently tagged α-syn constructs

Transfection with fluorescently tagged aggregation-prone proteins is a common method for investigating cellular aggregation, including that of α-syn (McLean et al. 2001, Outeiro et al. 2006), and was therefore first explored in the search for a robust cell model of α-syn aggregation. Constructs encoding α-synWT or the disease-associated mutant α-synA53T, C-terminally fused to a fluorescent EGFP tag, were used to determine whether α-syn aggregates and forms inclusions in cells. First, the expression of the α-syn-EGFP fusion proteins was confirmed via immunoblotting of transfected cell lysates (Figure 5.2A). This revealed a band at approximately 44 kDa immunoreactive against the α-syn antibody in both the α-synWT-EGFP and α-synA53T-EGFP samples, consistent with the expected mass of 41.5 kDa resulting from fusion of EGFP (27 kDa) to α-syn (14.5 kDa). However, a significant amount of smaller bands (ranging from 27 – 37 kDa) were also immunoreactive against the α-syn antibody in the α-synA53T-EGFP cell lysate, suggesting this protein is prone to degradation.

The expression and localisation of the α-syn-EGFP proteins was examined via confocal microscopy. Cells expressing the aggregation-prone TDP-43-tGFP were used as controls in this experiment, as TDP-43 aggregation has been extensively examined both in vitro and in vivo, displaying characteristics of amyloid oligomer and fibril formation (Guo et al. 2011, Fang et al. 2014). Importantly, TDP-43-tGFP has previously been demonstrated to form small intracellular inclusions in N2a cells when treated with a proteasome inhibitor (MG132) and an inducer of ER stress (thapsigargin) (Dr. Daniel Whiten, University of Wollongong, personal communication) similar to those previously detected for α-syn (McLean et al. 2001, Outeiro et al. 2006). Imaging of cells expressing TDP-43-tGFP revealed the presence of
small fluorescent puncta corresponding to inclusions in approximately 30% of GFP-positive cells (Figure 5.2B). No clearly defined fluorescent puncta were detected in cells expressing either WT or A53T isoforms of α-syn-EGFP (Figure 5.2B). However, accumulations of fluorescence were evident in some cells expressing high levels of α-syn-EGFP, which may represent aggregated α-syn-EGFP (Figure 5.2B, inset).

**Figure 5.2:** Fluorescently tagged α-syn variants produce visible inclusions. N2a cells were transiently transfected with constructs encoding EGFP-tagged α-synWT or α-synA53T, or the aggregating control protein TDP-43-tGFP. Cells transfected with TDP-43-tGFP were treated with proteostasis inhibitors (10 µM MG132 and 3 µM thapsigargin), and all cells were incubated for a further 48 h. (A) Cells were harvested and lysed such that whole cell protein extracts could be analysed via SDS-PAGE. Following transfer to nitrocellulose membrane, EGFP-tagged α-synWT (lane 1) or EGFP-tagged α-synA53T (lane 2) were detected using a mouse monoclonal anti-α-syn primary antibody and a HRP-conjugated anti-mouse IgG secondary antibody. The position of molecular weight markers in kDa are indicated by the arrows. Transfected cells were also grown on glass coverslips and (B) imaged via confocal microscopy. Scale bars represent 10 µm (or 5 µm in inset). Arrows indicate regions of concentrated fluorescence.

To evaluate whether the inclusion-like particles observed in cell expressing α-syn-EGFP were detectable via PulSA, N2a cells were transfected to express α-syn-EGFP (WT or A53T) or TDP-43-tGFP and compared to cells expressing EGFP-alone (which acted as a negative control). The forward and side scatter signals of events were used to select live, viable cells (Figure 5.3A), and to exclude events corresponding to cellular debris and cell-doublets. Cells expressing the proteins of interest were then selected based on their GFP fluorescence.
Chapter 5: sHsp chaperone activity in cellular models of aggregation

compared to untransfected cells (Figure 5.3B), and these live GFP-positive cells were then analysed via PulSA by plotting their pulse height and pulse width signals. Cells expressing EGFP alone were used to identify the inclusion-negative population of cells (n_{ipop}) and to set the gate to identify cells with inclusions (i_{ipop}) (Figure 5.3C). The proportion of cells expressing α-synWT-EGFP or α-synA53T-EGFP in the i_{ipop} gate was very low (<5%) (Figure 5.3D and E). However, even in cells expressing TDP-43-tGFP, which had previously been observed to contain fluorescent aggregates in approximately 30% of cells (see Figure 5.2), the proportion of cells in the i_{ipop} was low (4.5%) (Figure 5.3F). Thus, PulSA does not appear to be suitable for detection of cells with smaller inclusions, such as those formed by α-syn in cells. It was therefore concluded that fluorescence-based microscopy would likely be a more suitable method for assessing α-syn aggregation in cells.

**Figure 5.3:** Fluorescently tagged α-syn variants do not produce inclusions detectable via PulSA. N2a cells were transiently transfected with constructs encoding the control protein EGFP, EGFP-tagged α-synWT or α-synA53T, or TDP-43-tGFP, and incubated for 48 h. Cells transfected with TDP-43-tGFP were treated with proteostasis inhibitors (10 µM MG132 and 3 µM thapsigargin) and all cells were incubated for a further 48 h before being analysed via flow cytometry. Scatter plots of the forward and side scatter signals were used to select (A) live cells, and GFP fluorescence of untransfected cells was used to select (B) EGFP-positive cells. These cells were then analysed using PulSA. (C) The population of cells with inclusions (i_{ipop}) was identified based on the PulSA plot of cells expressing EGFP alone (very few of these cells develop inclusions). Cells expressing (D) α-synWT-EGFP, (E) α-synA53T-EGFP or (F) TDP-43-tGFP with inclusions were then identified. The proportion (as a percentage) of GFP-positive cells in the i_{ipop} is given in each plot.
Given the importance of the proteostasis network in modulating cellular aggregation, targeting members of this network is a common method of inducing the aggregation of proteins into inclusions, including in cellular models of α-syn aggregation (Outeiro et al. 2006, Bertoncini et al. 2007, Wan and Chung 2012) and as observed for TDP-43 (see Figure 5.2). To determine the effect of disrupting elements of proteostasis on the number of fluorescent puncta observed in N2a cells transfected to express α-synWT-EGFP, α-synA53T-EGFP, EGFP alone (negative control) or TDP-43-tGFP (positive control), cells were incubated in the absence or presence of MG132 (10 µM, proteasome inhibitor), thapsigargin (3 µM, inducer of ER stress) or FeCl₂ (10 mM, inducer of oxidative stress). Cells were imaged via confocal microscopy (Figure 5.4A), and the number of EGFP-positive cells containing diffuse fluorescence (left box, 1) or fluorescent puncta (right box, 2) was determined for each treatment. The percentage of transfected cells (diffuse + puncta cells) containing inclusions following treatment was then compared with the untreated EGFP expressing control cells (Figure 5.4B). As expected, there was a significantly higher (p<0.001) proportion of cells expressing TDP-43-tGFP with inclusions (41.5 ± 7.20%) compared to those expressing EGFP (2.92 ± 1.14%). However, treatment of cells expressing either of the α-syn-EGFP variants with these agents had no significant effect on the percentage of cells containing inclusions compared to those expressing EGFP alone. Moreover, the proportion of cells expressing α-syn with inclusions remained very low (<10%). To determine whether the lack of aggregation of α-syn into inclusions was due to the cell line being used in this work (i.e. N2a cells), this experiment was repeated using HEK293 cells (Figure 5.4C). As expected, treatment of HEK293 cells expressing TDP-43-tGFP with MG132 and thapsigargin significantly (p<0.001) increased the proportion of cells with inclusions (36.1 ± 8.56%) compared to treated cells expressing EGFP alone (5.40 ± 2.45%). However, as seen for N2a cells, there was no significant difference in the percentage of α-
syn-EGFP expressing HEK293 cells containing fluorescent puncta following treatment with MG132, thapsigargin or FeCl₂ compared to cells expressing EGFP alone. In all cases the percentage of transfected cells with inclusions remained very low (<7%). As such, it was concluded that expression of EGFP-tagged α-syn variants was not sufficient to produce a robust model of α-syn aggregation in cells, and therefore an alternate model was pursued.

**Figure 5.4:** Expression of fluorescently tagged α-syn isoforms does not result in robust inclusion formation in N2a or HEK293 cells. N2a or HEK293 cells were transiently transfected with constructs encoding EGFP (as a negative control), or EGFP-tagged variants of α-synWT or α-synA53T, or TDP-43-tGFP. Cells were treated with proteostasis inhibitors (10 μM MG132, 3 μM thapsigargin, or 10 mM FeCl₂) as indicated and incubated for 48 h before being mounted on glass slides and imaged via confocal microscopy. Images were randomly collected according to the brightfield channel, and the number of GFP-positive cells containing diffuse or aggregated fluorescent protein was manually quantified using (A) an overlay of the brightfield and fluorescent images. Examples of cells counted as (1) diffuse fluorescence and (2) punctate fluorescence are shown. Scale bars represent 25 μm. The percentage of GFP-positive cells containing inclusions for (B) N2a or (C) HEK293 cells following each treatment is displayed as mean ± S.E.M. (n=3), and was analysed via a one-way ANOVA with a Dunnett’s post-test compared to the untreated EGFP control, where *** indicates p<0.001.
5.3.1.2 Transfection with α-synA53T*

Previous work attempting to develop a cell model of α-syn aggregation in which a fluorescently tagged α-syn was expressed in human H4 neuroglioma cells led to the discovery of intracellular puncta which were non-fluorescent but immunoreactive to α-syn (McLean et al. 2001). This work identified a cleavage product of the expressed α-syn-EGFP in these inclusions. This product consisted of α-syn with a C-terminal fragment of EGFP in which the last 155 residues of EGFP has been cleaved from the protein. According to the sequences provided for these cleavage products, a construct was designed for the expression of α-syn fused to a fragment of EGFP (designated α-synA53T*). Using custom PCR primers, the appropriate region of the α-synA53T-EGFP construct was amplified and the product analysed via agarose gel electrophoresis (Figure 5.5A). The generation of a product of 850 bp was consistent with the expected size (849 bp) of the target sequence to be amplified. This PCR product, along with the original construct containing the α-synA53T-EGFP gene, were digested and ligated, before being transformed into chemically competent E. coli. Colonies were selected from the transformation and screened for the insert of interest via PCR amplification, which was analysed using agarose gel electrophoresis (Figure 5.5B). Clones displaying a prominent band of approximately 1400 bp correspond to amplification of the original, full length α-synA53T-EGFP sequence (which has an expected size of 1347 bp) and are therefore consistent with re-ligation of the original construct. Conversely, clones positive for the truncated construct were identified by the appearance of a band at approximately 900 bp, consistent with the expected size of 884 bp, due to the deletion of 155 amino acid residues of EGFP from the C-terminus. To confirm the presence of the construct of interest, clones were subjected to enzymatic digestion alongside the original construct, and analysed via agarose gel electrophoresis (Figure 5.5C). Digestion of the original construct liberates two linearised fragments of 4200 bp and 800 bp, corresponding to the vector and insert,
respectively. This is consistent with the expected size of 752 bp for α-synA53T-EGFP. In contrast, double digestion of clones predicted to contain the sequence of interest (i.e. α-synA53T*) liberated fragments of 4200 bp and 300 bp, corresponding to the vector backbone and the truncated insert (which has an expected size of 284 bp), respectively. Two positive clones were therefore selected for sequencing and both were found to contain the α-synA53T* insert, which was seen to be in frame and did not contain any mutations (data not shown).

Figure 5.5: Cloning and expression of α-synA53T*. (A) Using custom-designed primers, the α-synA53T* gene was amplified from the original α-synA53T-EGFP construct via PCR and analysed via agarose gel electrophoresis. (B) Following enzymatic digestion and ligation of the α-synA53T* gene into the original pN3 vector in place of α-synA53T-EGFP, E. coli were transformed with the ligation mixture. Colonies were screened via PCR, such that colonies containing the α-synA53T-EGFP gene (lane 1) were distinguishable from those containing the α-synA53T* gene of interest (lane 2). (C) Enzymatic digestion of DNA extracted from positive colonies was analysed via agarose gel electrophoresis, such that the α-synA53T-EGFP construct (800 bp, lane 1) is distinguishable from the α-synA53T* construct (300 bp, lane 2). (D) Following transfection of N2a cells with a construct encoding for expression of α-synA53T-EGFP (lane 1) or α-synA53T* (lane 2), whole cell lysates were analysed via immunoblot in which α-syn was detected via a monoclonal mouse anti α-syn antibody and a HRP-conjugated anti-mouse secondary antibody. For agarose gel electrophoresis, the position of Hyperladder II and Hyperladder I bp markers are shown where appropriate to left and right of gels respectively. For immunoblot analysis, arrows indicate the position of molecular weight markers in kDa.
The successfully cloned construct containing α-synA53T* was used to transfect N2a cells and, following 48 hours incubation to allow overexpression of the protein, lysates were analysed via immunoblotting (Figure 5.5D). As observed previously, transfection of cells to express α-synA53T-EGFP (positive control) resulted in an immunoreactive band at approximately 44 kDa, consistent with the full length α-synA53T-EGFP. In contrast, in cells transfected to express α-synA53T* an immunoreactive band at approximately 27 kDa, consistent with the expected size of the α-synA53T* protein was evident. As expected, cells expressing α-synA53T* were non-fluorescent.

Having established that α-synA53T* is expressed in N2a cells following transfection, its propensity to form intracellular inclusions was investigated. N2a cells transfected with the α-synA53T* encoding construct were immunostained for α-syn and mounted on glass slides for imaging via confocal microscopy (Figure 5.6A-D). Qualitative analysis of cells positive for α-syn revealed the formation of fluorescent puncta, which appeared to be both distributed throughout the cytosol (Figure 5.6B, D) and at the periphery of cells (Figure 5.6C, D). The presence of these puncta in transfected cells was quantified, and the percentage of transfected cells containing inclusions found to be 31 ± 5.5%. Following treatment with the proteostasis inhibitors 3-MA (autophagy inhibitor) or MG132 (proteasome inhibitor), there was a significant increase in the percentage of cells containing inclusions compared to the untreated or DMSO-vehicle treated samples (p<0.05; Figure 5.6E).

