A probe for conformational change of Hsc70 in mammalian cells under stress conditions

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Abstract

Hsc70 is a constitutively expressed member of the heat shock protein family. It has an important role in proteostasis and can stabilise nonnative proteins before being further matured in refolding, disaggregation, and degradation processes. Hsc70 switches between ATP and ADP bound states, and the hydrolysis of ATP drives the reaction for folding of the unfolded peptide. Förster resonance energy transfer (FRET) was used to report this conformational shift.

Creation of the FRET probe used variant 3T of Hsc70, which has three cysteines. One is buried in the conformation, and two are surface exposed on different domains. Maleimide Alexa Fluor (AF) dyes were applied to make covalent additions to the reduced cysteines, and conformational change was tracked by FRET. A luciferase refolding assay showed that although these mutations do not interfere with conformational change, they hinder refolding capabilities with substrate peptides.

Double labelled Hsc70 3T was transduced into HEK293 cells with varying conditions. It was found that unfiltered samples, imaged with Opti-MEM in the wells, and at the suggested PULSin concentration by the manufacturer was enough to detect fluorescence of AF488 and AF594. Heat shock can be used to determine how these conformations differ under stress and has been observed in live cell microscopy. This makes FRET a suitable technique to study conformational change of Hsc70 3T under cellular stress. All steps to establish a method to study FRET in live cells were optimised and could be done if a confocal microscope were available.

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Abbreviations

ABCA1	ATP binding cassette transporter 1
ADR	Acceptor donor ratio
AF	Alexa Fluor
APS	Ammonium persulfate
BAG-1	Bcl-2 associated athanogene 1 protein
BSA	Bovine serum albumin
C-IDR	C terminal intrinsically disordered region
CL	Clarified lysate
CP/CPK	Creatine phosphate/Creatine phosphokinase
CTD	C terminal domain
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
FlAsH	Fluorescein arsenical hairpin
FRET	Forster resonance energy transfer
HBV	Hepatitis B
HAP	Hydroxyapatite
HEPES	4-2-hydroxyethyl-1-piperazineethanesulfonic acid
Hsc70	Heat shock cognate protein 70
Hsp70	Heat shock protein 70
Hsps	Heat shock proteins
IPTG	Isopropyl β-d-1-thiogalactopyranoside
LB	Lysogeny broth
MDH	Malate dehydrogenase
MQ	Milli-Q
MW	Molecular weight

MWCO	Molecular weight cut off
NBD	Nucleotide binding domain
NEF	Nucleotide exchange factor
OD	Optical density
Opti-MEM	Opti-Modified Eagle Medium
PFA	4% v/v paraformaldehyde
PLL/PLO	Poly-L-lysine/ornithine
PMSF	Phenylmethylsulfonyl fluoride
RCF	Relative centrifugal force
RLU	Relative luminescence units
RMSD	Root mean square deviation
rpm	revolutions per minute
SBD	Substrate binding domain
SDS-Page	Sodium dodecyl sulphate polyacrylamide
SERT	Serotonin receptor
sHsps	Small Hsps
siRNA	Small interfering RNA
smFRET	Single molecule fret
TCA	Trichloroacetic acid
TCEP	Tris(2-caroxyethyl)phosphine
TEMED	Tetramethylethylenediamine
TF	Ribosome associated trigger factor
TLR4	Toll like receptor 4
TNF-α	Tumour necrosis factor alpha
TPR	Tetratricopeptide repeat
wt	Wild type

Chapter 1: Introduction

Homeostasis in the cell is important to ensure cell processes function normally. This includes protein homeostasis, or proteostasis. Fluctuations in proteostasis can result in disease, misfolding and aberrant assembly of proteins, and impaired trafficking of proteins. Heat shock proteins (Hsps) play an important role in proteostasis, where they are able to stabilise non-native proteins before proceeding to refolding, disaggregation or degradation processes (Fernandez-Fernandez & Valpuesta, 2018). Hsps interact with a wide array of polypeptides, to both fold and transport them. The main response that Hsp are involved in is the heat shock response, where Hsp are able to restore proteins to their native state from denaturation.

1.1 Heat Shock Proteins in Humans

The heat shock protein family are a very diverse range of proteins which are highly conserved in both eukaryotes and prokaryotes. They are characterised into families by their molecular weight. These families are Hsp100, Hsp90, Hsp70, Hsp60, Hsp40, and Hsp27 (Lu et al., 2014). Members of each family are involved in protein folding. Hsp100 is involved in releasing proteins from aggregates. Hsp90 proteins are involved in the maturation and activation of proteins. Hsp70, Hsp60, and Hsp27 are involved in preventing protein aggregation, and aiding in protein folding. Hsp40s act as cochaperones to Hsp70s during protein folding.

1.2 Hsp70s as a family and Conservation

Hsp70s are highly conserved chaperones and are found in almost all intracellular compartments, the main being the cytosol and the nucleus. They are found in any eukaryotic intracellular or extracellular compartment, in bacteria, and in certain archaea. There are 13 isoforms for just Hsp70s in humans (Taylor et al., 2018). These are encoded by 13 genes, as well as 30 pseudogenes (Radons, 2016). These genes are HSPA1A/B (Stress inducible Hsp72), HSPA1L (HspA1 - Like), HSPA8 (constitutively expressed Hsc70), HspA8 - 2 (Isoform of Hsc70), HSPA2, HSPA6, HSPA5 (Grp78), HSPA7 (Putative Hsp70 protein 7), HSPA9 (Grp75), HSPA14, HSPA4 (Hsp70 protein 4, APG-2), HSPA13 (Hsp70 protein 13, STCH), and HSPA4L (APG-1) (Stritcher et al., 2013). Names in brackets are other names which are used in this thesis. These proteins are between 70 – 78 kDa in size (Stritcher et al., 2013) and is the main chaperone protein to facilitate in peptide substrate folding. These isoforms will be further explained in later

sections. Other Hsp70 isoforms in other eukaryotes are also explained. In yeast, there are 8 Hsp70 homologues where all but two are located in the cytosol. These are Ssa1, Ssa2, Ssa3, Ssa4, Ssb1 and Ssb2. Ssc1 is in the mitochondria and Ssd1/Kar2 in the endoplasmic reticulum (ER). It was found that four Ssa proteins can compensate for each other, but if they are deleted, promote a lethal phenotype.

The well-studied *E. coli* Hsp70 bacterial homologue DnaK serves as a reliable control to compare Hsp70 proteins from different organisms due to the large amount of research behind it. DnaK shares 50% homology with eukaryotic Hsp70, Heat shock cognate protein 70 (Hsc70). The differences which result in the decreased identity do not rely on the core domains - the conserved ATPase domain, the middle region with protease sensitive sites, a peptide binding domain, and a G/P rich C terminal region with an EEVD motif which allows for cochaperones and other Hsps to bind (Dugaard, Rohde & Jaattela, 2007).

Dugaard, Rohde & Jaattela (2007) summarised why there are multiple highly homologous Hsp70 proteins among different species. This could be due to having individual and tissue specific expression, showing that they are required for certain biochemical reactions.

All eukaryotes have more than 1 gene encoding Hsp70s, and in some cases like yeast, substitutions with other Hsp70 proteins when one is deleted does not save the lethal phenotype. This shows that there are overlapping functions, but there are Hsp70s with tissue specific or biochemical specific functions (Werner – Washburne, Stone & Craig (1987). It has been found that other Hsps from other organisms are able to rescue an organism with an Hsp deletion.

Triple mutants were first made of the SSA1, SSA2 and SSA4 genes, which produced inviable spores. It was found that the lethal phenotype was able to be rescued by transforming a ssa1/ssa1 ssa2/+ ssa4/+ diploid with a yeast plasmid containing wild type (wt) SSA4. From this, it could be concluded that SSA4 is important in bud formation and may be essential in order for spore germination to occur. The vegetative growth of the spores was then tested by transforming the triple mutant diploid into a plasmid where wt SSA1 is controlled under a GAL1 promoter. Cells were then put onto galactose or glucose media. Growth, sporulation and germination were

observed. Triple mutants were unable to be found, even when transferred to a glucose-based media. Haploid cells containing mutants in genes ssa1, ssa2 and ssa4 were grown on glucose-based media. These cells were unable to form colonies. Therefore, it could be confirmed that SSA1, SSA2 and SSA4 are essential for normal growth in yeast.

The same experiments were then done, with SSA3. When transforming a yeast plasmid encoding SSA3 into a triple mutant, the cells were still unable to grow. Using a SSA2 promoter, the SSA3 gene was able to be transcribed at the same level as SSA2 at 23℃. Small colonies were able to be formed at 23℃ and were temperature sensitive for growth at 37℃, which was also shown in the ssa1 ssa2 double mutant. From this, it was found that ssa3 does not fully cover the loss of ssa1 or ssa2 but shows that ssa3 instead has a different function to that compared to ssa1 or ssa2. Finally, the functions of ssa1, ssa2, ssa3 or ssa4 were able to be determined through triple mutants. It was found that ssa1, ssa2 and the ssa4 triple mutant was able to be stimulated for thermotolerance, showing that these genes are not required for thermotolerance induction.

The Hsp protein family is a highly conserved protein family which has many functions. Members which are crucial for the heat shock response, while others are important in clearance of peptides, and others are to control proteostasis under normal conditions in the cell, such as Hsc70 and SSA4.

1.3 Tissue Localisation

Most studies rely on crystallography or *in vitro* techniques, but few on tracking live cells. For example, Kumar and Sourjik (2012) were able to analyse protein localisation and interactions of protein folding systems *in vivo* and could compare these results to what had previously been known about the interactions of the systems. Woo et al., (2009) was additionally able to do this, by analysing crystal results of NBD conformational changes. Because crystal structures cannot show full ranges of the conformational changes, fluorescence of the only Trp in bovine Hsp70 (Trp90) was measured, as well as molecular dynamics (MD) experiments. The NBD contains 2 lobes, which each have 2 subdomains. These subdomains are IA, IIA, IB and IIB. These lobes form a cleft with a nucleotide binding cassette, which binds ATP. It was identified that NBDs have more conformations than what had previously been known, as well as subdomains IB and

IIB being more closely associated to Trp90 (IB) and Arg261 (IIB). This is explained further in section 1.6.1.

Hsp70s are expressed in many tissues of the human body. Many studies have analysed Hsc70 localised in the brain, such as the cerebellum, neurons, and synaptic bouton (Wilhelm et al., 2014), due to their therapeutic role in neurodegenerative disorders, as they are crucial for endocytosis at synapses (Sala et al., 2013, Lu et al., 2014).

Additionally, Hsp70s play a role in cancers, with overexpression of Hsp70s providing a protective effect for cells which are supposed to die, thus resulting in cancerous effects (Tanaka et al., 2014). Due to this, Hsp70s are located in tissues all over the body. Although Hsp70s are located everywhere, expression can vary. For example, Radons (2016) showed that in HspA1L and HspA2 have high expression in the testis, while HspA2 is also highly expressed in the brain. Isoforms like HspA12A and B have different expression levels in different tissues. HspA12A is highly expressed in the brain, kidney, and muscle, while HspA12B is highly expressed in the muscle, heart, liver and lower levels in the kidney.

Additionally, this high expression could negatively influence the tissue. Upregulation of HspA14 has been linked to tumour tissues of Hepatitis B (HBV**)** related early stage liver carcinoma. HspA12B has been linked to contributing towards pathogenesis of endothelium associated processes. Hu et al., (2006) analysed the expression of HspA12B during development in human umbilical vein endothelial cells and zebrafish. Northern blot analysis and real-time PCR identified high expression in both zebrafish and humans. Knock down of HspA12B resulted in less wound healing, decreased cell migration and decreased tube formation in blood vessels. Therefore, it can be stated that HspA12B is important in angiogenesis and can be identified as a therapeutic treatment for atherosclerosis, sepsis, and preeclampsia.

1.4 Hsc70 in stress

Although it is very important to have a chaperone protein to protect degradation of proteins under stress conditions, failure to degrade key proteins may result in negative consequences. Tanaka et al., 2014, found that Hsc70 almost acts as a protective effect for Rab1A, a protein

which is part of the Ras superfamily. Rab1A regulates signal transduction, cell proliferation, and vesicle transport, which are many factors which are over expressions in cancer cells. Under stress conditions (serum depletion, 5-FU treatment), Hsc70 was in complex with Rab1A and so it was refolding the damaged protein, allowing it to continue to remain functional throughout the treatment. Therefore, targeting Hsc70 as an anti-cancer drug can be reasonable.

1.5 Expression of Hsp70s

Expression of Hsp70s has been localised within the cytosol, nucleus, cell membrane, extracellular vesicles, ER, blood microparticles, mitochondria, intracellular vesicles, endothelial cells, and blood plasma. Hsc70 is localised within the cytosol, nucleus, cell membrane and extracellular vesicles (Radons, 2016).

When the cell undergoes a stress response, such as ATP depletion, heat shock, or serum depletion, the heat shock protein must act in the nucleus rather than the cytoplasm, although it needs chaperones to assist with moving into the nucleus. Experimental evidence of Hsp70 localised in the ER was done by Kasaby et al., 2014. While investigating how Hsp70-1A interacts with the serotonin receptor (SERT), it was additionally found that Hsp70 is involved in the ER. Hsp70-1A and Hsp90 were found in a GST pulldown with the C terminus of SERT (which is a DnaK binding site). Hsp70-1A was then transfected into HEK293 cells. With Hsp70-1A YFP tagged, and the ER resident SERT with CFP, Forster Resonance Energy Transfer (FRET) signals indicated that there was an interaction between them both. Further co immunoprecipitation found Hsp70-1A, Hsp90B and SERT complexed together, which suggests that Hsp70 is involved in the ER, and involved in the folding trajectory of SERT.

1.5.1 Cochaperones - Hsp40 and BAG-1

Hsc70 interacts with a large variety of proteins, due to its protein folding capabilities. However, as well as unfolded proteins, there are those which facilitate the protein folding functions. The *E. coli* homologue of Hsc70, DnaK, (Dugaard, Rohde & Jaattela, 2007) has two main cochaperones, DnaJ and GrpE, which act in concert. DnaJ is important in binding client substrates and bringing them to DnaK. Ohtsuka (1993) used a cDNA expression approach to find the human homologue, Hsp40, of DnaJ. Hsp40 shares 34% homology with DnaJ, and so is

loosely related. DnaJ is made up of a J domain, G/F domain, and C domain. The J domain is highly conserved across homologues, while the C domain is not as conserved. The J domain is important in binding Hsc70 and stimulating its ATPase activity, while the accessory domains are important and binding and presenting substrates to Hsc70 (Jiang et al., 2007). The J domain is a helical bundle, made up of 4 α helices, and a loop domain, rich in His, Pro and Asp, making the HPD motif. This motif interacts with Hsp70 α helices II and II to stimulate the ATPase domain (Nillegoda et al., 2017). Hsp40 has both the J domain and the G/F domain, but no C domain. Additionally, their localisation is different: DnaJ is located in the membrane, while Hsp40 is in the cytosol and nucleus (Ohtsuka, 1993). The function of DnaJ is to facilitate substrate transfer to DnaK. DnaJ is able to bind heat induced aggregates and rapidly transfers them to DnaK. DnaJ plays an important role in ATP hydrolysis, by stimulating it and increasing substrate affinity. This is also observed in Hohfeld and Jentsch, 1997.