Filter trap assays have been used extensively in the past to assess inclusion formation in cells (Chang and Kuret 2008, Juenemann et al. 2015, Nasir et al. 2015). Typically, soluble protein moves through the membrane pores, however, aggregated protein is retained on the membrane such that proteins in the aggregates can be detected by immunoblotting. Thus, in order to confirm the immunoreactive puncta represent aggregated α-syn in inclusions, lysates
from cells transfected to express α-synA53T* (or an EGFP control) were subjected to filter trap analysis (Figure 5.6D). When recombinant monomeric α-syn was added into buffer at concentrations that exceed the level of α-synA53T* expressed in cells (see Appendix VIII for quantification), it was not trapped by the filter and therefore was not detected by immunoblotting (Figure 5.6F). Moreover, no signal was detected when recombinant monomeric α-syn was added to the lysate from untransfected cells, thus demonstrating that monomeric α-syn does not aggregate in the cell lysate as a result of the lysis procedure. Importantly, recombinant fibrillar α-syn added to buffer or spiked into the lysate from untransfected cells was trapped by the filter and detected when present at levels as low as 0.04 µM. As expected, no α-syn signal was detected in lysates from mock transfected or EGFP transfected cells (Figure 5.6G). However, α-syn was detected in cells transfected to express α-synA53T*, confirming that these cells contained aggregated forms of α-syn. Moreover, there was a significant increase in the amount of aggregated α-syn detected in lysates from cells following treatment with the proteostasis inhibitors, thus correlating with the increase in the number of cells with inclusions following this treatment (Figure 5.6E). Given the formation of α-syn immunoreactive fluorescent puncta corresponded to the accumulation of aggregated α-syn, and that this accumulation was sensitive to treatment with inhibitors of proteostasis, it was concluded that expression of α-synA53T* in N2a cells is a robust model of α-syn aggregation.
5.3.2 sHsps inhibit the deposition of α-syn into inclusions in cells

Having developed a cellular model of α-syn aggregation, it was used to examine the ability of the sHsps αB-c and Hsp27 to prevent α-syn aggregation in cells. N2a cells were co-transfected to express α-synA53T* and αB-c (using an αB-c encoding IRES construct). Following immunohistochemical labelling of both the α-syn and αB-c, cells were imaged via confocal microscopy. The interaction between α-syn and αB-c can be qualitatively observed. As expected, cells containing diffuse EGFP (expressed from the IRES construct and

Figure 5.6: α-synA53T* forms punctate inclusions in N2a cells. N2a cells were transiently transfected with a construct expressing α-synA53T* and incubated for 48 hours before being treated (or not) with 3-MA (10 mM), MG132 (10 µM) or an equivalent volume of the vehicle control (DMSO) and incubated for a further 48 hours. Intracellular α-syn was fluorescently labelled via immunohistochemistry, using a monoclonal mouse anti-syn antibody and a DyLight650-conjugated anti-mouse secondary antibody. (A-D) Fluorescent images of cells containing both cytosolic intracellular (B, D) and membrane-localised (C, D) inclusions are presented. Scale bars represent 5 µm. (E) The percentage of cells containing inclusions was quantified by manually counting at least 50 cells and the number of α-syn positive cells containing fluorescent puncta. Data are displayed as mean ± S.E.M. (n=4), and was analysed via one-way ANOVA with a Dunnett’s multiple comparison post-test, where * indicates p<0.05 compared to untreated or DMSO-treated controls. (F) Monomeric or fibrillar recombinant α-syn was added at (i) 5 µM (ii) 1 µM, (iii) 0.2 µM and (iv) 0.04 µM to either 50 mM phosphate buffer (pH 7.4) or 200 µg of untransfected cell lysate. Samples were analysed via filter trap using a 0.2 µm membrane, and detected using a monoclonal mouse anti-α-syn followed by a HRP-conjugated secondary antibody. (G) Cell extracts prepared from (i) mock transfected cells, and cells transfected with (ii) an EGFP vector control or (iii) the α-synA53T* construct. Cells were incubated in the absence (Untreated) or presence (Treated) of proteostasis inhibitors (10 µM MG132 and 3 µM thapsigargin). Samples were analysed via filter trap using a 0.2 µm membrane, and detected using a monoclonal mouse anti-α-syn followed by a HRP-conjugated secondary antibody. Samples were analysed on one membrane, and the image from this membrane has been truncated for clarity.
indicative of the presence of αB-c; Figure 5.7) were also immunoreactive for αB-c. Notably, the subcellular localisation of the EGFP and αB-c fluorescence was not correlated. In particular, EGFP was diffuse throughout the cytosol and nucleus, while αB-c immunoreactivity was contained to the cytosol. Moreover, some cells had fluorescent puncta that contained both αB-c and α-syn. However, not all α-syn inclusions contained αB-c; co-localisation of αB-c only occurred in approximately 10% of α-syn fluorescent puncta.

**Figure 5.7:** αB-c co-localises with α-synA53T* in some intracellular inclusions. N2a cells were transiently transfected with a construct expressing α-synA53T* and an IRES construct resulting in the expression of EGFP and αB-c. Forty-eight hours following transfection, cells were treated with MG132 and thapsigargin (10 μM and 3 μM respectively), then incubated for a further 48 h. Cells were immunolabelled for intracellular αB-c and α-syn using polyclonal rabbit anti αB-c and monoclonal mouse anti α-syn antibodies, which are detected using DyLight650- and DyLight550-conjugated secondary antibodies respectively. The emitted fluorescence of the conjugates and EGFP were then detected using a confocal microscope with fluorophore-specific excitation and emission wavelengths. Arrows indicate co-localised staining of α-syn and αB-c in inclusions. Scale bars represent 5μm.

Having established the potential for αB-c to interact with α-syn in this cell model, the ability of αB-c and Hsp27 to influence the aggregation and deposition of α-syn in cells was examined. N2a cells co-transfected with the α-synA53T* and IRES constructs encoding EGFP and either αB-c, Hsp27 or EGFPinv were treated with proteostasis inhibitors. To enable imaging via confocal microscopy, cells containing αB-c or Hsp27 (or the EGFPinv control) were identified through expression of the fluorescent reporter EGFP (Figure 5.8A, green), and cells expressing α-synA53T* were identified by immunohistochemical labelling of the α-synA53T* with DyLight-650 (Figure 5.8B, red). This allows identification of cells containing only α-syn (red only), those containing only αB-c or Hsp27 (or the EGFPinv control; green
only), and cells of interest, which contain both the α-syn and chaperone (or control) protein (yellow) (Figure 5.8C). The number of cells containing inclusions was then manually quantified using randomly imaged fields of view and semi-automated cell counting scripts custom written for ImageJ. This involved cells being outlined using the brightfield image following manual selection, and then overlayed with the red image in grayscale (Figure 5.8D) to allow cells containing red fluorescent (α-synA53T*) inclusions to be manually identified. Co-transfected cells were then automatically determined as those displaying fluorescence above the threshold in both the EGFP and red fluorescent channels, and only those co-transfected cells were considered for further analysis. The percentage of co-transfected cells containing inclusions was then calculated and normalised to the EGFP control (Figure 5.8E).

Expression of αB-c was found to significantly reduce the number of cells containing inclusions in untreated cells (p<0.05) and in those treated with the proteostasis disruptors thapsigargin and MG132 (p<0.01). Whilst αB-c also reduced the proportion of cells containing inclusions following treatment with 3-MA, this effect was not found to be significant (p>0.05, n=4). Co-transfection with Hsp27 was found to significantly reduce inclusion formation in both the untreated and treated cells expressing α-synA53T* by approximately 30% (p<0.05, n=4).
5.3.3 Hsps do not prevent the accumulation of Htt inclusion bodies

Having demonstrated that the sHsps αB-c and Hsp27 can inhibit the intracellular aggregation of α-syn, experiments were conducted to examine whether they are also capable of preventing the intracellular aggregation of another disease-related protein, the exon 1 fragment of Htt. Expression of the fluorescently tagged exon 1 fragment of Htt is a well characterised intracellular aggregation model (Olshina et al. 2010, Arrasate and Finkbeiner 2012, Ramdzan et al. 2012, Ormsby et al. 2013) and represents a robust, high throughput model in which to test the efficacy of Hsps in preventing the formation of inclusions. Thus,
the ability of αB-c and Hsp27 to prevent Htt aggregation in cells was compared to other Hsps, namely Hsp40, Hsp70 and Hsp90, that have previously been shown to influence Htt aggregation into inclusions in cells (Wytenbach et al. 2000).

Following transfection of N2a cells with control (Htt25Q) or aggregation-prone (Htt46Q) polyQ variants of Htt fused to the fluorescent protein mCherry, the cells were analysed via PulSA. The forward and side scatter signals of events were used to select live, viable cells, and to exclude events corresponding to cellular debris and cell-doublets. Live cells expressing Htt were selected based on their mCherry fluorescence (Figure 5.9A), and were then analysed via PulSA. The PulSA of the Htt25Q mCherry fluorescence (Figure 5.9C) was used to define the population of cells not containing inclusions as described previously (Ramdzan et al. 2012) (cells expressing Htt25Q do not develop a significant number of inclusions under the experimental conditions). Some cells expressing Htt46Q exhibited a narrower pulse width and higher pulse height, indicative of cells with inclusions (Ramdzan et al. 2012) (Figure 5.9D). When considering the proportion of mCherry-positive cells containing inclusions, there was a significant effect of co-transfection of the cells with an IRES construct on the proportion of cells with inclusions (F(3,8) = 10.26, p<0.0001) (Figure 5.9E). Co-transfection of cells with the Htt-encoding and IRES constructs led to a significant decrease in the proportion of cells with inclusions compared to when cells were transfected only to express Htt46Q-mCherry alone (p<0.0001). However, there was no significant difference in the proportion of cells containing inclusions in cells co-transfected to express any of the Hsps compared to those co-transfected to express the control proteins EGFPinv or LacZ.

By only accounting for cells expressing Htt-mCherry, the above analysis assumes that cells expressing Htt-mCherry are co-transfected and therefore, in this case, express proteins from
the IRES constructs. Moreover, it does not take into account potential differences in co-
transfection efficiency between samples. The bicistronic constructs, in which the expression
of the Hsps (or control proteins, EGFP\textsuperscript{inv} or LacZ) is correlated with the expression of a
fluorescent reporter (in this case EGFP) (Assoc. Prof. Heath Ecroyd, personal
communication), enable the identification and selection of co-transfected cells (i.e. cells
expressing both the Hsp or control protein and Htt46Q) for subsequent analyses. Thus,
mCherry-positive cells and EGFP-positive cells (Figure 5.9A, B) were selected and analysed
by PulSA (Figure 5.9C, D) in order to quantify the relative proportion of cells containing
inclusions. There was a significant effect of treatment on the proportion of cells containing
inclusions (F (7, 24) = 7.338, p<0.0001). Hsp90 significantly increased the proportion of cells
containing inclusions (2.10 ± 0.24 fold) relative to the EGFP\textsuperscript{inv} control (p<0.0001, Figure
5.9F). Whilst there was trend towards cells expressing Hsp70, Hsp40 or Hsp70 and Hsp40
having a higher proportion of cells with inclusions, this was not significantly different to cells
expressing EGFP\textsuperscript{inv}. There was no effect of αB-c or Hsp27 expression on the proportion of
cells with Htt-mediated inclusions.

Finally, it has previously been demonstrated that the propensity of an aggregation-prone
protein to form inclusions is strongly correlated with its level of expression in cells, i.e. cells
expressing higher levels of protein are more likely to develop inclusions (Ramdzan et al.
2012, Ormsby et al. 2013). The levels of expression of Htt46Q following co-transfection with
the various IRES constructs was therefore considered by using the median mCherry
fluorescence (Figure 5.9G) as a measure of the relative Htt content in cells. The median
mCherry fluorescence varied significantly with co-transfection with the different IRES
constructs (F (7, 3) = 5.684, p = 0.0009). Cells co-transfected to express Htt46Q and Hsp40
or Hsp90 had significantly higher levels of Htt than those co-transfected to express Htt and
the EGFP\textsuperscript{inv} control. Interestingly, the relative levels of Htt in cells following co-transfection
correlated with the trends observed in the relative proportion of cells with inclusions, such that higher Htt levels were associated with a higher proportion of cells containing inclusions (Figure 5.9F, G). By taking into account the levels of Htt in cells when comparing the effect of Hsp expression on the proportion of cells with inclusions (Figure 5.9H), it was found that there was no significant effect of the Hsps on the aggregation propensity of the Htt46Q in N2a cells (F(7, 24) = 1.057, p = 0.4199).
Chapter 5: sHsp chaperone activity in cellular models of aggregation

Figure 5.9: Hsps do not prevent the cellular deposition of Htt into inclusions. N2a cells were transiently transfected with constructs encoding the aggregation prone Htt46Q-mCherry or control Htt25Q-mCherry protein, in the absence or presence of a 1:4 (IRES: Htt) ratio of IRES constructs encoding EGFP and Hsps (Hsp40, Hsp70, Hsp90, αB-c, or Hsp27), or control proteins (EGFPinv or LacZ). Following transfection, cells were incubated for 36 h, before being analysed via flow cytometry. Scatter plots of the FSC and SSC signals were used to select live cells, and levels of mCherry fluorescence or EGFP fluorescence of untransfected cells were used to select (A) mCherry and (B) EGFP-positive cells. These cells were then analysed using PulSA (Ramdzan et al. 2012). (C) The population of cells with inclusions (iipop) was identified based on the PulSA plot of cells expressing Htt25Q-mCherry (very few of these cells develop inclusions (Ramdzan et al. 2012)). (D) Cells expressing Htt46Q with inclusions were identified and enumerated. (E) The percentage of mCherry-positive (i.e. Htt-expressing) cells containing inclusions is expressed as a fold change relative to the EGFPinv control. Alternatively, live mCherry positive cells can be further gated to select cells co-transfected with the bicistronic construct and so expressing EGFP and a Hsp (or control protein), as shown in B. (F) The percentage of co-transfected cells with inclusions is expressed as the fold change relative to the EGFPinv control. (G) The median of mCherry fluorescence in co-transfected cells. (H) The proportion of co-transfected cells containing inclusions taking into account the relative level of Htt expressed in the co-transfected cells. In panels E-H the data is presented as mean ± S.E.M. (n=4) and was analysed via a one-way ANOVA and a Dunnett’s post-test, where *, ** and *** denotes a significant difference (p<0.05, p<0.01 and p<0.001 respectively) compared to the EGFPinv control sample.
5.4 Discussion

In order to capture the complex factors influencing aggregation in the cell, it is essential to complement *in vitro* studies with cellular models of aggregation. In addition, models which are robust and amenable to medium-to-high throughput analyses are invaluable when pursuing quantitative evaluation of modulators of the aggregation process, such as molecular chaperones. In this work several avenues were investigated to establish a robust and reliable cell-based model of α-syn aggregation that could be used to assess the ability of Hsps to prevent α-syn aggregation in cells. A significant finding of this work was that expression of the sHsps αB-c and Hsp27 significantly decreased the proportion of cells containing α-syn inclusions, even when the cells were treated with inhibitors of autophagy or the proteasome. An important aspect of these experiments was the incorporation of a non-chaperone protein as a control (in this case an ‘invisible’, non-fluorescent variant of EGFP, EGFPinv). This ensured that all samples received the same amount of DNA and had a comparable ‘load’ with regards to the number of proteins to produce due to co-transfection. This avoids confounding effects resulting from higher protein expression levels of an aggregation-prone protein, which itself influences the number of inclusions formed in cells (Appendix IX). Inhibition of α-syn aggregation by the sHsps is consistent with previous work in which Hsp27 was shown to decrease inclusion formation with similar efficacy to Hsp70 in a cellular model of α-syn aggregation (Outeiro et al. 2006). In this previous study, αB-c reduced the percentage of cells containing inclusions by approximately 10%, although this effect was not significant (Outeiro et al. 2006). Taken together with the results presented here, these data provide strong evidence that the sHsps are potent inhibitors of α-syn aggregation in cells.

It remains to be determined whether or not this inhibition leads to an increase in cell viability. There is still much debate concerning whether or not inclusions represent a protective end point of aggregation or a detrimental side effect of the process in cells. Sequestration of toxic
species through the active partitioning of aggregates and formation of inclusions is thought to be protective, with emerging theories relating the presence of Lewy bodies in healthy neurons with the removal of toxic oligomers from the cytoplasm (Gibb and Lees 1988, Gertz et al. 1994, Hindle 2010). In contrast, the accumulation of cellular proteostasis machinery inside these deposits likely depletes these essential components from the cytoplasm, leading to a loss-of-function which may therefore be detrimental to the health of the cell. Furthermore, correlations between increased \( \alpha \)-syn inclusions and reduced cell viability have been demonstrated in some cell models (Wan and Chung 2012). Therefore, it would be useful in future studies to investigate whether populations displaying decreased inclusion formation in the presence of sHsps have enhanced cell viability.

Fusion of a fluorescent protein to a protein of interest is a well described method for studying intracellular aggregation and a key element in facilitating high throughput analyses (Ramdzan et al. 2012). Thus, the first approach investigated in this work was to fuse EGFP to \( \alpha \)-syn variants. Transfection of cells and immunoblot analysis showed that the \( \alpha \)-syn-EGFP-fusion protein was expressed in cells. Whilst no obvious inclusions were observed in cells expressing \( \alpha \)-syn-EGFP via confocal microscopy (compared to cells expressing TDP-43-tGFP in which inclusions were readily observed), there was some evidence of fluorescent protein accumulations which may represent protein aggregates. However, in cells expressing \( \alpha \)-syn-EGFP, no cells with inclusions were able to be detected by PulSA. Importantly, cells containing TDP-43 inclusions were also not able to be detected by PulSA. This is likely to be because PulSA relies on relatively large changes in the pulse height and pulse width in cells with inclusions compared to those in which the fluorescent protein is evenly distributed in the cytosol. It therefore readily detects cells with inclusions when the inclusions are large (such as those formed by Htt46Q), however, it is unable to detect cells that contain smaller inclusions. This suggests that even if a significant proportion of cells expressing \( \alpha \)-syn-EGFP
developed inclusions, it is unlikely that the PulSA technique would be useful in detecting them.

As an alternative approach to quantitatively evaluate the aggregation of α-syn into inclusions, cells were assessed by fluorescence microscopy. Despite evidence that some cells expressing α-syn-EGFP contain aggregates, the level of cells with inclusions remained very low (5 – 10% of cells) and did not increase even in the presence of proteostasis inhibitors or inducers of cell stress, both of which have been previously reported to increase α-syn aggregation in cells (Bertoncini et al. 2007). Thus, it was concluded that the fusion of the large fluorescent protein EGFP is unsuitable for developing a robust cellular model of α-syn aggregation. The addition of this relatively large (approximately 27 kDa) fluorescent protein to α-syn (14.5 kDa) is likely to interfere with the physical properties of α-syn, significantly altering the structure, conformation and/or chemical properties of the protein, and, in this case, its aggregation-propensity in cells (Crivat and Taraska 2012).

A cell-based model using untagged α-syn would be ideal, however, previous work has demonstrated that expression of α-syn alone does not result in significant levels of aggregation in cells (Paxinou et al. 2001, Matsuzaki et al. 2004). Previous work has reported that whilst α-syn-EGFP did not readily aggregate in cells, a cellular cleavage product consisting of α-syn-EGFP lacking the last 155 residues of EGFP, does readily aggregate into inclusions in cells (McLean et al. 2001). Of note, a 27 kDa cleavage product immunoreactive with the α-syn antibody was also observed in N2a cells overexpressing α-synA53T-EGFP in this work (see Figure 5.2). Consistent with these previous studies (McLean et al. 2001), this α-synA53T* protein was found to readily form inclusions in cells, which were detectable via immunolabelling of α-syn and by filter-trap assay. Importantly, a significant proportion of cells (~30%) developed inclusions under the experimental conditions used in this work and
Chapter 5: sHsp chaperone activity in cellular models of aggregation

this could be significantly increased by treatment with inhibitors of proteostasis pathways (i.e. the proteasome and autophagy). Moreover, it has been previously demonstrated that the aggregation of α-synA53T into inclusions is mediated by the α-syn portion of the protein, since when the same region of EGFP was fused to other non-aggregation-prone proteins that were expressed in cells, these cells did not develop aggregates (McLean et al. 2001). Thus, it is likely that the additional EGFP fragment at the C-terminus of α-syn acts to destabilise it and that this is key to recapitulating the cellular aggregation of α-syn associated with disease.

Importantly, C-terminal cleavage products of α-syn (α-synΔC) have been identified in vivo. For example, human brain extracts containing pathological α-syn Lewy bodies have significant levels of α-synΔC which preferentially localises to detergent-insoluble fractions following extraction of protein aggregates (Li et al. 2005). Moreover, C-terminal truncation of α-syn significantly increases its aggregation propensity when compared to α-synWT (full length) in vitro using ThT-based aggregation assays (Hoyer et al. 2004, Li et al. 2005). In addition, the incorporation of substoichiometric ratios of α-synΔC variants significantly enhances the aggregation of α-synWT in vivo (Li et al. 2005). Finally, α-synΔC variants increase the vulnerability of cells in culture to toxic assault by oxidative stressors (Kanda et al. 2000). These effects have been attributed to the absolute net charge of α-syn, given the highly acidic nature of the C-terminal region. In fact, truncation of just 16 residues from the C-terminal region of α-syn decreases the net charge of the protein from 9.0 to 3.0 at pH 7.5 (Hoyer et al. 2004). Similar to other amyloid-forming proteins in which charge state constitutes a major determinant of aggregation rate, electrostatic effects resulting from interaction with or truncation of the C-terminal of α-syn lead to an increase in its aggregation-propensity (Chiti et al. 2002, Chiti et al. 2003, Schmittschmitt and Scholtz 2003). Together, these data indicate that the C-terminal region of α-syn plays a critical role in governing its propensity to aggregate. Thus, this may account, at least in part, for the increased propensity
of α-synA53T* to form inclusions in cells. A cell model that incorporates co-expression of full-length α-syn with an α-synΔC isoform may also result in a robust cellular model of α-syn aggregation.

There is conflicting evidence regarding the co-localisation of sHsps with α-syn inclusions in cell-based models of aggregation. Using a cell culture model in which co-transfection with α-syn and synphilin-1 produces intracellular inclusions of α-syn, McLean and colleagues (2002) failed to find any evidence for the co-localisation of Hsp27 with aggregated α-syn. Conversely, Outeiro and colleagues (2006), using a similar transfection model, found that both Hsp27 and αB-c co-localise to α-syn positive inclusions. Notably however, both these previous studies demonstrated the presence of Hsp27 (McLean et al. 2002, Outeiro et al. 2006) and αB-c (Outeiro et al. 2006) in α-syn positive Lewy bodies from diseased patient tissues, providing evidence that sHsps can co-localise with α-syn inclusions in vivo. In this work, αB-c was found to be co-localised in some, but not all, α-syn positive inclusions formed in N2a cells. Whether sHsps co-localise with α-syn in an inclusion may be due to the type of inclusion formed by α-syn. Although some inclusions are essentially static depositions of protein aggregates, many inclusions are dynamic structures in which accessory proteins, such as chaperones, may diffuse in to and out of over the lifetime of the structure (Kaganovich et al. 2008).

Several distinct types of protein inclusions have been identified to date including aggresomes, juxtanuclear quality control (JUNQ), insoluble protein deposits (IPODs), RNA interactor specific compartments/inclusions, aggresome-like structures, ER-associated degradation-associated vesicles and intranuclear protein quality control compartments. These represent discrete structures with diverse but specific mechanisms of formation. For example, aggresomes are cell-driven, microtubule-dependent juxtanuclear inclusions which organise
and sequester aggregates to regions of the cell where they are associated with chaperone and proteasome subunits (Johnston et al. 1998, Kopito 2000). JUNQ inclusions are thought to be closely related to aggresomes, as they are composed primarily of ubiquitinated proteins, proteasomal subunits and refolding chaperones such as Hsp70 (Kaganovich et al. 2008). These inclusions have been proposed to be central refolding or degradation hubs, and it appears chaperones and proteases are able to freely diffuse in to and out of these structures (Kaganovich et al. 2008), although how they maintain the aggregated proteins within the inclusion is yet to be established. In contrast, IPODs consist mainly of aggregated proteins (most commonly insoluble amyloid fibrils) which are not ubiquitinated, and are characterised as extremely stable, dense, immobile compartments (Kaganovich et al. 2008). Partitioning of aggregates appears to depend largely on their aggregation state and ubiquitination status, such that soluble misfolded proteins are targeted for compartments capable of degradation and refolding (e.g. aggresomes and JUNQ), whereas terminally aggregated proteins are sequestered into compartments (e.g. IPOD) to prevent toxicity (Kaganovich et al. 2008).

Many of the intricate processes actively promoting formation of these structures in cells remain to be established and the specific types of inclusions formed by many aggregating proteins remains to be characterised. For example, TDP-43 has been shown to localise to JUNQ inclusions (Farrawell et al. 2015), while Htt partitions to IPODs (Polling et al. 2014). Little is known, however, of the types of inclusions formed by aggregation–prone α-syn in cells. The co-localisation of sHsp with α-syn in some of these inclusions may be reflective of a dynamic type of inclusion which evolves over the lifetime of a cell.

A range of potential mechanisms by which sHsps interact with α-syn have been explored in the preceding chapters of this thesis. For example, sHsps may interact primarily with monomeric species to prevent the formation of oligomeric nuclei. Alternatively, sHsps may bind to small α-syn oligomers and, in doing so, prevent their growth into fibrillar species that
would otherwise lead to their deposition. sHsps may also interact with fibrillar α-syn in order to prevent secondary nucleation processes that would otherwise increase the amount of aggregated material and enhance deposition of α-syn into inclusions. Finally, sHsps may prevent the release or uptake of α-syn aggregates that seed aggregation and propagate inclusion formation to neighbouring cells. Most likely, the inhibition of α-syn inclusion formation by the sHsps relies on a combination of these mechanisms. Development of techniques that enable the aggregation state of α-syn to be tracked and related to specific biological outcomes in intact cells would greatly assist future work in this area. For example, the development of conformational sensors of α-syn capable of distinguishing monomeric, oligomeric and fibrillar species, such as those developed for Htt (Ormsby et al. 2013), would facilitate investigation into the precise mechanism(s) by which sHsps act to prevent α-syn aggregation in cells.

This work also tested whether the sHsps, αB-c and Hsp27, inhibited the intracellular aggregation of proteins other than α-syn. Previous work has demonstrated that Htt undergoes nucleation-dependent, fibrillar aggregation (Scherzinger et al. 1999) in vitro and in vivo. Fusion of exon 1 of the Htt protein to a fluorescent protein enables Htt aggregates to be distinguishable from diffuse cytosolic protein (Ramdzan et al. 2012). In this work the impact of a range of Hsps, including Hsp40, Hsp70, Hsp90 and the sHsps Hsp27 and αB-c, on the formation of Htt-mCherry inclusions in N2a cells was investigated. An important aspect of this work was the use of non-chaperone proteins (i.e. EGFPinv and LacZ) as controls in these experiments. Whilst both the EGFPinv and LacZ proteins significantly reduced the number of cells containing aggregates when compared to cells transfected to express Htt46Q alone, this was seen to be simply due to the decrease in the amount of Htt DNA used to transf ect cells in the co-transfection samples (Appendix IX).
Overall, PuLSA of cells expressing Htt revealed that the Hsps had little effect on the proportion of cells with inclusions. In analysing these flow cytometry data, the first approach taken was that used in previous studies (Ramdzan et al. 2012), i.e. the analysis of cells that express the aggregation-prone protein and comparison of the proportion of cells with inclusions across treatments. However, whilst this approach has been used extensively in the past, it does not take into account differences in co-transfection efficiencies that can occur between treatments. In this work, this limitation was overcome by exploiting IRES constructs which, through the expression of a fluorescent reporter protein (in this case EGFP), enabled selection and analysis of only cells that had been co-transfected. Moreover, whilst cells expressing the Hsps (or control proteins) could be identified, the Hsps themselves were not tagged with a bulky fluorescent protein which could compromise their chaperone activity. Analysis of co-transfected cells using this approach revealed that expression of Hsp90 led to an increase in the proportion of cells containing Htt inclusions compared to cells expressing EGFP<sup>inv</sup>.

An important regulator of the aggregation-propensity of a protein is the levels at which it is expressed in cells. For example, a previous study demonstrated that the aggregation of Htt in cells is ‘concentration-dependent’, i.e. that cells containing more Htt were more likely to develop inclusions (Ormsby et al. 2013). In this work the levels of Htt in co-transfected cells was found to vary between treatments, with higher levels of Htt expressed in cells co-expressing Hsp40 and Hsp90. This may therefore account for the higher proportion of co-transfected cells expressing Hsp90 with inclusions. When these differences in Htt levels between treatments were taken into account in the analysis, there was no significant difference in the proportion of cells with inclusions when any of the Hsps were co-expressed in cells compared to the controls.
Little is known about the interaction of the sHsps or Hsp90 with Htt, however, the findings of this work that Hsp27 and Hsp70 do not reduce the aggregation of Htt in cells is consistent with earlier studies (Wyttenbach et al. 2000, Ormsby et al. 2013). However, in contrast to the findings of this work, the previous studies by Wyttenbach et al. (2000) and Ormsby et al (2013) reported that expression of Hsp40 reduces the proportion of Htt-expressing cells with inclusions. The most likely reason for the discrepancy between the findings of this work and these previous studies is the differences in the ratios of Htt to Hsp DNA used to transfect cells. In this work a 4-fold excess of Htt DNA over IRES DNA was used to transfect cells, however, an equal volume of both Htt and Hsp-encoding DNA constructs were used in these previous studies (Wyttenbach et al. 2000, Ormsby et al. 2013). It is therefore likely that the lower proportion of Hsp40-encoding DNA used in this work led to a lower ratio of Hsp40 to Htt in cells and this was insufficient to prevent Htt aggregation in the cells. This suggests that higher stoichiometric ratios of the Hsps to Htt may be necessary in order for the Hsps to prevent the aggregation of Htt in cells.