Nillegoda et al., 2017 aimed to find the evolutionary conservation of how J proteins function in a complex and how they function to disaggregate proteins. This led to the discovery of a eukaryotic specific signature in J proteins. Alterations in conservation has resulted in some J proteins being unable to interact with each other, which is why correct J protein pairing is important. Class A and B proteins have different substrate binding preferences at CTDs (which is in the J protein) and have different interaction sites for the J domain. The CTD also has interaction sites for opposite class members during J protein complex formation. Nillegoda et al., 2015 labelled J domains in class A and B with AF488, and CTDs of class A and B J proteins with Fluorescein Arsenical Hairpin (FlAsH). No interactions between J domains and CTD of the same class (e.g. J domain (JB1) – CTD (JB1)) were found, but interactions between J domains of JA2 and CTD of JB1 were found. This is beneficial, because J complexes with the two classes can interact with Hsp70 and Hsp110 to target a broader range of aggregates, rather than when J complexes of the same class, when interacting with Hsp70 and Hsp100, can target only aggregates of specific sizes.

GrpE is a nucleotide exchange factor (NEF) which has been found to be important in protein folding. Without GrpE, there is a 50% decrease in protein refolding (Wisen & Gestwicki, 2008). GrpE induces substrate release from DnaK. Bcl-2 associated athanogene 1 protein (BAG-1) has GrpE-like functions which interact with Hsc70. As found by Hohfeld and Jentsch, 1997, GrpE

facilitates in protein folding by binding the ATPase domain of Hsc70 and stabilising the ATP in the NBD. This stimulates the release of polypeptide substrate from the protein. Additionally, BAG-1 can speed up the Hsc70 substrate cycle by getting rid of ADP out of the NBD, which Hsp40 is not able to do. BAG-1 is only able to undergo these functions when Hsp40 stimulates ATP hydrolysis, which BAG-1 is not capable of doing. It is not stimulated by the nucleotide state of Hsc70. A competing protein, Hip, was found to additionally bind to the ATPase domain and essentially do the opposite of what BAG-1 does. It is stimulated by the nucleotide state of Hsc70, and stabilises ADP in the NBD, to slow down the ADP release. Although there are many other proteins which interact with Hsc70, Hsp40, BAG-1 and Hip are highly studied with a large amount of knowledge about them, which is why they have been included.

1.5.2 Interplay of hsp70s and other protein folding families (other systems)

Kumar and Sourjik (2012) analysed how Hsp70 systems of *E. coli* interact with other protein folding systems during heat shock. It was found that Hsp70 is able to both accept substrates from other chaperone systems, as well as transferring processed substrates to other chaperone systems. The systems which were analysed are ribosome associated trigger factor (TF), GroES/GroEL chaperonin system, Hsp90, Hsp100, and small Hsps (sHsps). The Hsp70 system included DnaK, DnaJ, and GrpE.

TF is the first chaperone to interact with nascent polypeptide chains after they leave the ribosome. This is to protect the polypeptide which could be degraded by proteases, and to promote their cotranslational folding. However, TFs have a low affinity for substrate proteins, meaning that another system would be required.

The GroE system is important in maturation of polypeptides which have a high energy barrier of folding. GroE has an oligomeric GroEL ring which acts as a cage-like environment around the polypeptide, allowing the complex structure to be formed. This system acts downstream of Hsp70. Knock out of GroE reduces cell viability under all growth conditions.

The Hsp90 system is involved in maturation and conformational remodelling and receives substrates from the Hsp70 system. Using FRET, cells were induced with heat shock and protein

localisation could be tracked. It was found that DnaK and DnaJ had interactions with TF, IbpA/B (sHsps) ClpB (a hexameric AAA protein involved in reactivating aggregated proteins with ATP hydrolysis), and HtpG, of the Hsp90 system. Although DnaK interacted with both DnaJ and GrpE, no direct interactions between DnaJ and GrpE were found. GrpE interacted with HtpG and ClpB. These findings could show that these proteins are acceptors of DnaK processed substrates.

It was concluded from the research that TF, Hsp70, Hsp100 and GroEL/GroES are involved in protein folding and maturation, while other systems, such as sHsps, Hsp70 and Hsp100 are dependent on substrate availability (Figure 1). Hsp70s rely on the catalysis of ATP and cochaperones to drive the refolding of peptides. The type of peptide also depends on substrate availability, as Hsp100s need peptides which are able to interact with the central pore of the chaperone.

Kasaby et al., 2014 analysed the interaction of the Hsp70 family and the proteolytic system, using proteins Hsp70-1A and Hsp90B in eukaryotes. The serotonin receptor, SERT, first matures in the ER before going to the cell surface. Point mutations in the gene results in the export and folding of the protein either becoming truncated or being stalled in the ER. Through co-immunoprecipitation and GST pulldown, it was found that Hsp70-1A and Hsp90B were in complex with SERT. Further analysis with computational methods resulted in the discovery of a putative binding site in the TM12 domain (in the C terminus), recognised by Hsp70. This binding site, YRLIITP, is similar to NRLLLTG, which is already known to be recognised by DnaK. This binding site has a peptide which covers the Hsp70/DnaK binding site. By introducing point mutations, Hsp70 is no longer able to bind to the binding site.

Kasaby et al., 2014 additionally analysed Hsp90. Hsp90 further matures protein substrates received by Hsp70, which was found co immunoprecipitated with Hsp70 and SERT. Drugs and siRNAs were used to knock down Hsp90. As a result, there were increased SERT and SERT F604Q receptors on the cell surface. In JAR cells, it was found that Hsp90 gets inhibited. Because Hsp90 regulates SERT, SERT levels increased, and it could be concluded that Hsp90B is important in controlling expression.

Nillegoda et al., 2015 gave a good understanding of how the Hsp70 system is linked to the proteolytic system. Using FRET, the system as to how both Hsp families interact with each other was able to be determined. Hsp100, which is a nuclear and cytosolic disaggregase system, has a hexameric ring with a central pore. This central pore has flexible aromatic loop residues inside, where protein aggregates are loaded by Hsp70. Protein aggregates (protruding polypeptides or surface loops of trapped polypeptides) are drawn into the central pore to interact with the internal residues. Once bound, Hsp70 must use its unfoldase activity to remodel the surface of the protein to make surface loops which will be able to interact with the internal pore residues. Hsp40 aids in this by controlled substrate binding cycles. ATP hydrolysis of Hsp100 then moves the aromatic residues in the central pore, and the polypeptide is pulled into the pore, and disentangled. Without a functional Hsp100, the yeast or bacteria will have very limited disaggregase activity. Hsp100 is also needed to be in complex with Hsp70, J proteins, and the aggregate protein substrate in order for the system to work.

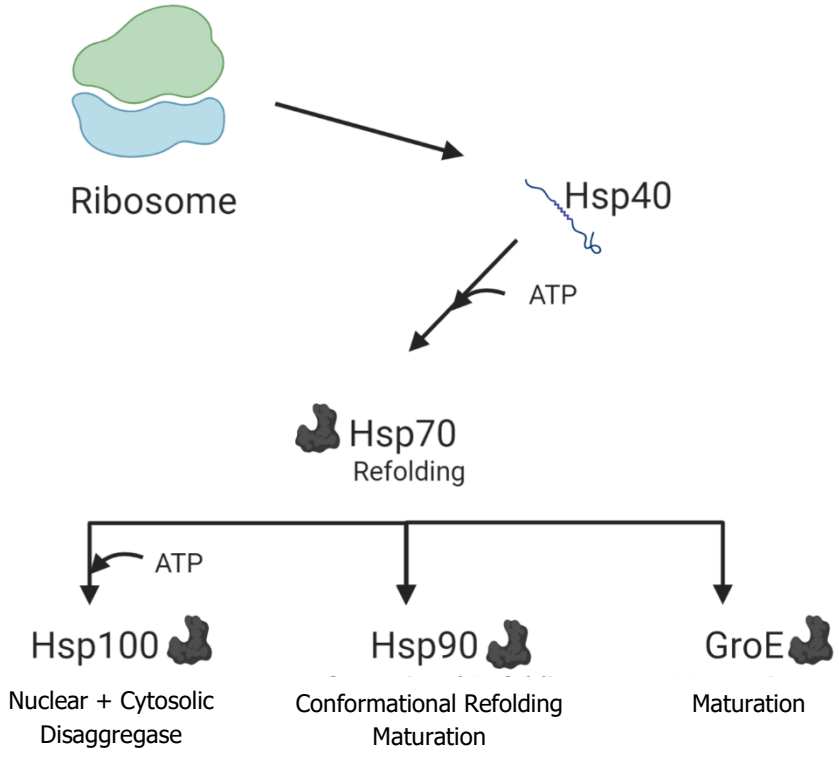

Figure 1. Interplay of Hsp systems. Polypeptides are released from the ER. TFs then bind to the unfolded peptides to protect against protease degradation. Hsp40 then binds the unfolded peptide and is transferred to Hsp70. Hsp70 can then refold the non-native peptide or transfer it to further systems if it requires a higher energy of folding.

1.6 Structure

1.6.1 Structure of NBD

Hsp70 proteins contain two domains, the nucleotide binding domain (NBD) and the substrate binding domain (SBD). The conformational changes of the NBD were analysed by Woo et al., 2009. Using molecular dynamics and comparing to crystallography results it was found that the NBD samples many conformations, due to the flexible interfaces among subdomains. This contrasts with the crystallography results, which showed no change This is due to not being able to analyse changes around Trp which is identical in structures, regardless of the nucleotide state of the NBD. The root mean square deviation (RMSD) of each residue was calculated, and it was found that aside from the flexible N and C terminal residues, 3 hinge regions were found. The first, (residues 186 - 191, and 357 - 366) forms loops at the interface between IA and IIB. The third hinge region was located at the interface between IIA and IIB (residues 226 - 231). Overall RMSD of whole NBD compared with those calculated for single or collections of subdomains were calculated. It was found that for both nucleotide free and ATP bound NBDs, subdomain IB shows the largest internal conformational change and flexibility, due to a partial unfolding of the β-sheet. The rest of the subdomains show RMSD values of around 1.4 Å, indicating that subdomain folds are very rigid. Measuring fluorescence from Trp90, it was found that in a closed conformation, Trp90 (located in the NBD) forms an H bond with Arg261. Quenching had occurred, and it could be hypothesised that this occurred when Arg261 was able to stablise the ATP bound conformation. When mutated to alanine, the quenching is no longer able to occur, and additionally caused a decrease in Trp fluorescence.

1.6.2 Structure of SBD

The SBD is smaller than the NBD, at 25 kDa (Lu et al., 2014). It is located at the C terminus and has a β-sandwich subdomain. Within the β-sandwich domain contains the substrate binding site (βSBD), which is covered by the helical lid (Umehara et al., 2018).

1.6.3 Helical lid

When ADP is bound to Hsp70s, the NBD “unlocks” from the SBD. Reducing affinity for substrates is achieved by the α helical lid (αLid) closing over the substrate binding cleft, thus resulting in no substrates accessing the binding site (English et al., 2017). The αLid is made up

of 5 α helices, and 30 amino acids in length (Sharma and Masison, 2009). The first helix rests on top of the SBDβ, and the remaining 4 helices form a helix bundle structure which acts as a lid to cover the SBDβ (Gong et al., 2018). In *C. elegans*, Hsc70 has a higher turnover compared to human Hsc70, but has limited ADP release after hydrolysis. The αLid domain was found to be important in influencing the rate limiting step of the ATPase cycle. In Sun et al., (2012), mutations were made by deleting the whole SBD (Δ384), deleting the whole αLid (Δ512), and lacking the C terminal helix bundle of the αLid (Δ545). ATPase rates (steady state) were then measured. An isolated ATPase had 0.21min k_{cat}, Δ512 had 0.14min k_{cat}, and Δ545 had 0.09min k_{cat}. The single turnover ATPase rates were then analysed, with Δ384 having a k_{cat} of 0.32min. This is for the isolated ATPase domain. Because these results are similar, these findings suggest that the ATP hydrolysis step is the rate limiting step for the protein. Looking at the single turnover results for Δ512 and Δ545, Δ512 had an increase in the hydrolysis rate (making it slower), of a rate of 1.03min. Δ512 has no αLid, and so it can be concluded that the αLid is what accelerates the ATP hydrolysis step.

Interaction with co chaperones, such as BAG-1 and DNJ-13 (a J protein) was analysed. Sun et al., (2012) showed that BAG-1 bound to the ATPase domain, and as with Hsp40, additionally competes with DNJ-13 for binding. The deletion of the αLid in Δ512 did not affect binding to the ATPase domain, and so it can be concluded that the αLid is not needed for co chaperone binding. Gong et al. (2018), analysed the C terminal domain in response to stress in yeast. The C terminal domain does not only consist of the αLid but includes the C terminal intrinsically disordered region (C-IDR). This region is a highly variable region and is typically made up of 30 - 40 aas. This region is found in both bacteria and eukaryotes. Located within the C-IDR are motifs involved in mediating binding of cochaperones. This involves a conserved tetratricopeptide repeat (TPR), GGAP and a 20 residue GGAP-like tetrapeptide motif. Following the C-IDR is the "extreme" C terminal EEVD motif. Gong et al., (2018) analysed the interaction between Ure2 and Ssa1. Ure2 is a yeast protein which can convert to the prion state, Ure3. Ssa1 can inhibit this conversion in both Ure2 and α syn formation. It is known that the SBD binds directly to Ure2, and thus inhibits fibril formation. Ssa1 is an Hsp70 which has a 45% sequence identity of the α helical bundle with human Hsc70 and is more like the human homologue than DnaK.

Following the C-IDR is an extreme C terminal EEVD motif. This second C terminal motif was found to enhance Hsp function by interacting with unfolded proteins. This motif has been investigated to establish how it plays a role in fibril formation.

1.6.4 Conformational Change and ATP Hydrolysis

The NBD has ATPase activity. ATP hydrolysis plays an important role in the refolding of client peptides, which the SBD uses to drive the process. This process is briefly highlighted by Figure 1. Initially, the Hsp40 brings the client peptide to Hsp70, and binds to the J domain of the ATPase. This initiates the ATPase to hydrolyse the ATP into ADP and P_i. Hsp70s undergo 3 conformational changes. These stages are when 1. ATP binds to the NBD, 2. ATP is hydrolysed into ADP, and 3. When ADP is released. The release of ADP depends on cochaperones. As mentioned in section 1.5, BAG-1 has been found to increase the rate of protein folding, by speeding up the reaction and allowing ADP to be removed from the nucleotide binding site.

Based on this system, it can be hypothesised that when there are low levels of ATP, no protein folding will occur.