Despite the limitations discussed above, this work was successful in using cellular models of aggregation to investigate the activity and relevance of molecular chaperones in a cellular environment. A robust cell-based model of α-syn aggregation was developed, and was subsequently exploited to demonstrate that the sHsps Hsp27 and αB-c prevent the aggregation of α-syn in cells. Ongoing investigation of the sHsps and their interaction with α-syn using models amenable to high throughput single cell analysis is critical in order to provide a complete picture of their potential as mediators of the cellular aggregation and toxicity of this protein. However, the results presented here highlight that the sHsps are viable therapeutic targets in the context of the α-synucleinopathies. In addition, examining sHsp activity against a suite of other aggregation-prone proteins, as done in this work with Htt, will be essential when evaluating their protective capacity against other protein aggregation-
related diseases. The use of chaperone-encoding IRES constructs will be invaluable in this
dependence, as they enable cells expressing Hsps to be identified without the addition of a
fluorescent tag, which may otherwise compromise Hsp activity.
Chapter 6:
Conclusions and future directions
The maintenance of proteostasis is crucial for cell and organism survival. The sHsps are essential components of the proteostasis network, protecting the cell from pathogenic aggregation of misfolded or non-native protein intermediates. However, the abundance of neurodegenerative diseases linked with aberrant aggregation highlight that, under some circumstances, the sHsps fail to prevent protein aggregation, and this facilitates the onset and progression of disease. One such group of diseases, the α-synucleinopathies, are characterised by the aggregation and deposition of α-syn in cells. Uncovering the mechanism(s) by which sHsps interact with aggregation-prone target proteins, such as α-syn, is therefore of great importance. Thus, the basis of the work described in this thesis was to determine how sHsps interact with α-syn to prevent aggregation and mitigate the cellular toxicity associated with this process.

6.1 sHsps interact transiently with aggregation-prone monomeric α-syn

A primary aim of this work was to establish the mechanism by which the sHsps inhibit aggregation of monomeric α-syn into fibrils. As reported in Chapter 3, the sHsps αB-c and Hsp27 interact transiently with aggregation-prone, monomeric α-syn to prevent its aggregation. Thus, the mechanism of αB-c and Hsp27 chaperone action in preventing α-syn aggregation does not involve the formation of high molecular mass complexes. Instead, the sHsps are able to briefly stabilise the aggregation-prone intermediate, presumably such that it can re-enter the protein folding-unfolding pathway. Thus, despite their traditional classification as holdase chaperones, this work demonstrates that this does not fully describe the mechanism of chaperone action of sHsps. It is yet to be determined whether the transient interaction between the sHsps and α-syn results in a conformational change in the aggregation-prone intermediate of α-syn. Previous work investigating the transient interaction between αB-c and α-lac (a model amyloidogenic protein) demonstrated that, when the αB-c
was removed from the sample, amyloid fibril aggregation of α-lac proceeded. Thus, these results suggest that the transient interactions between sHsps and aggregation-prone proteins do not substantially change the aggregation-propensity of amyloidogenic target proteins (Kulig and Ecroyd 2012). Further characterisation of the effect of the transient interactions between sHsps and α-syn would enhance our understanding of the fate of aggregation-prone target proteins in the cell once they are stabilised by the sHsps.

This work also sought to identify, for the first time, factors that may account for aggregation-prone proteins ‘escaping’ the chaperone action of sHsps, leading to disease. The work presented in Chapters 3 and 4 demonstrates that the rate of aggregation is a significant factor in determining how effectively the sHsps, αB-c and Hsp27, can prevent protein aggregation. This was consistently found to be the case regardless of whether the kinetics of α-syn aggregation were altered via increases in the monomeric α-syn concentration, fibril seed concentration or the presence of disease-associated mutations. Given the association of PD with duplication and mutation of the α-syn gene, all of which act to increase the rate at which α-syn aggregates (Bruinsma et al. 2011), these investigations highlight the physiological relevance of aggregation rate with respect to the failure of sHsps in neurodegenerative disease.

Identifying the rate of aggregation as a crucial factor in the chaperone efficacy of sHsps also serves to highlight the importance of subunit exchange dynamics with regards to the chaperone action of sHsps. The release of subunits from oligomeric reservoirs formed by αB-c and Hsp27 governs the amount of chaperone-active species available to interact with aggregation-prone intermediates. As such, future work should investigate whether factors that lead to the dissociation of sHsp oligomers into smaller subunits also increase chaperone
activity. The identification of small drug-like molecules that induce dissociation of sHsps may have therapeutic potential to treat diseases associated with protein aggregation.

6.2 sHsps bind to α-syn fibrils

Examining the potential for the sHsps to target α-syn aggregation intermediates, other than monomeric species, was also a primary aim of this work. The results presented in Chapter 4 demonstrate that Hsp27 specifically inhibits the growth and structural conversion of oligomeric nuclei formed during the aggregation of α-syn. This implies an interaction between Hsp27 and small amyloid intermediates, and was also reflected in the ability of Hsp27 to prevent the elongation of fibril fragments. These small amyloid species are key mediators of elongation and secondary nucleation processes, and have a range of potentially toxic effects in the cell (Martins et al. 2008, Xue et al. 2009, Jakhria et al. 2014, Shvadchak et al. 2015). Therefore, the results presented as part of this work highlight another mechanism by which sHsps may act to prevent further protein aggregation in the cell i.e. by binding to oligomeric nuclei or fibril fragments, and, in doing so, protect the cell from the cytotoxicity associated with this process.

Recent work has demonstrated that αB-c can interact with mature amyloid fibrils formed by α-syn (Waudby et al. 2010), apo-CII (Binger et al. 2013) and Aβ (Shammas et al. 2011). However, the mechanism of this interaction, and whether other sHsps are also capable of binding to fibrils, has not been established. The results from this work show that both αB-c and Hsp27 are able to bind to α-syn fibrils. In addition, this interaction was found to involve the N- and/or C-terminal regions of the protein, as isoforms lacking these domains (i.e. core domain variants) were unable to bind to the α-syn fibrils despite retaining the ability to prevent the aggregation of monomeric α-syn. Thus, it is concluded that the N- and/or C-
terminal regions of these sHsps are directly involved in fibril-binding, however, the ability to prevent the aggregation of monomeric α-syn is inherent to the α-crystallin core domain.

A significant finding of this work was that the interaction of sHsps with α-syn fibrils decreases the hydrophobic nature of the fibril surface and inhibits the cytotoxicity of the fibrils. These findings are particularly important given the emerging theory of prion-like cell-to-cell spreading of α-syn aggregates and the associated potential for extracellular aggregated α-syn to exert toxic effects on neighbouring cells (Lee et al. 2005, Kordower et al. 2008, Lee et al. 2008, Lee et al. 2008, Li et al. 2008). It remains to be established precisely how, by binding to fibrils, sHsps mitigate the toxicity of the α-syn fibrils. Given the toxicity of aggregates is strongly linked with their relative hydrophobicity (Bolognesi et al. 2010), and the present study established the ability of sHsps to decrease the relative hydrophobicity at the fibril surface, then shielding surface hydrophobicity is a likely mechanism by which the sHsps inhibit toxicity. It is also possible that, by binding to fibrils, the sHsps prevent fragmentation and secondary nucleation events that would otherwise generate more cytotoxic oligomers. Recent work has shown that, by binding to fibrils formed by apo-CII, αB-c prevents their dilution-induced fragmentation (Binger et al. 2013). Thus, future work could explore whether, by binding to α-syn fibrils, sHsps stabilises them in order to prevent fragmentation. Determining whether the ability of sHsps to stabilise fibrillar forms of proteins is a generic property of the sHsps or specific to particular sHsps and fibrils, would represent a significant avenue for further investigation.

Another potential area for future study would be investigating whether the α-syn fibrils must be taken up by cells in order to exert their toxic effects or whether these effects are mediated by them binding to the cell membrane. This would assist in understanding how the sHsps inhibit the cytotoxicity associated with the addition of α-syn fibrils to cells, and guide the
development of drugs that could mimic the binding of sHsps to α-syn fibrils. For example, if the uptake of fibrils into cells is mediated by a specific surface receptor, it may be possible to target this receptor by mimicking how sHsps bind to the fibrils. Alternatively, developing a molecule to block the relevant receptor, and therefore prevent fibrils from binding to the receptor, may also have therapeutic potential.

This work specifically considered intracellular ROS generation as a measure of fibril-associated toxicity. However, many other toxic effects have been linked with amyloid fibrils (Luk et al. 2009, Pieri et al. 2016). It therefore would be beneficial to examine whether, by binding to fibrils, the sHsps inhibit other fibril-associated toxic effects, as this may provide other promising avenues and pathways to target in the development of therapeutics to treat diseases associated with protein aggregation.

6.3 sHsps prevent the aggregation of α-syn into inclusions in cells

The work presented in Chapter 5 aimed to develop a cell model of α-syn aggregation that could subsequently be used to determine whether sHsps can prevent the aggregation of α-syn into inclusions in cells. It was found that, when over-expressed in cells expressing aggregation-prone α-syn, both Hsp27 and αB-c decrease the aggregation of α-syn into inclusions in a neuronal cell line (N2a). This provides evidence that over-expression or boosting the activity of sHsps may be a valid way to prevent amyloid fibril aggregation in cells. However, the inability of the chaperones to prevent the intracellular aggregation of Htt highlights the substrate specificity of the sHsp-target protein interaction in cells. Importantly, the sHsps have previously been shown to prevent the cellular toxicity of Htt aggregates without directly inhibiting aggregate formation (Wyttenbach et al. 2000). This again highlights that the sHsps play a role in multiple protective pathways in cells in the context of
amyloid aggregation. Elucidating each of these molecular mechanisms would significantly enhance our understanding of the role sHsps have in cells.

One possible method for further developing a cell-based model of α-syn aggregation would be the introduction of a functional fluorescent tagging system to allow real-time tracking of α-syn in cells. The introduction of a C-terminal tag that replaces the truncated EGFP used in this work, but still serves to destabilise the protein (and therefore enhance its aggregation propensity), is a possibility. For example, the use of a series of self-labelling enzymes, known as Halo or SNAP/CLIP tags, which are fused in frame with the protein of interest, enables the introduction of small fluorescent ligands (Lin and Wang 2008). Alternatively, a tetracysteine-biarsenical approach, which requires the introduction of a short peptide sequence (Cys-Cys-X-X-Cys-Cys, where X is any amino acid other than cysteine) that reacts with a biarsenical dye, most commonly fluorescein arsenical hairpin binder (FlAsH; emitting green fluorescence) or resorufin arsenical hairpin binder (ReAsH; emitting red fluorescence), enables specific labelling of proteins in cells with small fluorophores (Gaietta et al. 2002). In fact, the latter approach has previously been used to investigate α-syn aggregation in cells, however, only a small number of cells were found to contain inclusions (<10%, corresponding to only 8 – 15 cells per treatment) (Bertoncini et al. 2007). Extending this approach to encompass a larger population of cells may prove beneficial in the search for a high-throughput model of intracellular α-syn aggregation.

**6.4 A model of how sHsps interact with aggregation-prone α-syn**

Based on the results presented in this thesis, it is clear that the sHsps target multiple aggregation-prone species in order to inhibit amyloid accumulation and mediate its toxic effects. These aspects of sHsp activity, in the context of the α-syn aggregation pathway, are summarised in Figure 6.1. The sHsps prevent the aggregation of monomeric α-syn through...
transient interactions, without forming stable high molecular mass complexes (Chapter 3). The sHsps also prevent the growth of small oligomeric nuclei, inhibiting the formation of mature fibrils (Chapter 4). In addition, the sHsps form stable complexes with amyloid fibrils, decreasing their surface hydrophobicity and inhibiting their toxicity (Chapter 5). Finally, by inhibiting the aggregation of α-syn, the sHsps decrease the appearance of inclusions in a neuronal cell model (Chapter 5).

A focus of future work should be defining the relative importance of the interactions of sHsps with specific α-syn intermediates in a cellular context. This is because it is still unclear which of the interactions observed in vitro (i.e. transient interactions with monomeric α-syn or stable interactions with fibrillar α-syn) occur in cells. Studies utilising fluorescently labelled Htt proteins (e.g. mCherry fusion proteins) coupled with the introduction of a tetracysteine motif have enabled the subcellular localisation and oligomeric state of Htt to be monitored in cells (Ramdzan et al. 2010). When the protein is monomeric, binding of the biarsenical dyes is unperturbed and fluorescence from both the bound dye and fluorescent protein may be detected; however, the formation of oligomers occludes the binding site of the biarsenical dye and thus the loss of dye-associated fluorescence reports on the presence of oligomers. Cells with large inclusions can be identified by the PulSA flow cytometry. Thus, together this work has facilitated the distinction between monomeric, oligomeric and fibrillar forms of Htt in cells and allows the presence of different conformations to be related to cellular outcomes (Ormsby et al. 2013). Moreover, changes in the relative abundance of these forms were able to be determined upon addition of aggregation modifiers such as chaperones. A similar approach could be pursued in order to determine the forms of α-syn that exist within cells. This would provide a method by which to address forms of α-syn that the sHsps interact with in the cell.
Chapter 6: Conclusions

Figure 6.1: sHsps interact with various species formed during the fibrillar aggregation of α-syn. Unfolded α-syn aggregates through a nucleation-dependent mechanism, in which prefibrillar nuclei elongate via the addition of monomer to form amyloid fibrils. Fragmentation of mature fibrils can generate additional oligomeric nuclei, further perpetuating aggregation. Fibrils may be sequestered into inclusion bodies, or exert toxic effects on neighbouring cells following their release into the extracellular environment. (1) The sHsps prevent the aggregation of monomeric α-syn via weak, transient interactions, maintaining it in a monomeric state and preventing the growth of oligomeric nuclei. (2) The interaction of sHsps with prefibrillar intermediates or fibril fragments prevents their elongation into mature α-syn fibrils. sHsps can bind to fibrillar forms of α-syn with moderate (μM) affinity. In doing so, they decrease the surface hydrophobicity of fibrils and inhibit the cytotoxicity exogenous fibrils exert on neighbouring cells. Through these actions, sHsps are also able to inhibit the aggregation and deposition of α-syn in cells. It is as yet unclear whether sHsps can inhibit the fragmentation of α-syn fibrils, or inhibit the potential cytotoxicity of inclusion formation. Solid lines represent pathways well supported by the literature and/or the work described in this thesis. Dotted lines represent potential pathways that are yet to be fully characterised.

The results presented in this thesis contribute to a growing body of evidence that the term ‘holdase’ does not fully describe the molecular chaperone action of sHsps. Therefore, it is proposed that the term ‘protein stabilisers’ is a more accurate description of the molecular chaperone action of sHsps (Kulig and Ecroyd 2012). In this capacity, the sHsps monitor the cellular environment and maintain potentially aggregation-prone proteins in a folding-competent state via transient interactions. Proteins at risk of catastrophic hydrophobic collapse (commonly those destined for amorphous aggregation) are bound by the sHsps into a stable high molecular mass complex to prevent their aggregation. In addition, should aggregation-prone monomeric proteins (such as α-syn) escape interaction with the sHsps (e.g.
through factors that act to increase the rate at which they aggregate), the sHsps are capable of binding to oligomeric forms to prevent further aggregation. Finally, the sHsps are also able to bind to mature aggregates and in doing so mitigate the toxicity of these species.

6.5 Concluding remarks

The amyloid fibrillar aggregation and deposition of α-syn is a pathological hallmark of many neurodegenerative diseases, and represents a failure of the proteostasis network. It is therefore imperative that we understand how the components of this pathway interact with α-syn to prevent its aggregation. A common theme evident in this work is the multi-faceted nature of sHsp chaperone action. The results presented here significantly contribute to our understanding of how the sHsps αB-ε and Hsp27 interact with various species of α-syn formed during its aggregation into amyloid fibrils. The biological relevance of these interactions should now be pursued using more sophisticated cellular and animal-based models of α-syn aggregation. Doing so will help to establish ways to boost the activity of the sHsps and enable them to better protect against α-syn aggregation and the associated cytotoxicity. In the context of the α-synucleinopathies, this may reveal potential avenues for therapeutic intervention to treat these currently intractable and debilitating diseases.
Chapter 7: References


Ellis, R. J. (1997). "Do molecular chaperones have to be proteins?" Biochemical and Biophysical Research Communications 238(3): 687-692.


Chapter 8: Appendices
Appendix I:

**Figure 8.1: sHsp variants inhibit α-syn aggregation.** Recombinant α-synWT was incubated at 300 μM in 50 mM sodium phosphate buffer with 100 mM NaCl and 0.01% NaN₃ (pH 7.4), in the absence or presence of sHsp phosphomimicking (sHsp₃D) or core domain (sHsp₇core) variants. Fibril formation was monitored by the change in ThT fluorescence at 490 nm over time. (A) A representative trace is shown for α-syn in the absence or presence of αB-c₃D or Hsp27₇core at a 1:10 molar ratio (sHsp:α-syn). Data were fitted with a Boltzmann sigmoidal curve. (B) Values obtained for the maximum change in ThT fluorescence from these fits was used to determine the percent protection afforded by the sHsp variants. Results are presented as mean ± S.E.M.
Appendix II:
The following script is for the analysis of SAVE images, and was written and executed alongside Dr. Daniel Whiten using IgorPro v 6.3.4.1.

This script opens the source image stacks, thresholds these images to remove the background and detects spots above background in each channel (ThT and 647). It then determines the percentage of spots co-localised in the ThT and 647 channels. The script also calculates the likelihood of spots being colocalised by chance, by randomly distributing the pixels of one channel and determining the percentage of spots co-localised in the randomised and raw channels.

#pragma rtGlobals=1 // Use modern global access method.

DIRECTORY/THRESHOLD SECTIONDIRECTORY/THRESHOLD SECTION

function setthresholds()
make/o/n=1 thtthreshold,redthreshold,savedpatchsize
wave thtfiltered
imagedata thtfiltered
thtthreshold[0]=v_avg + 5*v_sdev // ThT threshold
wave af647filtered
imagedata af647Filtered
redthreshold[0]=v_avg+ 5*v_sdev // Red threshold
savedpatchsize=8 // Patchsize
end

// Macro section:
// Run set from index:
macro multipleimages()
folder()
end

macro see(folder,image)
string folder="1"
Prompt folder, "Scan number to see " // Set prompt for X param
variable image=0
prompt image,"Image to see"
setdatafolder root:$folder
seeimagecoincident(image)
end

// Run set of 9 images in each folder defined by index- i.e. list of folder names as numbers:
function folder()
LoadWave/J/D/W/K=0/A // Load index.
wave wave0
duplicate/o wave0.points // Call the folder list points.
killwaves wave0
string path=S_path
variable a,b,c,d
for(d=0;d<(dimsize(points,0));d+=1)
    setdatafolder root:
    string pathleveldown=S_path+num2str(points[d])
        // This is the folder that the 3 sets of folders containing 9 images are stored in.
    string foldername1=num2str(d+1)
        newdatafolder/s $foldername1
            // Make name of folder-
c=0
    for(a=1;a<4;a+=1)
    for(b=1;b<4;b+=1)
        string filename=pathleveldown+"":"Row"+num2str(a)+"Well"+num2str(b)+"_1_641andThT.tif"
            // This may need changing depending on the name of the files.
        string pathname=pathleveldown+"":"+
            string leveldownagain=num2str(c)
                // Name of folder that will contain each image
                information.
        //newdatafolder/s $leveldownagain
        print filename
        loadmulti(filename,c)
        // Function for spot detection:
        spots()
        coincidentspots()
        c+=1
    endfor
endfor
setdatafolder root:
consolidate()
endfor
end
function load()
setdatafolder root:
    // Load - will put up prompt to look for image:
    ImageLoad/T=tiff/S=0 /C=-1 /O /N=image
    ImageLoad/T=tiff/S=0 /C=-1 /O /N=imageforchance
    // Function to extract ThT and AF647 images
    averageimages()
    // Need a function to remove backgrounds:
    removebackground(0)
end
function loadmulti(filename,c)
string filename
variable c
    ImageLoad/T=tiff/S=0 /C=-1 /O /N=image filename
    // Function to extract ThT and AF647 images
    averageimages()
    removebackground(c)
end
// Function to extract averaged images from AF647 and ThT channels:
function averageimages()
wave image
duplicate/O/R=(10,480)(10,480)(4,6) image,AF647_pre
imagetransform averageimage AF647_pre
duplicate/o M_aveimage, AF647
duplicate/O/R=(10,480)(10,480)(101,200) image,tht
imagetransform averageimage ThT
killwaves th
killwaves image
killwaves m_stdvImage
duplicate/o m_aveImage,ThT
killwaves m_aveImage
killwaves AF647_pre
end
function removebackground(c)
variable c
wave tht,af647
variable a,b,d,e,f
duplicate/o ThT,ThTfiltered,ThTbackground // Make various waves of same size
for(a=0;a<(dimsize(ThT,0));a+=1) // Go through all of pixels
  for(b=0;b<(dimsize(ThT,1));b+=1)
    make/o temp=NAN
    for(d=-2;d<2;d+=1)
      for(f=-2;f<2;f+=1) / redimension/n=(e+1) temp
        temp[e]=tht[a+d][b+f]
e+=1
  endfor
endfor
e=0
wavestats/q temp
thtbackground[a][b]=V_min // Minimum pixel value
thtfiltered[a][b]=tht[a][b]-V_min // Remove background.
endfor
duplicate/o AF647,AF647filtered,Af647background // Make various waves of same size
for(a=0;a<(dimsize(AF647,0));a+=1) // Go through all of pixels
  for(b=0;b<(dimsize(AF647,1));b+=1)
    make/o temp=NAN
    for(d=-2;d<2;d+=1)
      for(f=-2;f<2;f+=1)
        redimension/n=(e+1) temp
        temp[e]=AF647[a+d][b+f]
e+=1
  endfor
endfor
e=0
wavestats/q temp
AF647background[a][b]=V_min // Minimum pixel value
AF647filtered[a][b]=af647[a][b]-V_min // Remove background.
endfor
string namertht="thtfiltered"+num2str(c)
string namerred="af647filtered"+num2str(c)
duplicate/o thtfiltered,$namertht
duplicate/o af647filtered,$namerred
killwaves thtfiltered
killwaves af647filtered
killwaves temp
killwaves ThTbackground
killwaves AF647background
killwaves tht
killwaves af647
make/o/n=1 counter=c
end
function spots()
wave
  counter,thtfiltered0,thtfiltered1,thtfiltered2,thtfiltered3,thtfiltered4,thtfiltered5,thtfiltered6,thtfiltered7,
  thtfiltered8
wave
  af647filtered0,af647filtered1,af647filtered2,af647filtered3,af647filtered4,af647filtered5,af647filtered6,
  af647filtered7,af647filtered8
variable a=counter[a]+1
variable b
variable i,j,k,l,m
make/o/n=1
redintensityfromtht,thtcoincidentintensity,thtnoncoincidentintensity,ratiointensity,logratiointensity
Make/O ThTX,ThTY,ThTIntensity
Make/O redX,redY,redIntensity
for(b=0;b<a;b+=1)
  string loadtht="thtfiltered"+num2str(b)
duplicate/o $loadtht,thtfiltered
  string loadred="af647filtered"+num2str(b)
duplicate/o $loadred,af647filtered
imagestats ThTFiltered
setthresholds()
wave thtthreshold,redthreshold,savedpatchsize

variable thresholdtht = thtthreshold[0]
variable thresholdred=redthreshold[0]
variable patchsize=savedpatchsize[0]
duplicate/o ThTFiltered,thtfiltered
  do
    redimension/n=(i+1) ThTX,ThTY,ThTIntensity // Increase length of waves
    ImageStats /M=1 thtfiltered // Statistics of image
    if(V_max>thtthreshold[0])
      ThTX[i]=V_maxRowLoc // Maximum intensity stored.
      ThTY[i]=V_maxColLoc
      ThTIntensity[i]=V_max
      variable row=V_maxRowLoc
      variable col=V_maxColLoc
      for(j=0;j<=(2*patchsize);j+=1)
        for(k=0;k<=(2*patchsize);k+=1)
          ThTWorking[V_maxRowLoc-patchsize+j][V_maxColLoc-patchsize+k]=0
        endfor
      endfor
    endif
  endfor
  if(ThT[i]==ThT[i-1]) // Control to prevent double counting of spots.
    for(j=0;j<=(patchsize);j+=1)
      for(k=0;k<=(patchsize);k+=1)
        ThTWorking[row-patchsize+j][col-patchsize+k]=0
      endfor
    endif
  i+=1
endif
while (V_max>thtthreshold[0])
killwaves thtfiltered
killwaves thtworking
duplicate/o Af647Filtered,redworking
do
redimension/n=(l+1) redX,redY,redIntensity // Increase length of waves for new data.
   ImageStats /M=1 redworking // Statistics of image
   if(V_max>redthreshold[0])
      redX[l]=V_maxRowLoc // Maximum intensity stored.
      redY[l]=V_maxColLoc
      redIntensity[l]=V_max
      row=V_maxRowLoc
      col=V_maxColLoc
   // This part is for straight-forward delete.
   for(j=0;j<=(2*patchsize);j+=1)
      for(k=0;k<=2*patchsize;k+=1)
         redworking[V_maxRowLoc-patchsize+j][V_maxColLoc-patchsize+k]=0
      endfor
   endif
   l+=1
   endif
while (V_max>redthreshold[0])
endfor
end
function coincidentspots()
   // Are there any coincident spots?
   wave counter
   variable number=8
   variable a,b,c
   variable u,q,h,x
   make/o/n=(number+1) numberoftht,numberofcoincident,numberofchance,coincidence
   make/o/n=1 coincidenttht,coincidentred,coincidentratio,noncoincidenttht,chancetht,chancred,chanceratio
   for(a=0;a<(number+1);a+=1)
      c=a-1
      //Make files to work with
      string thtname="thtfiltered"+num2str(a)
      duplicate/o $thtname,workingtht
      string redname="af647filtered"+num2str(a)
      duplicate/o $redname,workingred
      //For chance calculation:
      if(a<8)
         string redchance="af647filtered"+num2str(a+1)
      else
         redchance="af647filtered1"
      endif
      duplicate/o $redchance,workingredchance
      // Need to look for all of spots in the ThT image now. Same code as previously:
// Thresholding gubbings:
duplicate/o workingtht,thtfiltered
duplicate/o workingred,af647filtered
setthresholds()
wave thtthreshold,redthreshold,savedpatchsize
variable thresholdtht = thtthreshold[0]
variable thresholdred=redthreshold[0]
variable patchsize=savedpatchsize[0]
variable i=0,g=0
string thtx="ThTX"+num2str(a)
string thty="ThTY"+num2str(a)
string thtintensity="ThTIntensity"+num2str(a)
make/o/n=1 temp1=Nan,temp2=Nan,temp3=Nan,temp4=Nan,temp5=Nan,temp6=Nan
duplicate/o $thtname,thtworking
do
    redimension/n=(i+1) temp1,temp2,temp3 // Increase length of waves for new data.
    ImageStats /M=1 thtworking // Statistics of image
    if(V_max>thresholdtht)
        temp1[i]=V_maxRowLoc // Maximum intensity stored.
        temp2[i]=V_maxColLoc
        temp3[i]=V_max
        variable row=V_maxRowLoc
        variable col=V_maxColLoc
    variable j,k
    for(j=0;j<=(2*patchsize);j+=1)
        for(k=0;k<=2*patchsize;k+=1)
            ThTworking[V_maxRowLoc-patchsize+j][V_maxColLoc-patchsize+k]=0
        endfor
    endfor
    if(temp1[i]==temp1[i-1]) // Control to prevent double counting of spots.
        for(j=0;j<=(patchsize);j+=1)
            for(k=0;k<=patchsize;k+=1)
                ThTworking[row-patchsize+j][col-patchsize+k]=0
            endfor
        endfor
    endif
    i+=1
    endif
while (V_max>thresholdtht)
DeletePoints (dimsize(temp1,0)-1),1, temp1,temp2,temp3
duplicate/o temp1,$thtx
duplicate/o temp2,$thty
duplicate/o temp3,$thtintensity
numberoftht[a]=(dimsize(temp1,0))
// Check AF647 image just for spots:
string redx="redX"+num2str(a)
string reddy="redY"+num2str(a)
string redintensity="redIntensity"+num2str(a)
make/o/n=1 temp7=Nan,temp8=Nan,temp9=Nan
duplicate/o $redname,redworking
do
    redimension/n=(g+1) temp7,temp8,temp9 // Increase length of waves for new data.
    ImageStats /M=1 redworking // Statistics of image
    if(V_max>thresholdred) // Maximum intensity stored.
        temp7[g]=V_maxRowLoc
    endif
enddo
temp8[g]=V_maxColLoc
temp9[g]=V_max
row=V_maxRowLoc
col=V_maxColLoc