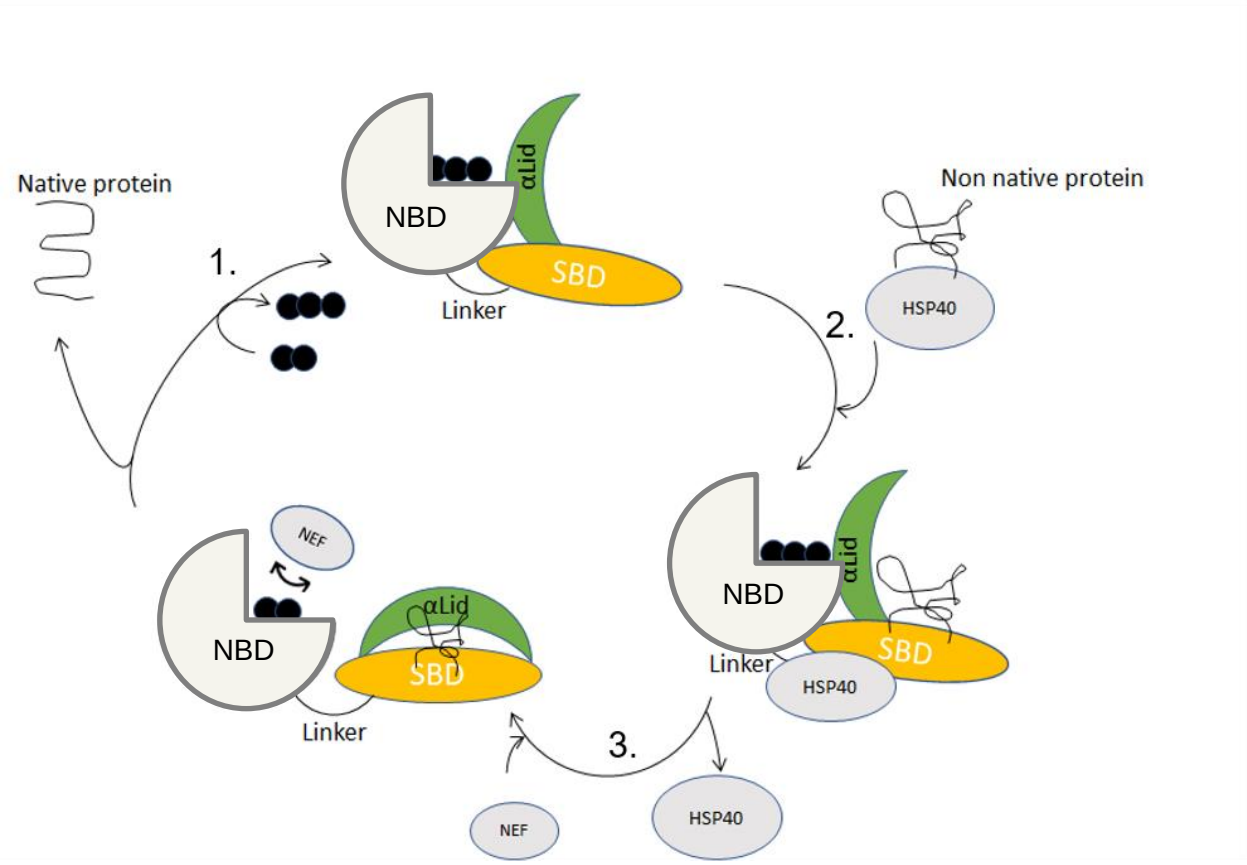

Figure 2. Conformational change of Hsp70 and the co chaperones which facilitate substrate processing. Three black circles together indicate ATP, and two corresponds to ADP. ATP binds to the NBD. There is a low affinity for the binding of the substrate to the SBD when the Hsp40 brings the client peptide. HSP40 stimulates nucleotide hydrolysis which then increases affinity for the substrate. Increasing affinity results in the αLid closing. The Nucleotide Exchange Factors (NEFs) then assist with the ADP release, which then causes substrate release.

Adapted from: Hartl F., Bracher A., Hayer-Hartl 2011. Molecular chaperones in protein folding and proteostasis. Nature. 475(7356): 324-332.

1.6.5 Short Flexible Linker Structure

The SBD and NBD are connected by a short flexible linker. This is a highly conserved sequence, made up of KSENVQDLLLLDV (Umehara et al., 2018), with the C terminal end of the linker, VQDLLLLDV, considered as the conserved sequence among Hsp70s (Jiang et al., 2005). It plays an important role in conformational change and interacting with cochaperones. Jiang et al., 2007 analysed how the flexible linker changes when interacting with the co chaperone, Hsp40.

Hsp40 has a J domain, which is highly conserved among J cochaperones, which can bind the linker. In the study, residues in the linker (V388 and L393) were mutated, and I181 in the NBD were mutated. This resulted in the J domain no longer being able to bind the linker, inhibiting the ATPase rate. This additionally showed that the J domain, when bound to the linker, stabilises the linker: I181 interactions. When ATP is bound to the NBD, this induces a change in the linker conformation. The linker is buried by the SBD but becomes exposed upon ATP binding. Crystallography revealed the interaction between the J domain, linker, and NBD. It was found that the linker approaches residues I181 and Y371 in the NBD. When I181A and Y371A mutants were made, ATPase rates were analysed. With J domain presence, the ATPase activity of the I181A was decreased 10-fold, showing that this residue is important in linker interactions. Y371A mutants showed inappropriate NBD: linker interactions, resulting in inhibition of the NBD, as well as constitutive and unresponsive interactions with the J domain.

The J domain is additionally important in transmitting a J signal to the NBD active site, to stimulate ATP hydrolysis. Using a slowly hydrolysable analogue which has identical base, ribose, and phosphate numbers to ADP, crystals of J domain interactions with the NBD domain and linker were able to be analysed. It was found that there was a 3.5Å shift in the complex towards E175, a residue located in the nucleotide binding site. Additionally, residues 172 - 181 had shifted by 0.6 - 1.3Å. This gave an indication that these residues interacted with the J domain. Previous studies had shown that E175 was catalytically important. By making a E175S mutant, the ATP rate was 100-fold lower than that of WT. Therefore, it could be concluded that E175 was important in transmitting the J signal to the nucleotide.

Mapa et al. (2010), analysed the mitochondrial yeast Hsp70, Ssc1, located in the mitochondrial matrix by using FRET efficiencies. Two sensors were made to analyse the distance of the SBD and the NBD from each other. A FRET sensor was in the SBD and the NBD, and another sensor was located between the base of the SBD, and the αLid. When Ssc1 was in the ATP state, there is a donor and acceptor separation of 42Å in between the SBD and the NBD, which indicates a narrow efficiency. In the ADP state, there is a separation between the donor and acceptor separation of 62Å, which indicates a very broad efficiency between the SBD and the NBD.

When Ssc1 is in the ATP state, the second sensor, between the SBD and the αLid, has a narrow efficiency (0.20) with a distance of 77Å. This indicates that the αLid is not covering the base of the SBD, or the SBD is open when ATP binds. In the ADP state, there is an acceptor - donor separation of 68Å. The distance and efficiency are not as broad compared to the first sensor. Therefore, it can be determined that the protein is more flexible in the ADP state.

Mdj1 is a mitochondrial DnaJ homologue which was found to aid in protein folding after denaturation and protect the yeast cell at temperatures above 37℃ (Rowley et al., 1994). Experiments were done with Mdj1 and a substrate peptide P5. These results could then be compared to the previous results generated by Ssc1. In the presence of Mdj1 only, results seen in FRET histograms and ensemble measurements are similar to results for Ssc1 in the ADP state. From this, it can be concluded that Mdj1 stimulates ATPase activity of Ssc2, which is a mitochondrial Hsp70 - type molecular chaperone which aids in organising Fe/S clusters, allowing the ability to store iron in the mitochondrial matrix (Voos & Rottgers, 2002). When Ssc2 is stimulated, the ATP form of Ssc1 is converted to the ADP form, which is what most J co chaperone proteins do.

FRET experiments with Mdj1 and substrate peptide P5 showed that the first sensor among the SBD and NBD has a lower acceptor to donor ratio (AD_R**)** compared to results with ADP or Mdj1 only. This is demonstrated by the FRET efficiency shifting to a single peak with a lower efficiency. Additionally, the distance between fluorophores increased from 62Å in ADP, to 75Å when the substrate peptide P5 was present. Therefore, when in the presence of substrate, the SBD and NBD undock from each other. The second sensor, among the base of the SBD and the αLid, a higher AD_R was found when substrate peptide P5 is present, compared to data from ADP or Mdj1 only. A dominant FRET peak was found, revealing an average donor acceptor separation of 52Å. Therefore, it can be determined that the αLid is in a closed conformation when substrate is present.

1.7 How has FRET been used to study protein interactions of Hsp70s?

FRET is used to study distances of protein-protein interactions and report conformational shifts. It is able to be used in live cells to identify these interactions in real time. FRET is a highly

sensitive technique which can measure distances typically between 3-6 nm (Sekar & Periasamy, 2003). FRET, the transfer of energy from one excited fluorophore, the donor, to another fluorophore, the acceptor, can be measured by the relative intensity of fluorescence from donor and acceptor.

Fluorophores which are used must have an overlap in the excitation and emission spectra in order to allow the transfer of energy from the donor fluorophore to the acceptor fluorophore. However, this overlap must be distinctive enough to be able to detect which fluorophore has been excited. One example of this is colour emitting at different wavelengths is typically used to identify these differences.

Kasaby et al., 2014 analysed the interaction of the Hsp70 family and the proteolytic system, using human proteins Hsp70-1A and Hsp90B. Mutations at the C terminus of the SERT results in disruptions in both the export and folding of the protein from the ER to the cell surface. Hsp70-1A binds to the C terminus. FRET live microscopy was used in HEK293 cells to observe the interaction between the C terminus SERT and Hsp70. Both SERT and Hsp70-1A were labelled with CFP and YFP, respectively. Genes for fusion proteins were transfected and expressed in the live HEK293 cells. FRET showed that SERT was localised in the ER with Hsp70-1A.

Hayashi et al., 2017 analysed interactions of Hsp70 and Hsp100. A fusion protein was produced by fusing in between A283 and Ser284 (loops of the NBD subdomain) in Hsp70. After Glu582, everything is deleted. When adding nucleotide, the ratio of fluorescence intensities was analysed. When no nucleotide was bound, the ratio was 1.4, increases when ATP is bound, and does not change when GrpE is added. However, in the K69A mutant, the ratio had increased to 1.5, and then increased to 2.8 with addition of ATP. GrpE influenced it further by increasing the ratio.

Therefore, this explains the mechanism of conformation, by ATP inducing the opening of the SBD. ATP hydrolysis then occurs, causing the SBD to close. GrpE then induces opening of the SBD, which promotes nucleotide exchange. This suggests the proteins are homologous to each other.

Ungelenk et al. (2016), analysed small heat shock proteins (sHsps) Hsp26 and Hsp42, and their role in refolding proteins. Hsp26 is important in promoting protein disaggregation, and Hsp42 facilitates both Hsp70 and Hsp100 post folding, but does not have aggregase activity like Hsp26. FRET showed that sHsps are able to separate aggregate malate dehydrogenase (MDH) molecules. This was done by labelling MDH with YFP, and other MDH molecules with 7-diethycoumarin-4-carboxylic acid. At normal temperatures, there was an aggregation of the donor and acceptor molecules, but when there was heat shock, the FRET signal increased. This showed that there was an increased Hsp binding distance between the MDH protein under heat shock or stress, and the spacing between the molecules allows for preventing the misfolded MDH protein.

Zou et al., 2008 explored how Hsc70 is released during ischemia reperfusion (an injury of heart tissue resulting from inadequate blood supply). It was already known that toll like receptor 4 (TLR4) was activated by Hsc70, but the mechanism of activation was unknown. TLR4 controls the inflammatory response to ischemia. Prior to FRET analysis, it had to be confirmed that Hsc70 does induce a TLR4-dependent inflammatory response. Isolated macrophages from TLR4-competent and TLR4-defective mice were treated with 1 µg/mL Hsc70. TNF-α levels were shown to increase in the TLR4-competent cells but did not show an interesting increase in the defective mice. With this confirmed, FRET could be observed. In untreated cells, there was no FRET signal between the TLR4 and Hsc70. Additionally, the Hsc70 had not moved from the cytoplasm. However, in the treated cells, Hsc70 had moved to the cell surface. The FRET signal showed an interaction between the receptor and Hsc70, and that TLR4 and Hsc70 were no more than 5 nm apart. Using FRET was reliable to see that Hsc70 does move within the cell and interacts with receptors on the cell surface.

Kumar and Sourjik, 2012 used FRET to identify which chaperones DnaK interacts with. This study looked at a large number of proteins, all of which are chaperones along the folding trajectory of DnaK. Using YFP and CFP at the C terminus, complexes were found in MG1655 *E. coli* cells even when there is not any complex formation, substrate folding occurred. Chaperones which were investigated included DnaJ, GrpE, TF, IbpA/B, ClpB and HtpG. The expected interactions among DnaK, DnaJ and GrpE were found, but interestingly, not between DnaJ and

GrpE themselves. However, DnaJ was found to interact with itself, but this is consistent with the idea that it is a dimer. GrpE, which is a dimer, did not have interactions between itself. Other protein interactions through FRET were found between TF, IbpA/B, ClpB and HtpG, which are found to be involved with DnaK after substrate processing. This gives evidence that FRET can be used to find interactions among Hsp70s.

Mapa et al., 2010 analysed the effects of nucleotides and co chaperones on the conformation of Ssc1, the mitochondrial yeast homologue of Hsp70. FRET based sensors in ensemble FRET/ smFRET were used to analyse how nucleotides affect the movement of two domains in Ssc1 and the opening of the SBD. The co chaperone Mdj1 was labelled with AF594, and Ssc1 labelled with Atto647N. FRET showed that the two domains in Ssc1 were closer to each other when ATP is present. The SBD was open in the ATP state, and closed in ADP, because the lid and base of the SBD are closer to each other when ADP present.

1.8 Aim for this project

To observe conformational change of Hsc70 under different conditions, including normal growth, heat shock, and ATP depletion in HEK293 and HepG2 cells.