// This part is for straight-forward delete.
for(j=0;j<=(2*patchsize);j+=1)
    for(k=0;k<=2*patchsize;k+=1)
        redworking[row-patchsize+j][col-patchsize+k]=0
endfor
endif

if(temp7[g]==temp7[g-1])
    // Control to prevent double counting of spots.
    for(j=0;j<=(patchsize);j+=1)
        for(k=0;k<=patchsize;k+=1)
            redworking[row-patchsize+j][col-patchsize+k]=0
        endfor
    endif
    g+=1
endif

while (V_max>thresholdred)
    DeletePoints (dimsize(temp7,0)-1),1, temp7,temp8,temp9
    duplicate/o temp7,$redx
duplicate/o temp8,$redy
duplicate/o temp9,$redintensity

    // Check AF647 channel for coincident spots
    j=0
    k=0
    make/o/n=(2*patchsize,2*patchsize) temp // temp for wavestats
    i=0
    variable p=0
    for(i=0;i<(Dimsize(temp1,0));i+=1)
        for(j=-(patchsize);j<=patchsize;j+=1)
            for(k=-(patchsize);k<=patchsize;k+=1)
                temp[j][k]=workingred[temp1[i]+j][temp2[i]+k]
            endfor
        endfor
    endfor
    wavestats/Q temp

    if(V_max>thresholdred)
        // Some stats on events.
        redimension/n=(x+1) coincidenttht,coincidentred,coincidentratio
        coincidentred[x]=V_max
        coincidenttht[x]=temp1[i]
        coincidentratio[x]=ln(V_max/temp1[i])
    p+=1
    x+=1
    else
        redimension/n=(q+1) noncoincidenttht
        noncoincidenttht[q]=temp1[i]
        q+=1
    endif
endfor
numberofcoincident[a]=p
// Now for chance:
j=0
k=0
make/o/n=(2*patchsize,2*patchsize) temp // temp for wavestats
i=0
variable t=0
for(i=0;i<(Dimsize(temp1,0));i+=1)
    for(j=(-patchsize);j<=patchsize;j+=1)
        for(k=(-patchsize);k<=patchsize;k+=1)
            temp[j][k]=workingredchance[temp1[i]+j][temp2[i]+k]
endfor
endfor
wavestats/Q temp
wave redthreshold // bring applied thr
if(V_max>thresholdred)
    // Some stats on events.
    redimension/n=(h+1) chancetht,chancered,chanceratio
    chancered[h]=V_max
    chancetht[h]=temp1[i]
    chanceratio[h]=ln(V_max/temp1[i])
    h+=1
    t+=1
endif
endfor
numberofchance[a]=t
coincidence[a]=(numberofcoincident[a]-numberofchance[a])/(numberoftht[a]-numberofchance[a])
print coincidence[a]
//print coincidence[a]
endfor
end
function seeoincident(num)
    variable num
    wave savedpatchsize
    variable patchsize=savedpatchsize[0]
    string tht_x="thtx"+num2str(num)
    string tht_y="thty"+num2str(num)
    string red_x="redx"+num2str(num)
    string red_y="redy"+num2str(num)
    string tht_img="thtfiltered"+num2str(num)
    string af647_img="af647filtered"+num2str(num)
    duplicate/o $tht_x,thtx
    duplicate/o $tht_y,thty
    duplicate/o $red_x,redx
    duplicate/o $red_y,redy
    duplicate/o $tht_img,tht
    duplicate/o $af647_img,af647filtered
    duplicate/o tht,thtfiltered
    setthresholds()
    wave thtthreshold,redthreshold
    variable redrange=redthreshold[0]*2
    variable thtrange=thtthreshold[0]*2
    wave redx,redy
    // THT part
    NewImage/K=0 tht
    SetDrawLayer ProgFront
    SetDrawEnv linefgc= (0,65535,0),fillpat= 0,xcoord= top,ycoord= left, save
    // Part to draw rectangles:
variable i
for(i=0;i<(Dimsize(thtx,0));i+=1)
    DrawRect thtx[i]-patchsize,thtY[i]-patchsize,thtX[i]+patchsize,thtY[i]+patchsize
endfor
ModifyGraph tick=3,noLabel=2
variable numberoftht=(dimsize(thtx,0))
TextBox/C/N=text0/F=0/A=MT "\K(65535,65535,65535)All spots in ThT image"
("+num2str(numberoftht)+")"
TextBox/C/N=text0/B=1
ModifyImage tht ctаб= {0,thtrange,Grays,0}
// Red Part
NewImage/K=0 af647filtered
SetDrawLayer ProgFront
SetDrawEnv linefgc= (65535,0,0),fillpat= 0,xcoord= top,ycoord= left, save
// Part to draw rectangles:
for(i=0;i<(Dimsize(redx,0));i+=1)
    DrawRect redx[i]-patchsize,redY[i]-patchsize,redX[i]+patchsize,redY[i]+patchsize
endfor
ModifyGraph tick=3,noLabel=2
variable numberofred=(dimsize(redx,0))
TextBox/C/N=text0/B=1
ModifyImage af647filtered ctаб= {0,redrange,Grays,0}
// Coincident part
NewImage/K=0 tht
ModifyImage tht ctаб= {*,*},Grays,0
SetDrawLayer ProgFront
SetDrawEnv linefgc= (65535,65535,0),fillpat= 0,xcoord= top,ycoord= left, save // Draw yellow spots in the tht image if the red is above threshold
// Part to draw rectangles:
make/o/n=(2*patchsize,2*patchsize) temp // temp for wavestats
variable j,k,t
for(i=0;i<(Dimsize(thtx,0));i+=1)
    for(j=(-patchsize);j<=patchsize;j+=1)
        for(k=(-patchsize);k<=patchsize;k+=1)
            temp[j][k]=af647filtered[thtx[i]+j][thty[i]+k]
        endfor
    endfor
wavestats/Q temp
wave redthreshold // bring applied threshold.
if(V_max>redthreshold[0])
    DrawRect thtX[i]-patchsize,thtY[i]-patchsize,thtX[i]+patchsize,thtY[i]+patchsize
    t+=1
endif
endfor
wave coincidence
variable percent=round(100*coincidence[num])
ModifyGraph tick=3,noLabel=2
ModifyGraph tick=3,noLabel=2
TextBox/C/N=text0/F=0/A=MT "\K(65535,65535,65535)Coincident spots in ThT image"
("+num2str(t)+", "+num2str(percent)+"% including chance)"
TextBox/C/N=text0/B=1
ModifyImage tht ctаб= {0,thtrange,Grays,0}
end
function consolidate()
wave points
setdatafolder root:
makero/n=1
allcoincidence,allchance,allthtcounts,allfractioncoincident,thtintensities,redintensities,coincidentthtintensities,coincidentredintensities,coincidentratios,noncoincidentthtintensities
variable a,b,c=0,d=0,e=0,f=0,g=0
for(a=0;a<(dimsize(points,0));a+=1)
    string folder=num2str(points[a])
    setdatafolder root:$folder
    wave coincidence,numbe
    rofchance,numberoftht,numberofcoincident
    for(b=0;b<(dimsize(coincidence,0));b+=1)
        redimension/n=(c+1) allcoincidence,allchance,allthtcounts,allfractioncoincident
        allcoincidence[c]=numberofcoincident[b]
        allchance[c]=numberofchance[b]
        allthtcounts[c]=numberoftht[b]
        allfractioncoincident[c]=coincidence[b]
        c+=1
    endfor
    wave thtintensity
    for(b=0;b<(dimsize(thtintensity,0));b+=1)
        redimension/n=(d+1) thtintensities
        thtintensities[d]=thtintensity[b]
        d+=1
    endfor
    wave redintensity
    for(b=0;b<(dimsize(redintensity,0));b+=1)
        redimension/n=(e+1) redintensities
        redintensities[e]=redintensity[b]
        e+=1
    endfor
    wave coincidentred,coincidenttht,coincidentratio
    for(b=0;b<(dimsize(coincidentratio,0));b+=1)
        redimension/n=(f+1) coincidentthtintensities,coincidentredintensities,coincidentratios
        coincidentthtintensities[f]=coincidenttht[b]
        coincidentredintensities[f]=coincidentred[b]
        coincidentratios[f]=coincidentratio[b]
        f+=1
    endfor
    wave noncoincidenttht
    for(b=0;b<(dimsize(noncoincidenttht,0));b+=1)
        redimension/n=(g+1) noncoincidentthtintensities
        noncoincidentthtintensities[g]=noncoincidenttht[b]
        g+=1
    endfor
endfor
setdatafolder root:
wavestats allfractioncoincident
makero/n=1 coincident_average=V_avg
makero/n=1 coincident_error=v_sdev
derend
Appendix III:
The following script is for the analysis of sPAINT images, and was written in Python 2.7 and executed using Spyder v2.3 (Spyder developer community, available from github.com/spyder-ide/spyder).

This script opens the source images, thresholds these images to remove the background and determines the pixels co-localised in the ThT and 647 channels. This script then collects the localisation wavelength data from the original sPAINT render process, and bins the localisations into low, medium and high ranges. These ranges are used to produce a colour-mapped sPAINT image and a histogram of localisations according to the low, medium and high ranges.

```python
import numpy as np
import PIL as pil
import Tkinter as tk
import tkFileDialog
import os
import cv2
import csv

def initialise():
    global bin, threshold1, threshold2
    bin = [100, 50, 3, 2] #will create histogram data for these number of bins
    frames_647 = 14 #images in tiff-stack to use
    frames_tht = 100 #images in tiff-stack to use
    threshold1 = 11 #change this for different thresholding
    threshold2 = 5 #change this after trying the one above
    image_opener(frames_647, frames_tht)

def image_opener(frames_647, frames_tht):
    root = tk.Tk()
    root.wm_attributes("-topmost", 1)
    directory = tkFileDialog.askdirectory()
    root.withdraw()

    im647 = '/647.tif'
    im647_path = directory + im647
    imtht = '/ThT.tif'
    imtht_path = directory + imtht

    im = pil.Image.open(im647_path)
    imtht = pil.Image.open(imtht_path)
    ashape = np.shape(np.array(im.seek(1)))
    max647_array = np.zeros(ashape)
    maxtht_array = np.zeros(ashape)
    avg647_array = np.zeros(ashape)
    avgtht_array = np.zeros(ashape)
```
frames = []
for frame in xrange(frames_647):
    im.seek(frame)
    im647_array = np.array(im)
    frames.append(im647_array)
for array in frames:
    max647_array = np.maximum(max647_array, array)
    avg647_array = avg647_array + array
avg647_array = avg647_array/frames_647
deleter = []
for x in xrange(352):
    deleter.append(x)
deleter = tuple(deleter)
max647_array = np.delete(max647_array, deleter, 0)
avg647_array = np.delete(avg647_array, deleter, 0)

frames = []
for frame in xrange(frames_tht):
    imtht.seek(frame)
    imtht_array = np.array(imtht)
    frames.append(imtht_array)
for array in frames:
    maxtht_array = np.maximum(maxtht_array, array)
    avgtht_array = avgtht_array + array
avgtht_array = avgtht_array/frames_tht
maxtht_array = np.delete(maxtht_array, deleter, 0)
avgtht_array = np.delete(avgtht_array, deleter, 0)

folder = directory + '/Colocalisation analysis'
if not os.path.exists(folder):
    os.makedirs(folder)
folder1 = directory + '/Colocalisation analysis/Histograms'
if not os.path.exists(folder1):
    os.makedirs(folder1)
folder2 = directory + '/Colocalisation analysis/Binned images'
if not os.path.exists(folder2):
    os.makedirs(folder2)

plots = []
plots.append(avgtht_array)
plots.append(avg647_array)
plots.append(maxtht_array)
plots.append(max647_array)
dirs = []
dirs.append(folder + '/avgtht.tif')
dirs.append(folder + '/avg647.tif')
dirs.append(folder + '/maxtht.tif')
dirs.append(folder + '/max647.tif')
count = 0
for plot in plots:
    thresh(plot, dirs[count])
    count += 1
colocalise(folder)

def thresh(arr, save_dir):
    global threshold1, threshold2
    arr = abs((arr/np.max(arr)*255)-255)
    cv2.imwrite(save_dir, arr)

    saver = save_dir[:-4]
saver = saver + '_thresholded.tif'
img = cv2.imread(save_dir,0)
th1 = cv2.adaptiveThreshold(img,255,cv2.ADAPTIVE_THRESH_GAUSSIAN_C,
cv2.THRESH_BINARY,threshold1,threshold2)
cv2.imwrite(saver, th1)

def colocalise(folder):
    pm647 = folder + '/max647_thresholded.tif'
    path = folder + '/avgtht_thresholded.tif'
    pa647 = folder + '/avg647_thresholded.tif'
    pmtht = folder + '/maxtht_thresholded.tif'

    im = pil.Image.open(pm647)
    arm647 = np.array(im)
    im = pil.Image.open(pmtht)
    armtht = np.array(im)
    im = pil.Image.open(pa647)
    arra647 = np.array(im)
    im = pil.Image.open(path)
    arratht = np.array(im)

    pos = 1
    condition1 = ((arra647 < pos) & (arratht < pos))
    condition2 = ((arm647 < pos) & (armtht < pos))
    condition3 = ((arra647 < pos) & (armtht < pos))
    condition4 = ((arm647 < pos) & (armtht < pos))
    conditiona = (arra647 < pos)
    conditionm = (arm647 < pos)
    conditionat = (arratht < pos)
    conditionmt = (armtht < pos)

    pos647a = len(arra647[conditiona])
    pos647m = len(arm647[conditionm])
    posthta = len(arratht[conditionat])
    posthtm = len(armtht[conditionmt])
    coloc6ata = len(arra647[condition1])
    coloc6atm = len(arra647[condition3])
    coloc6mta = len(arm647[condition2])
    coloc6mtm = len(arm647[condition4])
    pix = str(arra647.size)
    pix2 = str(arm647.size)

    results_path = folder + '/colocalisation_results.txt'
    with open(results_path, 'w+') as outfile:
        outfile.write('Comparison\t\tPxtThPx\tCol px\t%647px col\n')
        outfile.write('\nmax647 & avgtht:\t + pix + \t + str(posthta) +
                   \t + str(pos647a) + \t + str(coloc6ata) + \t + str(round(coloc6ata/float(pos647a)*100, 2)))
        outfile.write('\nmax647 & maxtht:\t + pix2 + \t + str(posthtm) +
                   \t + str(pos647m) + \t + str(coloc6mta) + \t + str(round(coloc6mta/float(pos647m)*100, 2)))
        outfile.write('\nmax647 & avg647 & maxtht:\t + pix + \t + str(posthtm) +
                   \t + str(pos647m) + \t + str(coloc6mtm) + \t + str(round(coloc6mtm/float(pos647m)*100, 2)))
        superres(folder)

def superres(folder):
folder = folder[:,-24]
nr = '/for_render.txt'
nr_path = folder + nr

diction = {}
with open(nr_path, 'rU') as tsv:
    for column in zip(*[line for line in csv.reader(tsv, dialect="excel-tab")]):
        column = np.array(column)
        label = column[0]
        column = np.delete(column, [0])
        column = column.astype(float)
        diction[label] = column

use_true = (diction['use']==1)
xposi = diction['xposi'][use_true].astype(int)
yposi = diction['yposi'][use_true].astype(int)
wavelength = diction['wavelength'][use_true]
sr = np.zeros((512,512))
yx = zip(yposi, xposi)
count = 0
for ynx in yx:
    sr[ynx] = wavelength[count]
    count+=1
deleter = []
for x in xrange(352):
    #THIS CROPS THE OF THE IMAGE
    deleter.append(x)
deleter = tuple(deleter)
sr = np.delete(sr,deleter,0)
srsave = np.copy(sr)

rdpath = folder+'/Colocalisation analysis/Histograms/raw data.csv'
np.savetxt(rdpath, sr, delimiter=",")