It was aimed to achieve this through fluorescent labelling of Hsc70, transduction of the fluorescent protein into tissue cells, and confocal fluorescent microscopic imaging of transduced cells.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Chemicals, Reagents and Standard Equipment

- Cell Culture
    - Trypsin ethylenediaminetetraacetic acid (EDTA) 0.25% Phenol Red (Thermofisher, USA). Life Technologies Pty Ltd. www.thermofisher.com
    - TrypLE Express Enzyme (Thermofisher, USA). Life Technologies Pty Ltd. www.thermofisher.com
    - Dulbecco's Modified Eagle Medium (DMEM) High Glucose (4.5 g/L D-Glucose, L-Glutamine, no sodium pyruvate) (Thermofisher, USA). Life Technologies Pty Ltd. www.thermofisher.com
    - Opti-Modified Eagle Medium (Opti-MEM) Reduced Serum Medium (HEPES, 2.4 g/L Sodium Bicarbonate, L-glutamine) (Thermofisher, USA). Life Technologies Pty Ltd. www.thermofisher.com
    - Amphotericin B (Thermofisher, USA). Life Technologies Pty Ltd. www.thermofisher.com
    - ProLong Gold Antifade Reagent (Thermofisher, USA). Life Technologies Pty Ltd. www.thermofisher.com
    - Lipofectamine 3000 Reagent (Thermofisher, USA). Life Technologies Pty Ltd. www.thermofisher.com
    - Penicillin-Streptomycin (10,000 U/mL) (Thermofisher, USA). Life Technologies Pty Ltd. www.thermofisher.com
    - Fetal Bovine Serum (FBS) (Thermofisher, USA). Life Technologies Pty Ltd. www.thermofisher.com
    - Poly-L-ornithine (PLO) www.sigmaaldrich.com
    - Poly-L-lysine (PLL) www.sigmaaldrich.com
    - PULSin Delivery Reagent (Polyplus transfection, France) www.polyplus-transfection.com

- Fluorescent Labelling
    - Pierce Immobilised TCEP Disulfide Reducing Gel (Life Technologies New Zealand Pty Ltd) www.thermofisher.com
    - Alexa Fluor 594 C5-maleimide (Thermofisher, USA). Life Technologies Pty Ltd. www.thermofisher.com
    - Alexa Fluor 488 C5-maleimide (Thermofisher, USA). Life Technologies Pty Ltd. www.thermofisher.com
    - Dimethylsulfoxide (DMSO), anhydrous Desiccate Life Technologies New Zealand Pty Ltd www.thermofisher.com

- Luciferase Assay
    - Steady Glo Luciferase Assay System (Promega,USA) www.lifescience.invitro.co.nz
    - QuantiLum Recombinant Luciferase (Promega, USA) www.lifescience.invitro.co.nz
    - Dithiothreitol (DTT) Life Technologies Pty Ltd. www.thermofisher.com
    - Creatine phosphate (Sigma Aldrich) www.sigmaaldrich.com
    - creatine phosphokinase (Sigma Aldrich) www.sigmaaldrich.com

- SDS-PAGE Gels
    - 30% Acrylamide/Bis Solution 37.5:1 (BIO-RAD Laboratories Pty Ltd) www.bio-rad.com/en-nz
    - Ammonium Persulfate (APS) (BIO-RAD Laboratories Pty Ltd) www.bio-rad.com/en-nz
    - Electran NNN'N'-Tetramethylethylenediamine (TEMED) Belgium www.sigmaaldrich.com/
    - Unstained SDS-PAGE Standards, Broad Range (BIO-RAD Laboratories Pty Ltd) www.bio-rad.com/en-nz

- Plasmid Expression

- E.N.Z.A. Plasmid DNA Mini Kit I (OMEGA Biotek, USA). www.omegabiotek.com
- Phenylmethanesuflonylfluoride (PMSF) Sigma Aldrich, Germany www.sigmaaldrich.com
- Syringe Filters CA ReliaPrep Sterile 0.2 µm and 0.45 µm 25 mm. (Ahlstrom, Germany) www.labsupply.co.nz

- Antibiotics
  - Sodium Ampicillin (Gold Biotechnology, USA) www.goldbio.com
  - Chloramphenicol (Gold Biotechnology, USA) www.goldbio.com

- Protein Purification
  - Adenosine 5’ -triphosphate disodium salt, Grade I (Sigma Aldrich, USA) www.sigmaaldrich.com
  - Adenosine 5’ -diphosphate (ADP). 95% HPLC (Sigma Aldrich, UK) www.sigmaaldrich.com
  - Snakeskin Dialysis Tubing 10,000 MWCO 22 mm (Thermofisher, USA). Life Technologies Pty Ltd. www.thermofisher.com
  - Amicon Ultra Centrifugal Filters Concentrator MWCO 30,000 (Millipore, Ireland) https://www.labsupply.co.nz/

2.1.2 Specialised Equipment

2.1.2.1 Centrifuge

- Beckman Coulter Avanti J 26 XP Centrifuge with JLA8.1 rotor
- Eppendorf 5810 R Centrifuge
- Beckman Coulter Microfuge 16 Centrifuge
- Thermoscientific CL10 Centrifuge

2.1.2.2 Chromatography Columns and Resin

All chromatography experiments were conducted on a AKTApure FPLC. (GE Healthcare)

- HiPrep Desalting 26/60 GE

- A-25-120 DEAE Sephadex, Sigma
- ATP agarose with C-8 linkage with nine-atom spacer, Sigma
- HiLoad 26/60 Superdex 200 prep grade GE
- HiPrep S-Sepharose, x2 5 mL
- HAP 40 mL

2.1.2.3 Mammalian Cell Culture

- HERSAFE KS Thermo Scientific Laminar Flow Hood
- BIORAD TC10 Automated Cell Counter
- BIORAD Counting Slides Dual Chamber for Cell Counter
- 13 mm cover glasses
- Nalgene Mr. Frosty freezing container

2.1.2.4 Microscopes

- Olympus CK40 Microscope
- Nikon Ti2-A Inverted Fluorescence Microscope

2.1.2.5 Purification

- Sonifier Cell Disrupter (Branson Sonic Power Co., Smith Kline) with 3cm probe
- Emulsiflex C3 Homogeniser Avestin

2.1.2.6 Spectrometers

- Biowave WPA CO8000 Cell Density Meter
- LKB Biochrom Ultrospec II, 4050 UV/VIS Spectrometer
- Cary 100 UV/VIS Agilent Technologies
- Cary Eclipse Varian Fluorescence Spectrometer
- CLARIOstar Plate Reader
- Nanodrop ND-1000 Spectrometer Thermo Fisher

2.1.2.7 Fermentation

- Microgen Fermentor New Brunswick Scientific Co. Inc

2.1.3 Software

- PyMol (Schroedinger Scientific)
- Fluoview (imaging mammalian cells) (Olympus)
- LabScan (SDS-PAGE) (HunterLab)
- Nanodrop software (Thermoscientific)
- Image J (Cell microscopy) (National Institutes of Health, Maryland USA)

2.2 Methods

2.2.1 Plasmids and Isolation of plasmid DNA

pMSK003 plasmids were used for expression and transformation of Hsc70 3T. The plasmid encodes a gene for ampicillin resistance. These plasmids were created by Malcolm Rutledge, a former member of the Wilbanks Lab.

A single colony of E. *coli* DH5α was grown on an ampicillin + chloramphenicol LB agar plate at 37ºC. One colony was isolated after overnight incubation and added to 5 mL LB and ampicillin. This was then incubated overnight at 37ºC, with aeration and agitation. Plasmid DNA was then purified using the E.Z.N.A Plasmid DNA Mini Kit Spin Protocol to isolate pMSK003. DNA was eluted with water, and the concentration determined with Nanodrop spectrometer. The plasmid DNA then stored at -80ºC until use.

2.2.2 *E. coli* growth

DH5α cells were used to isolate plasmid DNA, and to produce more pMSK plasmid for stores. DH5α competent cells do not carry any antibiotic resistance.

BB1553 DE3 cells are a DnaK knockout strain of *E. coli*. These cells carry chloramphenicol resistance. The DE3 lysogen in the cell carries the gene for T7 RNA polymerase and is under control of a *lac* promoter. The *lac* promoter controls the transcription of the Hsc70 gene, which was specifically engineered into the plasmid. The *lac* promoter was activated by IPTG, which promotes activation of T7 RNA polymerase, thus driving transcription of the Hsc70 gene.

2.2.2.1 Media and conditions

LB liquid medium: 0.5% yeast, 1% NaCl, 1% peptone.

LB agar: 0.5% yeast, 1% NaCl, 1% peptone, 1.5% agar.

Ampicillin was prepared in sterile water and used at a final concentration of 250 mg/mL.

Chloramphenicol was made in isopropanol, and used at a final concentration of 30 mg/mL
IPTG was made in sterile water and used at a final concentration of 100 µM.

BB1553 DE3 cells were spread on LB agar, and overnight samples were grown in LB media. Induction samples were grown in presence of just ampicillin in LB media. All stages of growth were incubated under 28 ºC, and in the presence of both amp and chloramphenicol.

DH5α cells growth on Amp/Chloro LB agar, overnights grown in LB Amp media. All stages of growth performed under 37ºC.

2.2.3 Transformation

2.2.3.1 Competent cells

Competent BB1553 (DE3) cells were produced by Malcolm Rutledge, a former member of the Wilbanks lab.

A single BB1553 (DE3) colony was grown at 28ºC overnight in 5 mL LB supplemented with 100 µg/mL ampicillin and chloramphenicol with agitation. To inoculate 40 mL 2 × YT, 400 µL of the overnight culture was added, and samples were analysed at OD_{600} until a value of 0.4 - 0.6 was reached. Cells were then centrifuged at 3000 *g* for 5 min at 4ºC. The supernatant was discarded, and the cell pellet resuspended in 25 mL ice cold 100 mM $CaCl_2$ for 30 min. The cell mixture was then centrifuged again, and the cell pellet is resuspended in 20% glycerol. This was then incubated on ice for 20 min, before being aliquoted and snap frozen in a mixture of dry ice and ethanol. Aliquoted cells were stored at -80ºC.

DH5α cells were made competent by the same method by Pauline Uyseco and Brooke Hayes, both members of the Wilbanks lab. However, chloramphenicol was not used, and 100 µg/mL ampicillin was used to supplement the agar and liquid media. Growth of cells was undertaken at 37ºC.

2.2.3.2 Heat shock transformation

Competent DE3 cells (50 µL) were thawed on ice and were then added on top of 1 µL plasmid DNA. The plasmid and cells were then incubated for 30 min on ice. Cells were then heat

shocked at 37ºC for 45 seconds. Cells were then left on ice for 2 min. LB media (800 µL) was added to each sample and incubated for 1 hour at 28ºC with agitation. Samples were then centrifuged at 14800 rpm for 2 min in a Beckman Coulter Microfuge centrifuge to pellet. Seven hundred and eighty µL of supernatant was removed, and cells were resuspended in the remaining media. Fifty µL was then spread onto LB agar plates (no antibiotics, ampicillin, and ampicillin + chloramphenicol). Plates were then incubated at 28ºC overnight.

The remaining BB1553 DE3 cells were used as a control to indicate no plasmid DNA transformation, as they lacked plasmid addition but were also heat shocked and streaked onto the agar plates.

Plasmid only samples and water only samples were also used to control for possible contamination of plasmid.

2.2.4 SDS PAGE

Pre and post induction samples were centrifuged for 5 min at 14000 rpm. The OD_{600} reading of the original sample was multiplied by 100, and this volume in µL of 5 x loading dye was used to resuspend pellet. Samples were then boiled at 95ºC for 10 min to lyse cells and denature proteins. Volumes of both 8 µL and 4 µL were loaded onto a 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gel for each sample.

Gels were prepared from a 4x stock of resolving and stacking gel buffer. Resolving gel was prepared in a 50 mL Falcon tube, and 26 mL was poured into a 4 gel multi caster. Isopropanol (800 µL) was pipetted on top of each gel and left to set. Isopropanol was poured off, and prepared stacking gel was pipetted on top. Lane combs were added, and gel was left to set. Gels were kept at 4ºC for up to 2 weeks.

Gels were run with electrophoresis buffer at 100 V for 10 min until stacked, and then at 250 V for 45 min until bands reached the bottom gel edge. Gels were then stained with Coomassie blue for protein visualisation, and then destained so only protein bands were visualised.

4 × resolving gel recipe: 10 mL acrylamide, 6.25 mL resolving buffer, 8.5 mL MQ, 250 µL 10% APS, 30 µL TEMED

4 × stacking buffer recipe: 0.5 M Tris-HCl pH 6.8, 0.4% SDS, MQ.

Resolving buffer recipe: 1.5 M Tris-HCl pH 8.8, 0.4% SDS, MQ.

Stacking gel recipe: 3 mL acrylamide, 4.5 mL stacking buffer, 10.5 mL MQ, 100 µL 10% APS, 30 µL TEMED

5 × high glycerol sample buffer: 0.6 mL 1 M Tris-HCl pH 6.8, 1 mL 20% SDS, 1 mL 1% Bromophenol Blue (filtered), 0.4 mL Sterile MQ, 6.5 mL 50% glycerol. Add 5% BME prior to use.

1 × SDS-PAGE running buffer: 25 mM Tris, 192 mM glycine, 0.1% SDS, MQ.

Coomassie blue recipe: 250 mL isopropanol, 100 mL acetic acid, 0.5g Coomassie Brilliant Blue R250. Make up to 1 L with MQ and filter through Whatman paper.

Destain buffer: 100 mL isopropanol, 140 mL acetic acid, 80 mL glycerol. Make up to 2 L MQ.

SDS-PAGE low range marker: 100 µL stock: 5 µL stock low range marker, 20 µL 5 × sample buffer, 75 µL sterile MQ. Aliquot into 5 µL.

2.2.5 Purification of Hsc70 3T

2.2.5.1 Expression of Hsc70 3T

Single colonies from heat shock transformation were added to 50 mL LB, and then incubated overnight at 28ºC with aeration. A New Brunswick Scientific Microgen Fermentor was used to grow BB1553 DE3 cells on a large scale. To sterilise the fermenter, 8 L of water was brought up

to 120ºC for half an hour, and then set at 28ºC for optimal growth. The cloudy LB overnight cultures (50 mL) were then added to fermenter, with 100 mg/mL ampicillin. Autoclaved 2 L 5 x LB was then added to the fermenter, and bacteria were grown with agitation. Every 2 hours 1 mL samples were taken, and once an OD_{600} of around 0.4 was achieved, plasmid expression was induced by 100 µM IPTG. Induced sample was left at 28ºC agitation overnight.

A post induction sample was taken around 17 hours later. Once the OD_{600} had doubled compared to the previous day's OD_{600} reading, cells were harvested. Cells were centrifuged in a Beckman Coulter with JLA 8.1 rotor centrifuge at 10,000 rpm for 30 min at 4ºC. Pellets were stored at -20ºC.

2.2.5.2 Cell homogenisation and lysis by sonification

The cell pellet must be homogenised and sonified before loading to the AKTA Pure FPLC.

Lysis buffer was used to resuspend the cell pellet, and then homogenised further in a 40 mL Dounce homogeniser. OD_{600} readings were taken, and the cell homogenate was diluted with lysis buffer until an OD_{600} reading below 1.0 was achieved. A 3 cm Sonifier probe was chilled on ice before use, and then used to lyse cells on 40% duty cycle for 6 min pulsed power output 8. The sample was then checked to ensure the sample had no bubbles or was clear, and then sonified for a further 6 minutes at the same settings. The sample was then centrifuged for 30 min at 14,000 rcf at 4ºC using a JA-14 rotor. Supernatant was then filtered through 0.45 µM and 0.2 µM syringe filters.