#Saving image with according to three colour bins
# These are ranges for wavelengths
short_range = 550
med_range = 600
med_range2 = 650
high_range = 700
binned_im = np.zeros((160,512))
short__ = ((sr > short_range) & (sr <= med_range))
medium = ((sr > med_range) & (sr <= med_range2))
long__ = ((sr > med_range2) & (sr <= high_range))

# Changing these numbers will shift colours up and down colormaps
binned_im[short__] = 220
binned_im[medium] = 160
binned_im[long__] = 100

# Other colormaps here -
http://docs.opencv.org/2.4/modules/contrib/doc/facerec/colormaps.html
binned_im_HOT = cv2.applyColorMap(binned_im, cv2.COLORMAP_HOT)
binned_im_OCEAN = cv2.applyColorMap(binned_im, cv2.COLORMAP_OCEAN)

# Images are saved in "...\Colocalisation analysis\Binned images/Wavelengths_blackwhite.tif"
Appendices

Var2 = folder + '/Colocalisation analysis/Binned images/Wavelengths_hot.tif'
Var3 = folder + '/Colocalisation analysis/Binned images/Wavelengths_ocean.tif'
cv2.imwrite(Var1, binned_im)
cv2.imwrite(Var2, binned_im_HOT)
cv2.imwrite(Var3, binned_im_OCEAN)

srsave = srsave/750*255
saver = folder + '/Colocalisation analysis/NRnew.tif'
cv2.imwrite(saver, srsave)
a647p = folder + '/Colocalisation analysis/avg647_thresholded.tif'
m647p = folder + '/Colocalisation analysis/max647_thresholded.tif'
m647 = cv2.imread(m647p)
m647 = cv2.cvtColor(m647, cv2.COLOR_BGR2GRAY)
a647 = cv2.imread(a647p)
a647 = cv2.cvtColor(a647, cv2.COLOR_BGR2GRAY)

allnr = (sr > 549)
acoloc = ((a647 < 255) & (sr > 549))
mcoloc = ((m647 < 255) & (sr > 549))
asepar = ((a647 == 255) & (sr > 549))
msepar = ((m647 == 255) & (sr > 549))

srall = sr[allnr]
srmxnc = sr[msepar]
srmxc = sr[mcoloc]
sravnc = sr[asepar]
sravc = sr[acoloc]

avgcolocalised_avg647 = str(round(np.mean(sravc), 2))
avgcolocalised_max647 = str(round(np.mean(srmxc), 2))
avguncolocalised_avg647 = str(round(np.mean(sravnc), 2))
avguncolocalised_max647 = str(round(np.mean(srmxnc), 2))

results_path = folder + '/Colocalisation analysis/Wavelength summary.txt'
with open(results_path, 'w') as outfile:
    outfile.write('Avg wvlth uncoloc avg647
    Avg wvlth coloc avg647
    Avg wvlth uncoloc max647
    Avg wvlth coloc max647
    
    Results are two rows, the histogram values (top) and
    bin edges (bottom).
    The last value in the histogram values should be
    deleted (only added to make length even).
    
    For bins in bin:
    histograms([srall, srmxnc, sravnc, sravc], bins, count, folder)
    print 'Done!''

def histograms(arrs, bin, count, folder):
    count = 0
    for arr in arrs:
if count == 0:
    array = 'All pixels - '
elif count == 1:
    array = 'Max 647 - not colocalised - '
elif count == 2:
    array = 'Max 647 - colocalised - '
elif count == 3:
    array = 'Avg 647 - not colocalised - '
elif count == 4:
    array = 'Avg 647 - colocalised - '

    rdpath = folder+'/Colocalisation analysis/Histograms/' + array + ' raw data.csv'
    np.savetxt(rdpath, arr, delimiter="",)

    path = folder+'/Colocalisation analysis/Histograms/' + array + str(bin) + ' bins.csv'
    count+=1

    histo, bin_edges = np.histogram(arr, bins=bin)
    histo = np.append(histo, 0.0)
    data = np.concatenate((histo, bin_edges), axis=1)
    data = np.reshape(data, (2, -1))
    np.savetxt(path, data, delimiter="",)

initialise()
Appendix IV:
The following script is for the analysis of DHE assay images, and was written in Python 2.7 and executed using Spyder v2.3 (Spyder developer community, available from github.com/spyder-ide/spyder).

This script allows cells to be selected according to the red fluorescent image, such that the mean fluorescence intensity for each of the cells can be determined from every image in the series, in both the red and blue channels. The script then calculates the ratio of red to blue fluorescence both before and after treatment, which is fit with linear regression. The difference in the gradient of the line before and after treatment is then calculated for each cell, and averaged over the population.

```python
import PIL
import os
import numpy as np
import matplotlib.pyplot as plt
from matplotlib.widgets import RectangleSelector
# import Tkinter, tkFileDialog
from matplotlib.patches import Rectangle
import csv

def initialise():
    global cells_to_analyse, start_coords, end_coords, images_per_channel,
    images_to_discard_mid, images_to_discard_start
    images_per_channel = 60
    cells_to_analyse = 15
    images_to_discard_mid = 2
    images_to_discard_start = 2
    start_coords = []
    end_coords = []

def opener():
    global files, directory
    directory = 'E:/Path'
    files = os.listdir(directory)
    files.sort()

    results_path = directory + '/Results'
    if not os.path.exists(results_path): os.makedirs(results_path)
    boxes()

def boxes():
    global files, directory, images_per_channel
    for f in files:
        if 'ch02' in f:
            x = 1
```
```python
y = 1
fig = plt.figure
ax = fig.subplot(111)
ax.plot(x, y)
red_image_path = os.path.join(directory, f)
img = PIL.Image.open(os.path.join(directory, f))
try:
    img.seek(images_per_channel - 1)
currentAxis = plt.gca()
for coord1, coord2 in zip(start_coords, end_coords):
    x, y1 = coord1
    x1, y = coord2
    upper_left = (x, y1)
    width = x1 - x
    height = y - y1
    currentAxis.add_patch(Rectangle(upper_left, width, height, facecolor="none", edgecolor='w'))
figManager = plt.get_current_fig_manager()
figManager.window.showMaximized()
plt.imshow(img)
toggle_selector.RS = RectangleSelector(ax, onselect,
drawtype='box')
def onselect(eclick, erelease):
    global cells_to_analyse
    'eclick and erelease are matplotlib events at press and release'
    start = (eclick.xdata, eclick.ydata)
    end = (erelease.xdata, erelease.ydata)
    start_coords.append(start)
    end_coords.append(end)
    plt.close(1)
cells_to_analyse -= 1
if cells_to_analyse != 0:
    boxes()
else:
    analyse()

def toggle_selector(event):
    if event.key in ['0', 'q'] and toggle_selector.RS.active:
        toggle_selector.RS.set_active(False)
    if event.key in ['A', 'a'] and not toggle_selector.RS.active:
        toggle_selector.RS.set_active(True)

def analyse():
    global files, directory, images_per_channel
    count = 1
    uv_dict = {}
    red_dict = {}
    ratio_dict = {}

    for coord1, coord2 in zip(start_coords, end_coords):
        uv = []
        red = []
        x, y = coord1
        x1, y1 = coord2
        x = int(x)
        x1 = int(x1)
        y = int(y)
        y1 = int(y1)
        for f in files:
            if 'ch02' in f:
```
redimg2 = PIL.Image.open(os.path.join(directory, f))
for i in range(images_per_channel):
    try:
        redimg2.seek(i)
        redarr2 = np.array(redimg2)
        print 'running'
        print '1', redarr2
        redarr3 = redarr2[y:y1,x:x1]
        print '2', redarr3
        red.append(np.mean(redarr3))
    except:
        pass
red_dict[count] = red
if 'ch01' in f:
    uvimg2 = PIL.Image.open(os.path.join(directory, f))
for i in range(images_per_channel):
    try:
        uvimg2.seek(i)
        uvarr2 = np.array(uvimg2)
        uvarr3 = uvarr2[y:y1,x:x1]
        uv.append(np.mean(uvarr3))
    except:
        pass
uv_dict[count] = uv

count += 1
print 'Working...

for key in red_dict:
    ratio = []
    for b,m in zip(red_dict[key], uv_dict[key]):
        ratio.append(b/m)
    ratio_dict[key] = ratio

before_treat = images_per_channel / 2
x_ax = []
before_treat_slopes = []
for x in xrange(images_to_discard_start, before_treat):
    x_ax.append(x)
for key, val in ratio_dict.iteritems():
    print key, val
for key in ratio_dict:
    y_ax = []
    for x in xrange(images_to_discard_start, before_treat):
        print key, x
        val = ratio_dict[key][x]
        y_ax.append(val)
slope, intercept = np.polyfit(x_ax, y_ax, 1)
before_treat_slopes.append(slope)

after_treat_start = images_per_channel / 2 + images_to_discard_mid
x_ax = []
after_treat_slopes = []
for x in xrange(after_treat_start, images_per_channel):
    x_ax.append(x)
for key in ratio_dict:
    y_ax = []
    for x in xrange(after_treat_start, images_per_channel):
        val = ratio_dict[key][x]
        y_ax.append(val)
slope, intercept = np.polyfit(x_ax, y_ax, 1)
after_treat_slopes.append(slope)
results_uv_path = directory + '/Results/uv_channel.csv'
results_red_path = directory + '/Results/red_channel.csv'
results_slopes_path = directory + '/Results/slopes.csv'
results_ratio_path = directory + '/Results/ratio.csv'

with open(results_uv_path, 'wb') as outfile:
    writer = csv.writer(outfile)
    writer.writerows(zip(*uv_dict.values()))
with open(results_red_path, 'wb') as outfile:
    writer = csv.writer(outfile)
    writer.writerows(zip(*red_dict.values()))
with open(results_slopes_path, 'wb') as outfile:
    writer = csv.writer(outfile)
    writer.writerow(['Before treatment', 'After treatment'])
    writer.writerows(zip(before_treat_slopes, after_treat_slopes))
with open(results_ratio_path, 'wb') as outfile:
    writer = csv.writer(outfile)
    writer.writerows(zip(*ratio_dict.values()))

print 'Done!'

initialise()
Appendix V:

Figure 8.2: Hsp27 interacts with α-syn fibril fragments. Recombinant monomeric α-syn was incubated in 50 mM phosphate buffer and 0.01% NaN₃ (pH 7.4) at 50 μM in the absence or presence of Hsp27 at concentrations ranging from 0.1 – 10 μM. After equilibration at 37°C, α-syn seeds were added at concentrations ranging from 0.5 – 5 μM (i.e. 1%-10% (w/w) when expressed as a percentage of the soluble protein concentration) and elongation monitored via the change in ThT fluorescence at 490 nm over time. (A, C, E) Representative traces of the change in ThT fluorescence normalised to the maximum value in the absence of Hsp27 are shown, in the presence of 1%, 2.5% and 10% seed. (B, D, F) The linear portion (0 – 2.5 h) was fit with linear regression such that the rate of elongation could be calculated. Data is representative of at least three independent experiments, consisting of duplicate samples.
Appendix VI:
The following script was written and executed using ImageJ’s internal macro editor.

This script utilises the GDSC PlugIn (University of Sussex, UK; available from http://www.sussex.ac.uk/gdsc/intranet/microscopy/imagej/gdsc_plugins), specifically the CellOutliner function, to produce a cell map based on a brightfield image. The script measures the cell area, then determines the minimum, maximum, and average fluorescence intensity of the cell. These parameters are assigned to each cell according to the cell map. The user is then responsible for assigning which cells contain inclusion(s), using only the red image (i.e. without information as to whether the cell also expresses the sHsp or control protein in these experiments, which is done to minimise treatment-related bias).

```
macro "InclusionAnalyser [o]" { 
    var path = File.openDialog("Select the merge image:");
    var dir = File.getParent(path);
    var name = File.getName(path);
    var merge = split(name,".");
    var simplefile = "Results"+File.separator
    var output = dir+File.separator+simplefile
    var channelRed = File.separator+merge[0]+'_ch02.jpg';
    var channelBright = File.separator+merge[0]+'_ch01.jpg';
    var redpath = dir+channelRed;
    var brightpath = dir+channelBright;

    open(redpath);
    run("Duplicate...", "title=OverlayRed");
    run("Duplicate...", "title=Red");
    run("32-bit");

    open(brightpath);
    run("Duplicate...", "title=DIC");
    run("Add Image...", "image=[OverlayRed] x=0 y=0 opacity=20");

    selectWindow("DIC");
    setTool("multipoint");

    waitForUser("Select centre of all displayed cells \n \nPress OK to continue");
    selectWindow("DIC");

    run("Cell Outliner", "cell_radius=18 tolerance=1.0 kernel_width=13 dark_edge kernel_smoothing=1 polygon_smoothing=1 weighting_gamma=3 iterations=3 dilate=0");
    selectWindow("DIC Cell Outline");
} 
```
setAutoThreshold("Default dark");
setThreshold(1, 255);
setOption("BlackBackground", true);
run("Convert to Mask");
run("Watershed");

selectWindow("DIC Cell Outline");
run("Set Measurements...", "area mean standard min limit display add redirect=[Red] decimal=3");
run("Analyze Particles...", "size=100-Infinity show=Outlines display clear");

selectWindow("Drawing of DIC Cell Outline");
run("Duplicate...", "title=CellOutline");
run("Add Image...", "image=[Red] x=0 y=0 opacity=60");
saveAs("Jpeg", output+merge[0]+"_CellOutline_Red.jpg");
close("Drawing of DIC Cell Outline");
saveAs("Results", output+merge[0]+"_Results_Red.txt");

print("Process Complete");
print("Results for "+merge[0]+" saved in directory: "+output);
selectWindow("Log");
}

macro "CloseAll [w]" {
    run("Close All");
}
Appendices

Appendix VII:
The following script was written in Python 2.7 and executed using Spyder v2.3 (Spyder developer community, available from github.com/spyder-ide/spyder).