Lysis buffer: 20 mM Tris pH 6.9, 50 mM NaCl, 100 µM EDTA, 100 µM PMSF.

2.2.5.3 Anion exchange chromatography

A A-25-120 DEAE Sephadex (Sigma) column was equilibrated overnight with low and high salt buffer. Samples were loaded into a 50 mL Falcon tube and kept on ice while being loaded through the sample pump. Waste was collected during loading, to capture any sample which may not have attached to column if overloading occurs. The flow rate was set at 3.0 mL/min. The column was rinsed for 100 mL of low salt buffer and rinsed on a linear gradient of 0 - 5% for 40 mL high salt buffer. The next 440 mL undergoes a linear gradient elution of 5 - 60% high salt buffer. Fractions are collected (40 mL) in 50 mL Falcon tubes and are then collected until the

end of the run. Fractions are then ran on a 12% SDS-PAGE gel (4 µL), and remaining fractions were stored at 4ºC for further purification.

2.2.5.4 Dialysis

Fractions which showed a band around 66 kDa and limited contaminants at other weights were then pooled in 10,000 MW dialysis snakeskin tubing and placed into dialysis buffer overnight with activated charcoal and stored at -4ºC with magnetic flea. This was to ensure nucleotide has been removed from the active site of Hsc70.

DEAE low salt buffer: 20 mM Tris pH 6.9, 50 mM NaCl, 100 µM EDTA

Anion exchange elution (high salt) buffer: 20 mM Tris pH 6.9, 1.0 M NaCl, 100 µM EDTA, 100 µM PMSF.

EDTA dialysis buffer: 20 mM Tris pH 6.9, 150 mM KCl, 4 mM EDTA.

All buffers were filtered and degassed before addition to column.

2.2.5.5 ATP agarose chromatography

An ATP-agarose column with C-8 linkage with nine atom spacer resin was used. No equilibration step was required, so a system and pump wash of low and high salt buffers followed by elution buffer and degassed MQ was done prior to sample loading. Dialysed sample moved from snakeskin tubing to 50 mL Falcon tubes. 1 M $MgCl^{2+}$ was added to the sample with 10 µL of Mg^{2+} to every 1 mL of sample and incubated at room temperature for 30 minutes. The sample was centrifuged at 20,000 rcf for 25 minutes. The supernatant is recovered and filtered through 0.2 µM syringe filter. The sample was kept on ice while loading via the sample pump, and waste was collected during loading in case any protein was eluted and not attached to the column. The flow rate was set to 1.0 mL/min, then increased to 3 mL/min. The column was washed with 10 mL of high salt buffer and then 10 mL of low salt buffer. Bound protein was then eluted with 40 mL elution buffer in 1 mL fractions, and then followed by low salt buffer.

Fractions were then run on a 12% SDS-PAGE, and then concentrated with a MW 30,000 concentrator to around 3 mL. Fractions were stored at 4ºC until further purification.

Low salt buffer: 20 mM Tris pH 6.9, 25 mM KCl, 3 mM $MgCl_2$

High salt buffer: 20 mM Tris pH 6.9, 1.0 M KCl, 3 mM $MgCl_2$

Elution buffer: 20 mM Tris pH 6.9, 25 mM KCl, 3 mM ATP, 3 mM $MgCl_2$

All buffers were filtered and degassed before addition to column.

2.2.5.6 Gel filtration chromatography

A Superdex 200 26/600 column was equilibrated overnight with 400 mL MQ and gel filtration buffer to ensure all EtOH was removed from the column. Additionally, a 5 mL sample loop was washed with ethanol, MQ, and gel filtration buffer before sample loading. The sample was then loaded by direct injection onto the column, and then the method was selected. Running at a flow rate of 3 mL/min, the protein was eluted by 1.5 column volumes using an isocratic elution. Fractions (1 mL) were collected from 80 – 360 mL.

If the UV elution peaks are below the conductivity trace, there is a chance that the protein band may not appear on SDS-PAGE. In order for bands to be present, a TCA precipitation was prepared. To make 100% TCA, 1 g of TCA and mixing with MQ up to 1 mL. From each fraction 100 µL was added to a 1.5 mL Eppendorf tube, and then 20 µL 100% TCA was added to each Eppendorf tube. Tubes were then chilled at -20ºC for at least an hour with a metal Eppendorf tube rack to ensure rapid cooling. Samples were then centrifuged for 5 min at 10,000 g. A cloudy precipitate should be present, which is kept, and the supernatant discarded. The precipitant was then resuspended in 30 µL 5 × sample buffer and boiled at 100ºC for 5 minutes. Samples were then loaded onto an SDS-PAGE gel and analysed.

Selected fractions were pooled into a concentrator (MW30,000) and condensed down to 1 mg/mL. The concentration was measured using a spectrometer and using the Beer Lambert

equation to determine protein concentration. Fractions were then aliquoted and snap frozen in liquid nitrogen before storing at -80ºC.

Gel filtration buffer: 20 mM HEPES pH 7.6, 100 mM KCl, 5 mM $MgCl_2$. Buffer was filtered and degassed before use.

2.2.6 Expression and Purification of DnaJ

2.2.6.1 Induction of pUH21

The expression and purification methods are based off the protocols from Linke, Wolfram, Bussemer et al., 2003. DnaJ is the *E. coli* homologue and acts as a cochaperone by delivering unfolded peptides to Hsc70 (see section 1.5 for more information).

A glycerol stock of *E. coli* W3110 cells containing a pUH21 plasmid was streaked (50 µL) onto LB agar plates with 100 mM ampicillin (see section 2.2.3.2). Plates were incubated at 28ºC overnight, for 16 hours. One colony was scraped onto a pipette tip and added to two 50 mL LB overnight cultures. These were incubated at 28ºC for 16 hours overnight. After overnight incubation, cultures which were clear did not indicate growth, while a foamy and cloudy culture indicates growth. Of each successful overnight culture indicating growth, 10 mL was added to 4 L YT broth supplemented with 100 mM ampicillin and left at 28ºC (in baffled flasks). First 1 mL samples were taken an hour after inoculation, a second 2 hours after, and the last one 3 hours after. This last sample was spun down at 14800 rpm for 1 minute in an Eppendorf 5810 R Centrifuge, the pellet was kept, and then frozen at -20ºC to keep. Once reaching an OD_{600} of 0.5, cultures were induced with 300 µL 1M IPTG (33 µM) and left to grow overnight.

The next day, the OD_{600} was checked. Seeing an increase (2-fold), the culture was pelleted by centrifugation for 30 min at 7000 rpm 4ºC (JLA 8.1). Pellets were then stored at -20ºC until purification. A post induction sample (1 mL) was taken from this sample prior to cell pelleting and frozen at -20ºC.

Before purification, it must be confirmed that the DnaJ plasmid was successfully induced. As for Hsc70, a 12% gel was used. The pre and post induction samples saved during growth and harvesting were thawed and then resuspended in 5 × sample buffer. The sample was then boiled

at 95ºC for 10 minutes, and then loaded onto the gel. If a prevalent band at 40 kDa in the post induction band was shown, purification can continue.

YT Broth: 8% Bacto Tryptone, 5% Bacto Yeast Extract, 2.5% NaCl. Adjusted to pH 7.0.

2.2.6.2 Cell Lysis

The whole frozen cell pellet was left to defrost in a cup of water, and then was resuspended in 50 mL lysis buffer (without the Brij58) using a 60 mL homogeniser. Protease inhibitors were added to the cell slurry (0.8 mg/mL lysozyme, 1 mM PMSF and 1 tablet protease inhibitor cocktail mix (complete, EDTA free, in lysis buffer)). Cells were then lysed by 6 cycles of the Emulsiflex homogeniser at 15,000 psi 4ºC. The Brij 58 is then added to the cell lysate, to ensure the Emulsiflex does not bubble over, and centrifuged for 30 min 7000 rpm at 4ºC to separate the soluble proteins. The supernatant was then kept, and diluted 1:2 with buffer A.

2.2.6.3 Ammonium sulfate precipitation

An ammonium sulfate precipitation was used to bring out proteins which aggregate easily from very soluble proteins (Duong-Ly & Gabelli, 2014). This makes it a good method prior to purification and removes DnaJ from the insoluble fraction. Ammonium sulfate was added to the diluted supernatant, 10% at a time over 30 min. The supernatant was saturated to a 65% final concentration (w/v 399 g/L). The saturated mixture was then left to stir at the lowest rate for 30 min at 4ºC. A 1 mL sample was then kept to run on a gel for later use. Precipitated proteins were then removed by centrifugation, for 30 min, 7000 rpm, 4ºC (J-LA 17). The pellet was then resuspended in 220 mL buffer B. This was then dialysed overnight against 5 L buffer B (changing from 3 L to 2 L each 5hrs) to remove excess salt.

2.2.6.4 Purification of DnaJ

Two 5 mL HiTrap S-Sepharose columns were equilibrated with 5 column volumes degassed buffer B (75 mL). Half the sample was loaded onto the column, then washed with 5 column volumes of buffer B. A gradient elution of 0 to 666 mM KCl (B1: Buffer B + KCl) eluted DnaJ into 5 mL fractions. The column was washed again with 5 column volumes of Buffer B. The second half of the sample was then loaded onto the column and eluted the same way. S-

Sepharose columns were stored in 20% EtOH after use. Fractions were then run on an 12% SDS-PAGE gel to determine which fraction contains the highest amount of DnaJ and was compared to the sample taken after ammonium sulfate precipitation to show any protein losses along the way. Fractions which contain highest concentrations of DnaJ were then concentrated down to 15 mL with 30 kDa exclusion volume concentrator. The 15 mL sample was then dialysed against 2 L buffer C at 4ºC overnight to remove excess salt.

A 50 mL HAP column was equilibrated in 5 column volumes (250 mL) buffer C (A1). The 15 mL sample was then loaded onto the column, and the column was washed with 40 mL buffer C + 1 M KCl (A2). The column was then washed with 80 mL buffer C (A1), and a gradient elution of 0 - 50% buffer D (B1). An isocratic elution of 80 mL 50% buffer D (B1) eluted DnaJ into 3 mL fractions. The column was then equilibrated in 5 CV buffer C, and then stored in 0.1 M NaOH. A 12% SDS-PAGE gel was then used to determine the fractions with the highest DnaJ concentrations, and then were pooled. Pooled fractions were then dialysed against 2 L Buffer E overnight at 4ºC. Dialysed DnaJ was then concentrated down to 10 mL with 30 kDa concentrators, and concentration was determined by spectrometry and the beer lambert equation. Once a desired concentration was achieved, DnaJ was aliquoted, snap frozen in liquid nitrogen, and then stored at -80ºC.

Polyoxyethylene 10 tridecyl ether was used for Brij 58.

Lysis buffer: 50 mM Tris-HCl pH 8.0, 5 mM DTT, 0.6% (w/v) Brij 58

Sodium phosphate buffer: 1 M NaH_2PO_4 – 390 mL, 1 M Na_2HPO_4 – 610 mL, adjusted to pH 7.0

Buffer A: 50 mM sodium phosphate buffer, pH 7.0, 5 mM DTT, 1 mM EDTA, 0.1% (w/v) Brij 58

Buffer B: 50 mM sodium phosphate buffer, pH 7.0, 5 mM DTT, 1 mM EDTA, 0.1% (w/v) Brij 58, 2 M Urea

Buffer C (A1): 50 mM Tris-HCl pH 7.5, 2 M Urea, 0.1% (w/v) Brij 58, 5 mM DTT, 50 mM KCl A2 supplemented with 1 M KCl

Buffer D: 50 mM Tris-HCl pH 7.5, 2 M Urea, 0.1% (w/v) Brij 58, 5 mM DTT, 50 mM KCl, 600 mM KH_2PO_4

Buffer E: 50 mM Tris HCl pH 7.7, 100 mM KCl

All buffers were filtered and degassed before addition to column.

2.2.7 Fluorescent Labelling

Alexa Fluor (AF) maleimide dyes were used to label Hsc70 3T. They are able to bind by forming a covalent bond with the maleimide group and thiol groups (Kim et al., 2008). Due to cysteines not being widely present in proteins, this is a suitable option to ensure point labelling at certain residues. In Hsc70 3T, there are 3 cysteines present. One is buried in the conformation, so no binding is possible, but two are in the domains, which were mutated by Malcolm Rutledge. E318 has been mutated to a C in the ATP domain near the ATP binding site, and T427 in the β sheets near the substrate binding site has been mutated to a C (Rutledge, 2016). This ensures that dyes are bound in both domains, and a conformational change tracked by FRET can be achieved. It has been shown not to interfere with conformational change and only slightly hinders refolding capabilities with substrate peptides.

2.2.7.1 Labelling and separation of excess dye

In order to label, there must be an oxygen free environment and limited light exposure as the dyes can be photobleached and cysteines on the protein can be reoxidised. By using an amber vial during labelling, storing dyes in black Eppendorf tubes which were sealed by parafilm, and using argon purging vials and Eppendorf tubes before usage, this helps to limit these problems. Because TCEP has reactive thiol groups (Kim et al., 2008), immobilised TCEP is used to reduce the protein, but ensures that the slurry has been removed from the reaction before addition of dyes.

To calculate the amount of protein required for the labelling (1 mM), Equation 1 was used.
A nanodrop spectrometer reading will determine the amount of sample required for the experiment, and used in the following equation:

Equation 1. Calculating amount of protein for labelling
Amount of protein for experiment (μL) = 2500 / (Protein concentration/0.07)

This is derived from 0.07 moles per 1 mg/mL. This is then divided by 2500, as this is the amount of mL needed for the experiment.

First, fluorescent labelling buffer is degassed and filtered by using a vacuum pump. To reduce cysteines in the protein, 5 × the amount of immobilised TCEP was then added to an Eppendorf tube, and the same amount of degassed fluorescence buffer is added. The TCEP was then spun at 1000 *g* for 1 minute so that the slurry is at the bottom of the Eppendorf tube. The labelling buffer was then removed, and the protein was added into the Eppendorf tube and vortexed before adding onto a rocker for 1hr at room temp. The Eppendorf tube was then spun for 1 min at 1000 *g*, and the supernatant was kept.

Once reduced, the protein was added to an argon purged 4 mL amber vial. Next, 1 mL fluorescent labelling buffer was added, as well as 2 μL AF488. A magnetic flea was then added to the vial, and then left to stir in the dark on a magnetic plate at room temperature for 30 min. A sterilised Hamilton syringe was then used to add 50 μL AF594 and inserted into the vial through the lid. This was then returned to the dark magnetic plate at room temperature for 2 hours.

Because the acceptor dye is added to 10-fold in excess, it must be removed to ensure that only attached dye is being observed and so no misrepresentations of FRET are observed. This was done by using a desalting column, HiPrep Desalting 26/60, with an isocratic elution. Fractions were then pooled and dialysed against a charcoal slug to ensure no nucleotide was in the ATP binding site (see methods in section 2.2.5.3).

To ensure that dyes have been appropriately labelled at the right ratio, an efficiency of labelling experiment was carried out. The Cary spectrometer was used to determine the concentration of dye present. This method estimates all dye which is present in the reaction, not just bound dye, which is why it is important to remove excess before estimating the labelling efficiency of dyes. Ideally there would be more donor bound compared to acceptor dye. If there is a donor dye bound to the protein, the energy will be able to be passed onto the next dye. If there is more acceptor present, then the energy from the acceptor will not be present/analysed, due to a donor dye not being present to allow the transfer of energy to the acceptor dye.

Equation 2. Dye concentration per protein

$[594] = A_{590}/\varepsilon$

$[488] = A_{595} - 0.04 / \varepsilon$

AF594 $\varepsilon = 73{,}000$

AF488 $\varepsilon = 71{,}000$

This calculation was provided by Samuel Walsh, a past member of the Wilbanks Lab. The protein concentration is additionally able to be determined from the dataset using the Beer Lambert law and the A_{280}.

Fluorescent labelling buffer: (for storage and blanking): 250 mM Tris-HCl pH 8.5, 500 mM KCl, 25 mM EDTA pH 8.0, 5 mM $MgCl_2$, 500 mM glycerol.

I1 buffer (for desalting): 137 mM NaCl, 27 mM KCl, 43 mM KH_2PO, 14 mM Na_2HPO_4, 1 mM $MgCl_2$, 10% glycerol.

AF488 and AF594: Made up to 1 mM dry DMSO

All buffers made up with MQ.

2.2.7.2 Observing energy transfer with fluorescence spectrometer

To observe the energy transfer between the ATP and ADP state of Hsc70, 10 µL ATP, ADP and MQ was added to 900 µL of either fluorescent buffer (as a control) or double labelled Hsc70 in a quartz UV cuvette. The samples were mixed to combine and left for 5 minutes before measuring on a fluorescence spectrometer. Emission spectra was collected between 500 nm and 700 nm, with an excitation of 480 nm. Successful energy transfer is determined when the sample ATP trace is lower than the ADP/MQ trace around 525 nm, and then crosses over the ADP/MQ trace around 590 nm. This shows that the energy from the first dye (AF488) is being transferred to the acceptor dye (AF594).

2.2.8 Luciferase Refolding

The luciferase refolding method is based off of Moir (2015). Luciferase refolding is usually used in determination of promoter activity (Lyu et al., 2020), but instead was used to determine the activity of protein and has been used to determine the refolding capability of chaperones (Zippay, Place & Hoffman, 2004). The luciferase enzyme was denatured chemically with high concentrations of guanidine, by denaturing the α helix, and any β sheets which are either side of the denatured α helix. H bonds and isolated β sheets are not destabilised (Camilloni et al., 2008). BSA was added to the denaturation buffer shortly before starting the reaction to prevent adsorption of luciferase to the walls of the 96 well plate (Promega Luciferase Assay System Technical Bulletin).

The luciferase protein was denatured by adding 1 µL of the provided luciferase from the Promega Luciferase Assay System to the denaturation buffer and incubated at 25ºC for 10 min on a heat block. Phosphokinase was added to the refolding buffer, and then creatine phosphate was added. An aliquot was taken to make a negative control. DnaJ was then added, and an aliquot was taken to be the DnaJ only control. Labelled and unlabelled Hsc70 3T was added to their corresponding Eppendorf tube in varying concentrations, and then 49 µL refolding mix containing DnaJ and CP/CPK to each Eppendorf tube. These reactions are then added to a NUNC 96 opaque white 96 well plate. The denatured luciferase from the beginning of the protocol was added to each well (1 µL), and fluorescence was measured immediately after addition using a CLARIOstar plate reader. Refolding measurements were recorded every 2 min

with a 500 ms normalisation time and a gain of 3500. Mixing was carried out every 5s. All measurements were done in triplicate and the raw data were averaged to produce a luminescence readout which can be graphed. Each reaction was carried out in triplicate, alongside a DnaJ only control and a non-chaperone control.

Denaturation Buffer: 6 M Guanidine HCl (GdmCl), 25 mM HEPES (pH 7.2), 50 mM KOAc, 5 mM DTT, 1 mg/mL BSA (added immediately before use).

Refolding Mix: 75 mM KOAc, 50 mM glycine, 30 mM $MgSO_4$, 10 mM ATP, 4 mM DTT, 12 mM creatine phosphate (CP), 50 U.mL creatine phosphokinase (CPK), 2% SteadyGlo reagent. Hsc70 3T (labelled and unlabelled) added at concentrations of 1.2, 2.4 and 4.8 µM. DnaJ was added at a concentration of 48 nM.

Glycine buffer: 50 mM glycine, 30 mM $MgSO_4$, 10 mM ATP, 4 mM DTT, MQ. Buffer adjusted to be pH 7.8.

SteadyGlo reagent: Diluted in glycine buffer with 10% SteadyGlo.

2.2.9 Cell Culture

HEK293 and HepG2 cells were cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM), which were kindly provided from the McCormick liquid nitrogen dewar. To make full media, 10% fetal bovine serum (FBS), 1% Penicillin- Streptomycin (10,000 U/mL), 1% L-glu (200 mM) and fungizone/amphotericin B (25 µg/mL) was added to the DMEM. Opti-MEM Reduced Serum Medium (with HEPES, 2.4 g/L Sodium Bicarbonate and L-Glu) was used as a serum free medium during transfection and transduction protocols.

2.2.9.1 Thawing from cell stocks

Liquid nitrogen cell stocks were thawed in a 37ºC water bath and placed under a laminar flow hood. From the stocks, 1 mL of the cells were added to a 15 mL Falcon tube containing 5 mL full media. The Falcon tube was then centrifuged at 660 g for 5 min. The supernatant was discarded, and 5 mL full media was added prior to cells transferred to a T25 flask. Cells were

grown overnight at 37ºC with 5% CO_2, and media was replaced the following day. Once confluent, cells were passaged to a T75 flask.

2.2.9.2 Making frozen cell stocks

Cells should be trypsinised and then centrifuged at 1000 g for 5 min. Supernatant was then removed, and pelleted cells were resuspended in 2 mL full media. 20 µL of cell suspension is then added to a cell slide and added to a cell counter. Ideally, 2 million cells should be in each cryotube. Freezing media was then made up to a final concentration of 75% FBS and 10% DMSO. DMSO was added carefully, dropwise along the side of the Falcon tube. The tube was then carefully inverted to ensure cells and freezing media have mixed, due to DMSO being toxic to cells. The freezing media cell mixture was aliquoted to each cryotube (1.25 mL). Cryotubes were then added to a Nalgene Mr. Frosty freezing container and placed at -80ºC overnight. This is to ensure cells are frozen at a rate of 1ºC per min. Cryotubes were then be transferred to a liquid nitrogen dewar for future use.

Full media: High glucose DMEM, 10% FBS, 1% Pen-Strep, 1% L-glu, 1% fungizone/amphotericin B.

Serum free media: DMEM, 2 mM L-glu, 100 U Pen-strep, 0.25 µg/mL amphotericin B.

Freezing media: 67.5% FBS, 10% DMSO, 2.5% full media.

10 × PBS: 80g NaCl, 11.5g Na_2HPO_4, 2g KH_2PO_4, 2g KCl, 800 mL MQ.

1 × PBS: 100 mL PBS, 900 mL MQ.

PBS was filtered through a 0.2 µM filter and 1 × PBS was filtered and autoclaved.

2.2.9.3 Passaging

Both HepG2 and HEK293 cells follow the same passaging method but grow at different speeds and require different trypsinisation reagents. HepG2 cells can be passaged 1:2 or 1:3, while HEK293 cells can be passaged up to 1:10. However cells can be over passaged by passaging when cells are not confluent enough. Trypsin-EDTA is used for HepG2 cells as they are more adherent than HEK293 cells.

Once cells were around 80% confluent, media was removed from the T-75 flask and washed with PBS which is then removed. Trypsinisation reagents Tryple X (HEK293) and Trypsin-EDTA (HepG2) were added at 3 mL to the flask and returned to incubator for 5 min. Full media was added to stop the reaction, and then all contents are moved to a 15 mL Falcon tube. Cells were then pelleted by centrifuging for 5 min at 800 x *g*. Supernatant was then discarded, and pellet was resuspended in 10 mL full media. Depending on the passage ratio, an amount of cell suspension was moved to a new T75 flask and made up to volume with full media. Flask was then returned to the incubator for future use.

2.2.9.4 Plating cells

Before seeding cells to a 24 well plate for fixed imaging, coverslips must be treated with Poly - L - Lysine (PLL). Animal cell membranes (and thus, mammalian) have a net negative charge (Blau, 2013), while coverslips are negatively charged. By treating coverslips with positively charged PLL, coverslips were now positively charged, allowing for promoted cell adhesion due to the electrostatic interactions now present (Hoshiba, Yoshikawa & Sakakibara, 2018).

Autoclaved coverslips were added to each well in a 24 well plate. A PLL over 30,000 kDa stock was prepared in PBS to 15 mg/mL. The PLL mixture was then diluted to 15 µg/µL, and then added to each well and incubated for 1hr at 37ºC 5% CO_2. PLL was then removed from each well and then washed with PBS 3 times to ensure all PLL is no longer present. Coverslips then should be allowed to dry for 30 min or kept in PBS at 4ºC prior to use. Coverslips should be at least room temperature before seeding cells.

Once cells were 80 - 90% confluent, cells were trypsinised for 5 min. Cells were then transferred to a 15 mL Falcon tube and full media was made up to 10 mL and centrifuged for 5 min at

1000 g. Supernatant was then discarded and pelleted cells were resuspended in 2 mL full media. Cells were counted by adding 20 µL of cell suspension was then added to a counting slide (BIO-RAD) and measured on a TC20 BIO-RAD automated cell counter. To calculate the amount of cell suspension required, use Equation 3.

Equation 3. Cell Plating
Cell suspension required (mL) = Number of cells required/ (cells/mL)

The cell suspension was then multiplied by how many wells were used, and then added to a 15 mL Falcon tube. From the cell suspension, 1 mL added to per well, as well as 1 mL full media. For an example, if using 10 wells, 10 mL is required. Therefore only 200 µL of cell suspension was added to the T75 flask, and then topped up with 9.8 mL of full media. This mix was then distributed equally into the 10 wells, where 1 mL of the mix was added to each well. Seeded cells were then left for at least 24 hours prior to transfection or transduction methods.

2.2.9.5 Transfection of ABCA1 into cells with lipofectamine

Once seeded cells were 70-90% confluent, peptide ABCA1 was transfected into cells with Lipofectamine 3000 (Invitrogen). This was to observe how Hsc70 3T interacts with the peptide, and due to the translocation of the peptides being well studied (Sorrenson et al., 2013), and to observe the translocation of Hsc70 3T. Using the manufacturer's instructions, 1 µg/µL ABCA1-GFP plasmid was diluted with serum free OptiMEM media and mixed with P3000 reagent. Lipofectamine 3000 was then diluted in serum free OptiMEM media and then mixed with the diluted DNA. Diluted DNA was then incubated for 15 min at room temp under a laminar flow hood, and 50 µL was added to each well, dropwise. The 24 well plate was then returned to the incubator for 2 days, and translocation can be observed under a microscope or further transduced.

2.2.9.6 Transduction of Hsc70 into cells

PULSin (PolyPlus) allows for non-toxic delivery of proteins and peptides into the cytoplasm of cells. The mechanism of action is achieved by the PULSin reagent and the protein interacting to form positively charged complexes. These complexes are then able to form electrostatic

interactions with HSPGs which are on the cell surface. This binding induces endocytosis by aggregating HSPGs. Once inside the cytoplasm, the endosome is unpackaged, and the protein is released.

From the T75 flask, 70,000 - 100,000 cells were on on a 24 well plate and incubated at 37ºC 5% CO_2 for 16 – 24hrs. Once 70 -80% confluent, cells were washed with PBS and then supplemented with 900 µL OptiMEM media. As per the manufacturer's instructions, 1 µg double labelled Hsc70 3T was diluted in 20 mM HEPES. PULSin was added at a volume of 4 µL, and then incubated for 15 minutes. The protein-PULSin mixture was added to each well, dropwise (100 µL), and then swirled gently. The plate was then returned to the incubator for 4 hours at 37ºC 5% CO_2. After 4 hours the Opti-MEM media was replaced for full media and returned to the incubator. Cells were then fixed immediately and imaged on an inverted microscope.

Rachel Moir, a past member of the Wilbanks lab, previously had found that labelled Hsc70 clumped on the surface of cells when imaging through live cell microscopy. To decrease chances of clumping, protein was filtered prior to transduction, to remove any protein which may have aggregated together. A BIO-RAD Micro Bio-Spin Column with Bio Gel was inverted to resuspend the buffer and the top was snapped off. The column was then placed into a 2 mL microcentrifuge tube and the top cap was removed to drain the packing buffer stored in the spin column. Once drained, the column and microcentrifuge tube were centrifuged at 1000 x g for 2 minutes. The buffer was then discarded, and the equilibrated column was placed into a 1.5 mL microcentrifuge tube. The protein sample was added to the centre of the column (20 µL) and centrifuged at 1000 x g for 4 minutes. The filtered protein was able to be used in transduction experiments for further investigation.

Live cell microscopy experiments used a Cytation 5 Cell Imaging Multi Mode Reader, and a Griener 96 well plate was used to seed cells for the plate to be compatible with the reader. Cells were seeded (10,000 cells) onto a Griener 96 well plate and incubated at 37ºC 5% CO_2 for 16 hrs. Once 70 – 80% confluent, cells could be transduced with Hsc70 3T. Because PULSin can be harmful at high concentrations to cells at prolonged periods, two PULSin-protein concentrations

were used. This included the standard concentration (0.3 µg protein, 1.2 µL PULSin) and half the concentration (0.15 µg protein, 0.6 µL PULSin). Double labelled Hsc70 3T was diluted (0.3 µg) in 20 mM HEPES. PULSin was added (1.2 µL) and incubated at room temperature for 15 minutes. Opti MEM serum free media was added up to 200 µL to the microcentrifuge tube, and 200 µL of the protein – PULSin mixture was distributed to the well. The plate was then returned to the incubator for one hour prior to visualisation on the Cytation 5. 15 minutes before visualisation, Opti- MEM media was replaced with full media in selected wells. This was to identify if full media improved the longevity of the cells during prolonged time periods during cell imaging. Live microscopy imaging begun 2hrs after transduction.

2.2.9.7 Cellular stress

HEK293 cells were kept at a constant 37ºC 5% CO_2 environment throughout growth, and only were taken out during transfection, transduction, and fixing. Bellmann et al., 2010 used heat shock experiments with HEK293 cells to investigate cell stress by incubating cell plates in a water bath at 44ºC for 120 minutes. Live cell microscopy imaged with the Cytation 5 was used to track cellular stress. After one hour of imaging at 37ºC 5% CO_2, temperature was brought up to 44ºC and cells were tracked for an additional hour. Comparisons could then be drawn with images captured every 30 minutes.