This script collects output from Appendix VI and determines the following parameters: (1) the cells which are co-transfected, (2) the percentage of co-transfected cells containing inclusions, (3) the average fluorescence of co-transfected cells with and without inclusions.

```python
import Tkinter   # Allows closing of parent window
import tkFileDialog  # Allows user input for getting directory
import os        # Allows setting of default directory
import glob      # Allows specification of filenames with wildcard characters
import pandas as pd     # Allows parsing of files
import numpy as np
import re
import csv

# Create a reference to parent window
root = Tkinter.Tk()
# Close parent window
root.withdraw()

# Get user input to select folder
directory = tkFileDialog.askdirectory()
# Set selected directory as active directory
os.chdir(str(directory))

# To hard-code constant thresholds
red_thresh = float(0.5)
green_thresh = float(3)

# To get the names of the treatments and directories
folderlist = glob.glob('*/')
treatmentfolderlist = []
for x in folderlist:
    if 'Combined Results' not in x:
        treatmentfolder = x
treatmentfolder = treatmentfolder[0:-1]
treatmentfolderlist.append(treatmentfolder)
treatment = treatmentfolder
outputdirectory = directory + '/' + '/Combined Results/'

# To go through each folder in the directory, that is not the combined results folder
for dirName, subdirList, fileList in os.walk(directory):
    if 'Combined Results' not in dirName:
        if 'Results' in dirName:
            # To set the working directory to each folder in turn
            os.chdir(str(dirName))
            # To adjust 'treatment' value according to each folder
            resultsDir = re.split('\\\|\|\|\|', dirName)
            for x in treatmentfolderlist:
                if x in resultsDir:
```
treatment = x

print treatment

# Initialise empty lists - to clear lists that are appended to
appended_data_red = []
appended_data_green = []
cotrans_red = []
cotrans_green = []
appended_red = []
appended_green = []
rawdata_red = []
rawdata_green = []

for datafile in glob.glob("*Red.txt"):
    # Add entire file to pandas data frame
data = pd.read_table(datafile)
    # Create column showing where row came from
data["Filename"] = datafile
    # Adds data frame to the list
    appended_data_red.append(data)

    # Append each data frame to a new data frame
appended_red = pd.concat(appended_data_red, ignore_index = True)

    # Save appended data frames to a new file
appended_red.to_csv("concatenated_data_red.txt")

for datafile in glob.glob("*Green.txt"):
    # Add entire file to pandas data frame
data = pd.read_table(datafile)
    # Create column showing where row came from
data["Filename"] = datafile
    # Adds data frame to the list
    appended_data_green.append(data)

    # Append each data frame to a new data frame
appended_green = pd.concat(appended_data_green, ignore_index = True)

    # Save appended data frames to a new file
appended_green.to_csv("concatenated_data_green.txt")

rawdata_green = appended_green.rename(columns =
    {'Area': 'Green Area',
     'Filename': 'Green Filename',
     'Label': 'Green Label',
     'Max': 'Green Max',
     'Min': 'Green Min',
     'Mean': 'Green Mean',
     'StdDev': 'Green StdDev',
    })

rawdata_red = appended_red.rename(columns =
    {'Area': 'Red Area',
     'Filename': 'Red Filename',
     'Label': 'Red Label',
     'Max': 'Red Max',
     'Min': 'Red Min',
    })
'Mean': 'Red_Mean', 'StdDev': 'Red StdDev',
})
# selects only rows with mean values that exceed the threshold
thresholded_green = rawdata_green[rawdata_green.Green_Mean >=
green_thresh]
thresholded_red = rawdata_red[rawdata_red.Red_Mean >=
red_thresh]
# collects indexes of the rows that passed the threshold
green_trans_indexer = thresholded_green.index.tolist()
red_trans_indexer = thresholded_red.index.tolist()
# finds co-transfected cells, copies into new list
cotrans_indexer = []
for x in red_trans_indexer:
    if x in green_trans_indexer:
        cotrans_indexer.append(x)
else:
    pass
# appends data common to both indexers
for x in cotrans_indexer:
    cotrans_red.append(rawdata_red.xs(x))
    cotrans_green.append(rawdata_green.xs(x))
# converts appended data to data frame
cotrans_red = pd.DataFrame(cotrans_red)
cotrans_green = pd.DataFrame(cotrans_green)
# calculates the fluorescence load
cotrans_red['Red_load'] =
    cotrans_red['Red_Mean'] * cotrans_red['Red Area']
cotrans_green['Green_load'] =
    cotrans_green['Green_Mean'] * cotrans_green['Green Area']
# saves co-transfected data to excel files
cotrans_red.to_csv("cotransfected_Red.txt")
cotrans_green.to_csv("cotransfected_Green.txt")
# separating data into with and without inclusions
cotrans_green_inclusions =
    pd.DataFrame(cotrans_green[cotrans_red.Inclusions == 1])
cotrans_red_inclusions =
    pd.DataFrame(cotrans_red[cotrans_red.Inclusions == 1])
cotrans_green_noinc =
    pd.DataFrame(cotrans_green[cotrans_red.Inclusions != 1])
cotrans_red_noinc =
    pd.DataFrame(cotrans_red[cotrans_red.Inclusions != 1])
# calculating info about cells with and without inclusions
num_inc = cotrans_red['Inclusions'].sum()
num_cells = len(cotrans_red.index)
um_ni = num_cells - num_inc
inc_percent = num_inc / num_cells * 100
place_holder = 0
c_t_total_red_load = cotrans_red['Red_load'].sum()
c_t_av_red_load = c_t_total_red_load / num_cells
c_t_avmean_red = cotrans_red['Red_Mean'].sum() / num_cells
c_t_total_green_load = cotrans_green['Green load'].sum()
c_t_av_green_load = c_t_total_green_load / num_cells
c_t_avmean_green = cotrans_green['Green_Mean'].sum() / num_cells
inc_total_red_load = cotrans_red_inclusions['Red_load'].sum()
\[
\text{inc\ av\ red\ load} = \text{inc\ total\ red\ load} / \text{num\ inc}
\]
\[
\text{inc\ avmean\ red} = \text{cotrans\ red\ inclusions}'\text{Red\ Mean}'.\sum() / \text{num\ inc}
\]
\[
\text{ni\ total\ red\ load} = \text{cotrans\ red\ noinc}'\text{Red\ load}'.\sum() \quad \text{ni\ av\ red\ load} = \text{ni\ total\ red\ load} / \text{num\ ni}
\]
\[
\text{ni\ avmean\ red} = \text{cotrans\ red\ noinc}'\text{Red\ Mean}'.\sum() / \text{num\ ni}
\]
\[
\text{inc\ total\ green\ load} = \text{cotrans\ green\ inclusions}'\text{Green\ load}'.\sum() \\
\text{inc\ av\ green\ load} = \text{inc\ total\ green\ load} / \text{num\ inc}
\]
\[
\text{ni\ total\ green\ load} = \text{cotrans\ green\ noinc}'\text{Green\ load}'.\sum() / \text{num\ ni}
\]
\[
\text{ni\ av\ green\ load} = \text{ni\ total\ green\ load} / \text{num\ ni}
\]
\[
\text{ni\ avmean\ green} = \text{cotrans\ green\ noinc}'\text{Green\ Mean}'.\sum() / \text{num\ ni}
\]

#creating series to store the calculated info
whole_treatment = (\text{inc\ percent, num\ cells, ct\ total\ red\ load, ct\ av\ red\ load, ct\ avmean\ red, ct\ total\ green\ load, av\ green\ load, ct\ avmean\ green})

inclusions_only = (place\ holder, num\ inc, inc\ total\ red\ load, inc\ av\ red\ load, inc\ avmean\ red, inc\ total\ green\ load, inc\ av\ green\ load, inc\ avmean\ green)

no_inclusions = (place\ holder, num\ ni, ni\ total\ red\ load, ni\ av\ red\ load, ni\ avmean\ red, ni\ total\ green\ load, ni\ av\ green\ load, ni\ avmean\ green)

#list to store the index label for each row of stats
row_labels = ('\% cells w Inclusions', 'Number of cells', 'Red Load (Total)', 'Red Load (per cell)', 'Average Intensity', 'Green Load (Total)', 'Green Load (per cell)', 'Average Intensity')

#creates the dictionaries to link calculated values with row labels
summary_stats_whole = {'Whole Treatment': pd.Series(whole_treatment, index=row_labels)}
summary_stats_inclusions = {'Inclusions': pd.Series(inclusions_only, index=row_labels)}
summary_stats_noinc = {'No Inclusions': pd.Series(no_inclusions, index=row_labels)}

#turns series into dataframes
summary_stats_whole = np.round(pd.DataFrame(summary_stats_whole), decimals=2)
summary_stats_inclusions = np.round(pd.DataFrame(summary_stats_inclusions), decimals=2)
summary_stats_noinc = np.round(pd.DataFrame(summary_stats_noinc), decimals=2)
summary_stats = np.round(pd.DataFrame(summary_stats), decimals=2)

# saves outputs to excel files:
summary_stats.to_csv("Stats.txt")
summary_stats.to_csv(outputdirectory+treatment+"_Stats.txt")
summary_stats_whole.to_csv(outputdirectory+treatment+"_Stats_Whole.txt")
summary_stats_inclusions.to_csv(outputdirectory+treatment+"_Stats_Inclusions.txt")
summary_stats_noinc.to_csv(outputdirectory+treatment+"_Stats_NoInc.txt")

# Renaming columns to allow joining Red and Green data
co_green = cotrans_green.rename(columns = {'Area': 'Green Area', 'Filename': 'Green Filename', 'Label': 'Green Label', 'Max': 'Green Max', 'Min': 'Green Min', 'Mean': 'Green Mean', 'StdDev': 'Green StdDev'})

coop = coo.drop(coo.columns[[0]], axis=1)
co_green = co_green.drop(coo.columns[[0]], axis=1)
co_red = cotrans_red.rename(columns = {'Area': 'Red Area', 'Filename': 'Red Filename', 'Label': 'Red Label', 'Max': 'Red Max', 'Min': 'Red Min', 'Mean': 'Red Mean', 'StdDev': 'Red StdDev'})

coop = coo.drop(coo.columns[[0]], axis=1)

# joins cotransfected data, to save as excel file
cotrans = [co_green, co_red]
cotrans = pd.concat(cotrans, axis = 1)
cotrans.to_csv("cotrans.txt")

print treatment, 'Complete!'
for datafile in glob.glob("*Whole.txt"): # This does only 'whole' text files
    # Add entire file to pandas data frame, without the column header
data = pd.read_csv(datafile)
treatment = datafile.split('_')[0]
    # Create row showing where row came from
data = data.rename(columns = {'Whole Treatment': treatment})
    # Adds data frame to the list
appended_data_whole.append(data)

    # Append each data frame to a new data frame
appended_whole = pd.concat(appended_data_whole, axis = 1)
    # Save appended data frames to a new file
appended_whole.to_csv('Whole Treatment.txt')

    # Iterate through each of the text files
for datafile in glob.glob("*Inclusions.txt"): # This does only 'Inclusion' text files
    # Add entire file to pandas data frame, without the column header
data = pd.read_csv(datafile)
treatment = datafile.split('_')[0]
    # Create row showing where row came from
data = data.rename(columns = {'Inclusions': treatment})
    # Adds data frame to the list
appended_data_inclusions.append(data)

    # Append each data frame to a new data frame
appended_inclusions = pd.concat(appended_data_inclusions, axis = 1)
    # Save appended data frames to a new file
appended_inclusions.to_csv('Inclusions.txt')

    # Iterate through each of the text files
for datafile in glob.glob("*NoInc.xlsx"): # This does only 'NoInc' text files
    # Add entire file to pandas data frame, without the column header
data = pd.read_csv(datafile)
treatment = datafile.split('_')[0]
    # Create row showing where row came from
data = data.rename(columns = {'No Inclusions': treatment})
    # Adds data frame to the list
appended_data_noinc.append(data)

    # Append each data frame to a new data frame
appended_noinc = pd.concat(appended_data_noinc, axis = 1)
    # Save appended data frames to a new file
appended_noinc.to_csv('No Inclusions.txt')

print 'Experimental Summary Complete. All files saved to:', outputdirectory
writer.save()
Appendix VIII:
The concentration of α-syn detectable in cell extracts following transfection was determined via dot blot. There was seen to be no significant difference in the intensity of detected monomeric or fibrillar α-syn (Figure 8.3A), indicating the presence of aggregates in the cell extract samples would not influence the detected concentration. Samples of known recombinant monomeric α-syn concentration were therefore used to generate a standard curve (Figure 8.3B). The concentration of α-syn was found to be 0.4 µM and 0.6 µM in the treated and untreated cell extracts, respectively. This corresponds to ~ 0.009 µg/µL, or less than 1% of the total protein extracted from cells.

Figure 8.3: Quantification of α-synA53T* expression in whole cell lysate. (A) Monomeric (1-8) or fibrillar (9-10) recombinant α-syn was prepared at (1, 9) 50 µM, (2, 10) 25 µM, (3) 5 µM, (4) 1 µM, (5) 0.2 µM, (6) 0.04 µM, (7) 0.008 µM or (8) 0.00016 µM in 50 mM phosphate buffer (pH 7.4). Whole cell extracts were also prepared to a total concentration of 1 µg/µL from cells transfected with the α-synA53T* construct incubated in the (11) absence or (12) presence of inhibitors (10 µM MG132 and 3 µM thapsigargin). Samples (5 µL) were blotted onto nitrocellulose membrane and detected using a monoclonal mouse anti-α-syn followed by a HRP-conjugated secondary antibody. (B) The relative intensity of each spot was determined using the ImageJ gel analysis tool, and fitted with one-phase association.
Appendix IX:

N2a cells were transfected with a range of Htt46Q-mCherry DNA concentrations (expressed as a percentage of the standard 1 ug/mL) in the absence or presence of IRES constructs expressing EGFP and either EGFP\textsuperscript{inv} or LacZ. The proportion of live cells that were mCherry positive was determined, and the proportion of these cells containing inclusions was enumerated according to the PulSA gating strategy. Whilst decreasing the amount of Htt46Q DNA used to transfect cells did not significantly influence the percentage of cells transfected or the median fluorescence, it led to a decrease (by 22%) in the proportion of cells containing inclusions.

**Figure 8.4:** The concentration of DNA used to transfect N2a cells influences the proportion of transfected cells with Htt46Q inclusions. N2a cells were transiently transfected with Htt46Q-mCherry at various concentrations in the absence or presence of IRES constructs encoding EGFP and EGFP\textsuperscript{inv} or LacZ. Where necessary, in the absence of a second DNA construct the transfection volume was equalised using serum free media (SFM). Cells were incubated for 36 h, then analysed via flow cytometry. (A) The percentage of live cells which were mCherry-positive was quantified, and (B) the median mCherry fluorescence of these cells determined. (C) The proportion of mCherry-positive cells containing inclusions was determined using the PulSA gating strategy. DNA ratios are expressed as a percentage of the standard (1 µg/ml), and data is presented as mean ± S.E.M. (n=2).