2.2.9.8 Inverted fluorescence microscopy - fixed cells

Because cells are leaving the 37ºC 5% CO_2 environment, cells must be fixed prior to observation. Full media was removed from cells and washed gently with PBS. PFA was used to fix cells, and so 4% PFA is aliquoted to each well and let sit for 20 minutes at room temp. PFA was then discarded and another PBS wash was done to remove any excess PFA. A drop of ProLong Gold Antifade (+DAPI staining) was added to microscope slides, and coverslips were removed from the 24 well plate placed onto microscope slides cell surface down and left to cure for 24hrs. Once hardened, slides were able to be observed under an inverted fluorescence microscope.

2.2.9.9 Imaging

Fixed microscopy was analysed through a Nikon Ti2-A Inverted Fluorescence Microscope.

Filters Cy3 and Cy5 are used to identify AF488 and AF594 dyes, with the argon lamp. First, cells were identified with brightfield to ensure that they were the right morphology, were alive and properly adhered to the slide. The corresponding filter to the donor fluorophore used to identify the first dye was imaged, and then imaged with the second filter. Images were then superimposed to get the effect of the shared fluorescence.

2.2.9.10 Live microscopy

Live microscopy was analysed on a Cytation 5 Cell Imaging Multi Mode Reader with filters GFP and RFP which identify AF488 and AF594. Prior to visualisation on the Cytation 5, FITC and TRITC filters were used to identify AF594 and AF488 fluorescence, as well as cell health and abundance. Six images were taken in each well every 30 minutes at 37ºC 5% CO_2 for 2 – 3 hrs. To track cell stress, cells were imaged every 30 minutes for one hour, and then the temperature was raised to 44ºC for 1 hr and imaged.

Chapter 3: Results and Discussion

Past students have found that expression of the pMSK003 plasmid is relatively low and results in lower concentrations of purified Hsc70 3T. However, this purification of Hsc70 3T resulted in higher yields at each step of purification, as well as more cell colonies on agar plates after heat shock transformation. To ensure that what has been purified is in fact Hsc70 3T, rather than the bacterial homologue DnaK, which yields much higher concentrations of purified protein, mass spectrometry confirmed that Hsc70 3T was in fact purified.

3.1 Growth and expression of Hsc70 3T in BB1553(DE3)

Colonies found after 16 hours of growth at 27ºC were large (3 mm) and translucent. Cells are grown at the low temperature to ensure cells survival, due to BB1553 (DE3) being a DnaK knock out strain, and therefore struggle to grow at higher temperatures without the important protein. One colony was isolated and used to inoculate 50 mL LB media as an overnight culture. This 50 mL overnight culture, once no longer clear, was used to inoculate the growth media in the oxygen fermenter.

During culturing of BB1553 DE3 cells samples were taken to track microbial growth by monitoring optical density. After 40 min of growth in fermenter, an OD_{600} reading of 0.16 was the initial reading. At 80 minutes, an increase of 0.26 was shown. At 120 minutes, an OD_{600} reading of 0.42 determined that the plasmid could be induced with 100 mM IPTG (Figure 3).

Cells were harvested 17 hours later with an OD_{600} reading of 0.85, showing cell doubling. A distinct band in the post induction sample above 66 kDa suggested expression of Hsc70 3T (Figure 4). Weak bands above 66 kDa in the pre-induced samples indicates small expression of Hsc70 3T from the pMSK003 plasmid. Due to the high expression found in Figure 4, the morphology of colonies isolated from heat shock transformation may be noted for future experiments with the plasmid.

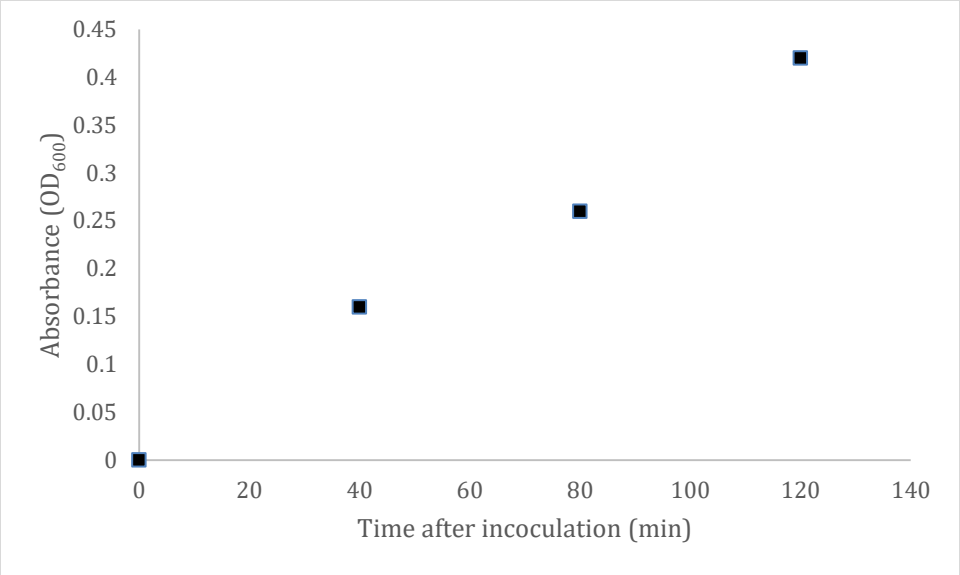

Figure 3. Growth of BB1553 DE3 after inoculation of fermenter. 1 mL samples taken at each time point, until an OD_{600} of 0.4 was reached, after which IPTG was used to induce pMSK003 plasmid.

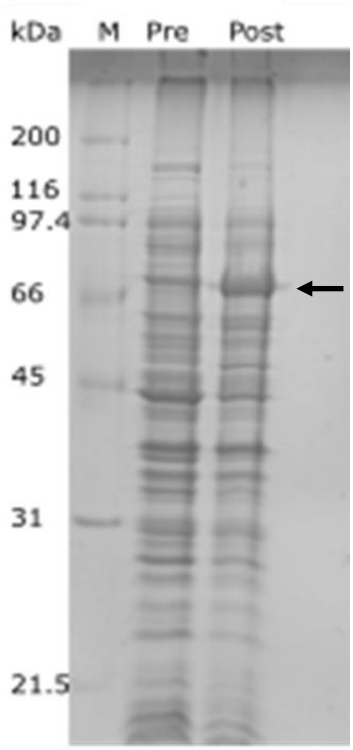

Figure 4. Induction in BB1553 DE3 *E. coli* expressing Hsc70 3T. Samples representing 100 µL of culture were separated on 12% SDS-PAGE. Pre indicates the pre induction sample, and the post indicates a sample taken 17 hours after addition of IPTG. Arrow indicates band of interest. SDS-PAGE standards, broad range (BIO-RAD) located on the left of the gel.

3.2 Purification of Hsc70 3T

3.2.1 Anion Exchange Purification

Separation over a DEAE column yielded several peaks. The first large peak indicates the flow through, which contained any components which did not bind to the column. The second peak can be viewed closer in panel B of Figure 5. Three distinct peaks are observed on the UV trace, indicating A_{280} for protein. From 0 - 300 mL, flow through from the equilibration step is present, where purified Hsc70 is not expected to be eluted. Peaks present within 400 - 450 mL include the fractions which were collected and analysed, however include weakly bound proteins and Hsc70 is not expected to be present. Figure 6 shows an SDS PAGE gel of the fractions eluted in peaks present between 250 - 700 mL. Fractions 8 and 9 had the most distinctive bands around 71 kDa, and so the peak at 550 mL contained the most Hsc70 containing fractions. These fractions were

pooled and dialysed with an EDTA buffer, and with activated charcoal to remove ATP or Mg^{2+}, which may be bound to the Hsc70 active site.

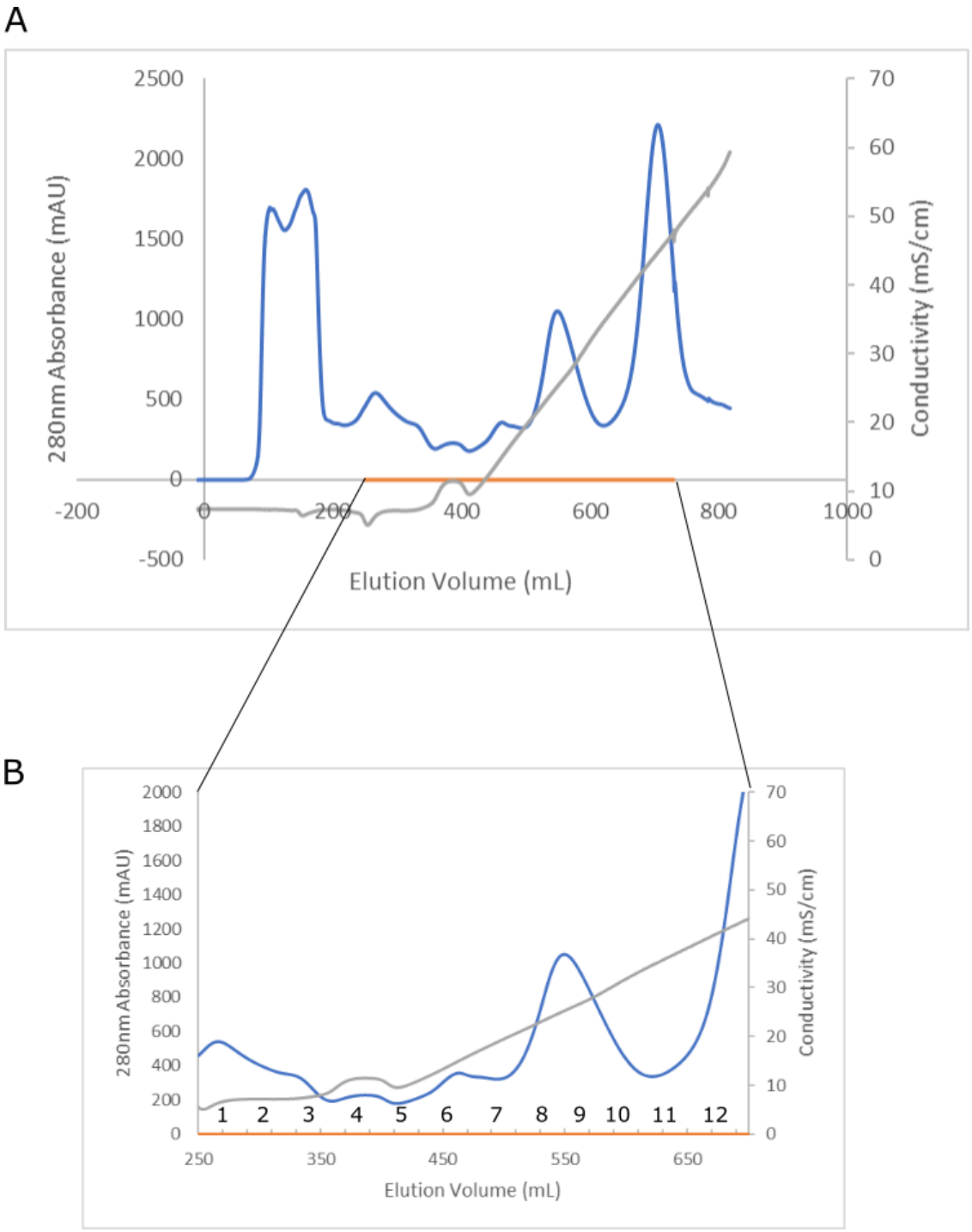

Figure 5. Anion Exchange Chromatogram (DEAE), of Hsc70 3T. A. Full trace indicates the UV trace (blue) and the conductivity trace (grey), and fractions for further analysis were highlighted in yellow. B. Detail of chromatogram corresponding to fractions analysed in Figure 6, with numbers for each fraction written along the x axis.

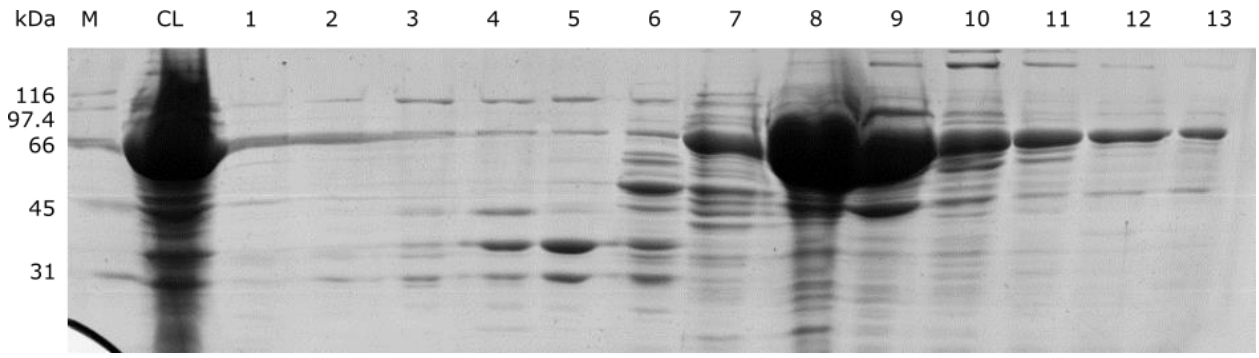

Figure 6. SDS-PAGE of selected DEAE fractions of Hsc70 3T. 5 µL 12% SDS- PAGE Molecular Weight Marker loaded to lane 1. CL: clarified lysate, sample prior to DEAE purification. Fractions shown in Figure 5 (4 µL samples with 1 µL sample buffer) were loaded to gel. M: SDS-PAGE standards, broad range (BIO-RAD)

3.2.2 ATP- Agarose Chromatography

A large peak in the UV trace is shown in ATP-Agarose chromatography (Figure 7). This peak between 97 - 120 mL corresponds to ATP elution and the UV absorbance of ATP, which partially obscures the trace for eluting protein, showed earlier in the peak. All fractions across this peak were collected and analysed on an SDS-PAGE gel (Figure 8). Fractions located within 145 - 150 mL, (B4, B5 and B6) had the most distinct bands just above 66 kDa, which were then pooled and concentrated for further purification. A small amount of Hsc70 3T was found in the flow through sample (not shown on gels), however was not loaded onto the next step of purification (S200), as this protein may not be able to bind ATP, which is important for the activity assays.

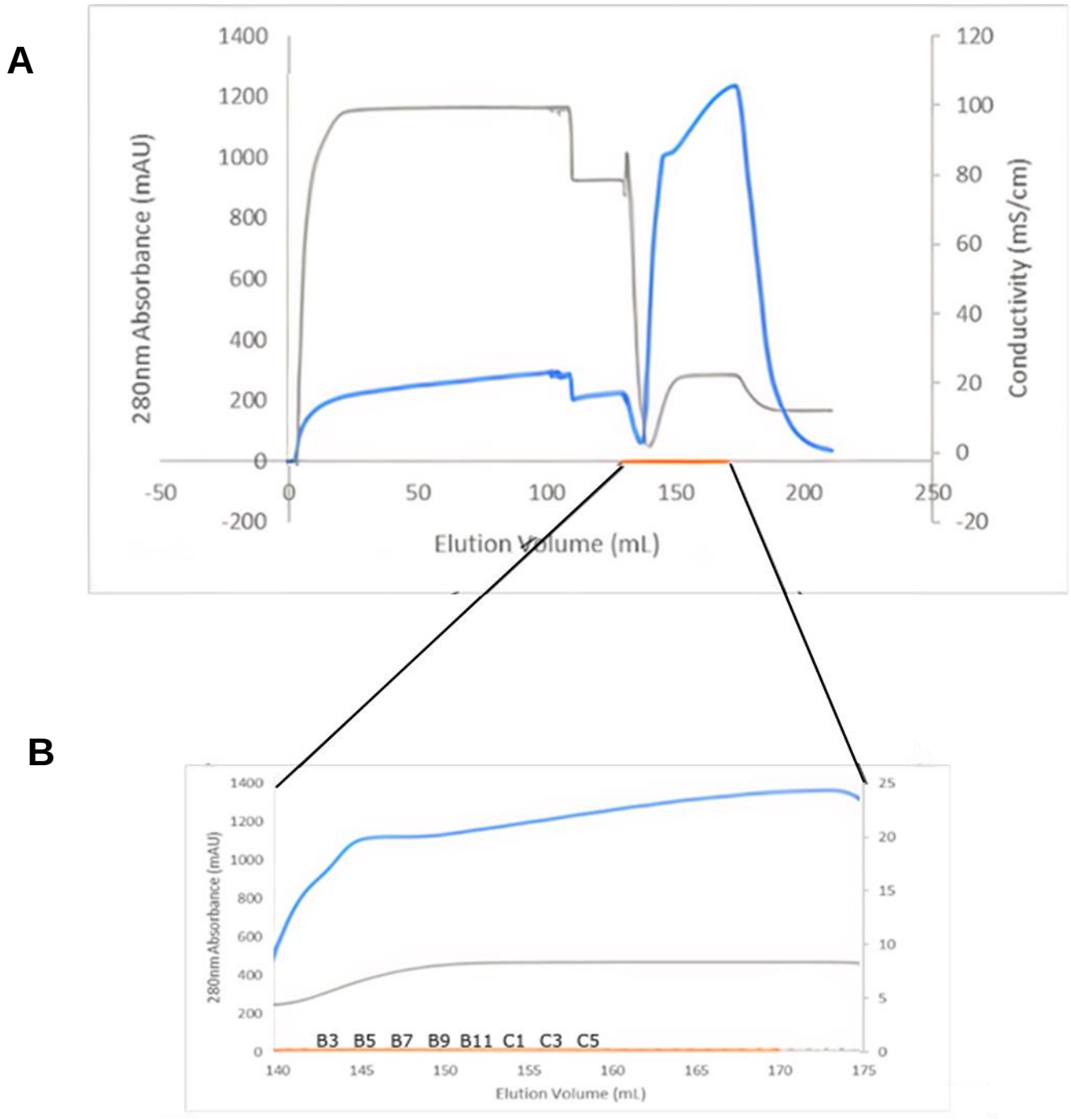

Figure 7. ATP-agarose Chromatogram of Hsc70 3T. A. Full trace indicates the UV trace (blue) and the conductivity trace (grey), and fractions for further analysis were highlighted in yellow. B. Detail of chromatogram corresponding to fractions analysed in Figure 8, with numbers for each fraction written along the x axis.

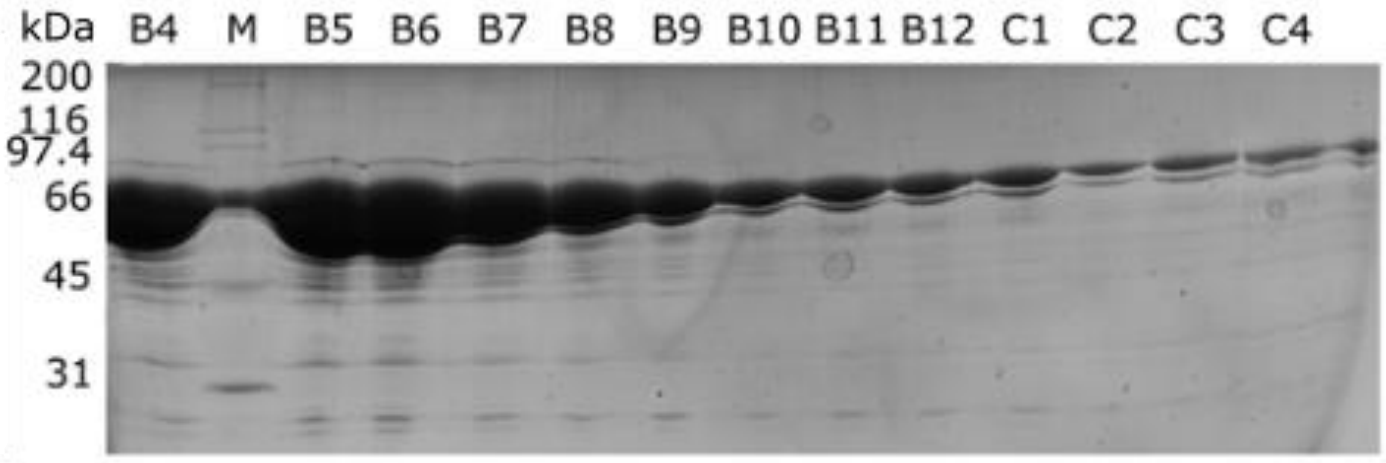

Figure 8. 12% SDS-PAGE of ATP-agarose purification of Hsc70 3T. Fractions shown in Figure 7 (4 µL samples) were loaded to gel. M: M: SDS-PAGE standards, broad range (BIO-RAD)

3.2.3 Gel Filtration

The pooled fractions for ATP agarose were concentrated and then further separated on a Gel filtration Superdex 200 column (Figure 9). The fraction peak is located between 115 – 245 mL with three small peaks, indicating a trimer, monomer, and truncated protein peak, based on the assumption that a trimer protein would be larger than shorter, truncated protein. It could be assumed that the peak located at around 190 mL indicates monomer protein, and so fractions between 160 – 215 mL were collected. Due to the low A_{280}, samples from fractions were precipitated with 100% TCA prior to analysis on SDS- PAGE gel. The fractions which contained the highest Hsc70 3T protein concentration were fractions F4 – F12, collected from 190 – 210 mL (Figure 10). These fractions correspond with the highest point in the assumed monomer peak.

Fractions were separated into trimer and monomer forms, by pooling fractions indicated by Figure 10. This yielded around 3 mL of 1.21 mg/mL Hsc70 3T, which was aliquoted and then kept stored at -80ºC.

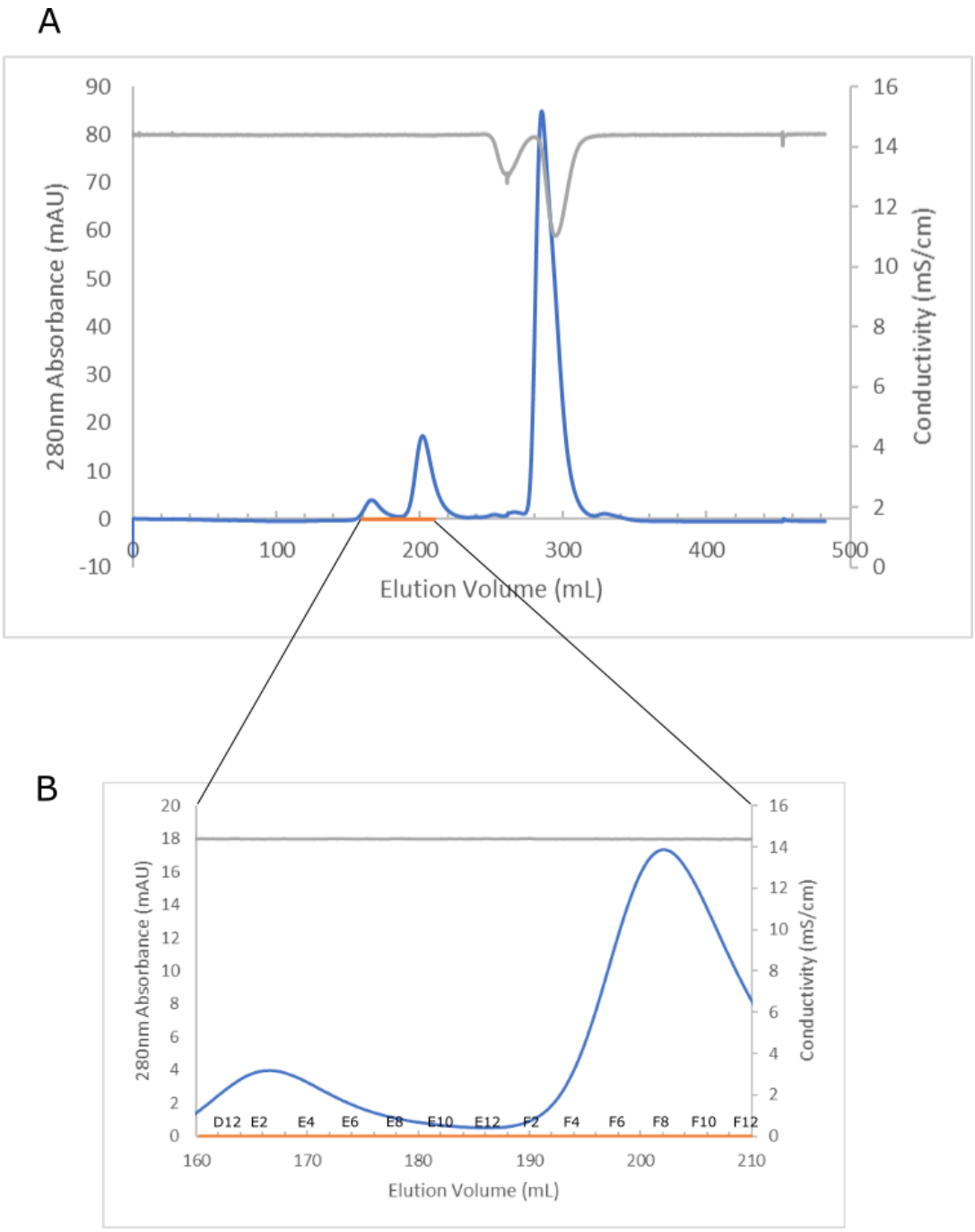

Figure 9. S200 gel filtration chromatogram of Hsc70 3T. A. Full trace indicates the UV trace (blue) and the conductivity trace (grey), and fractions for further analysis were highlighted in yellow. B. Detail of chromatogram corresponding to fractions analysed in Figure 10, with numbers for each fraction written along the x axis.

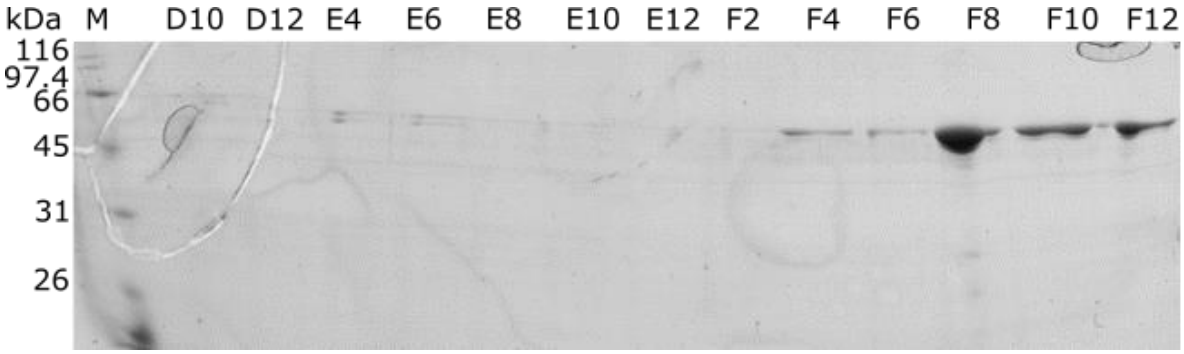

Figure 10. 12% SDS-PAGE of S200 purification of Hsc70 3T. Every second fraction shown in Figure 9 eluted between 160 and 210 mL underwent TCA precipitation (4 µL samples) were loaded to gel. M: SDS-PAGE standards, broad range (BIO-RAD)

3.3 Purification of DnaJ

DnaJ is a cochaperone of Hsc70 and was used during mammalian cell culture experiments and the luciferase assay. To purify DnaJ, the *E. coli* strain W3110 (made competent by Matthias Mayer) was used, containing transformed plasmid pUH21. In order to purify DnaJ, an ammonium sulfate precipitation was used to bring soluble proteins into solution, and two purifications with two HiTrap S-Sepharose columns, and a 40 mL HAP column.

3.3.1 Induction of DnaJ by IPTG

Samples during growth of *E. coli* W3110 cells with pUH21 plasmid were taken to track growth. After 1hr of inoculation, an OD_{600} reading of 0.09 was the initial reading. At 3 hours post inoculation, the reading was shown to be 0.15. At 4 hours post inoculation, an OD_{600} reading of 0.48 determined that the plasmid could be induced with 100 mM IPTG. At 16 hours post induction, an OD_{600} reading of 1.71 determined that cell harvesting was suitable. Figure 11 shows a DnaJ band in the post induction sample around 40 kDa.

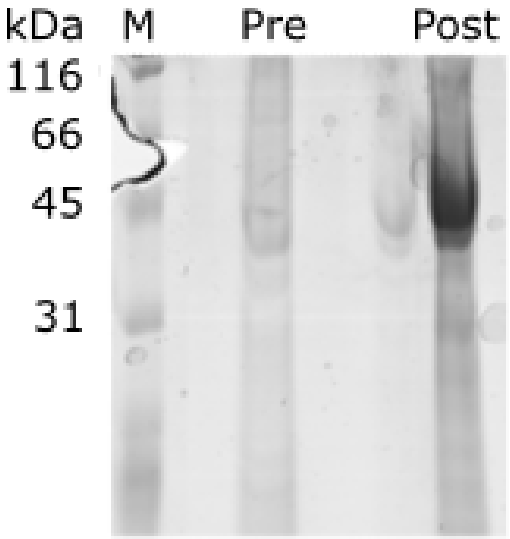

Figure 11. Induction in *E. coli* W3110 cells expressing DnaJ. 4 µL samples representing 100 µL of culture were separated on 12% SDS- PAGE. Pre indicates the pre induction sample taken 8 hours after inoculation, and post induction sample indicates sample taken 16 hours after addition of IPTG. M: SDS-PAGE standards, broad range (BIO-RAD) located on the left of the gel.

3.3.2 HiTrap S-Sepharose Chromatography

Cation-exchange HiTrap S-Sepharose chromatogram is observed in Figure 12. The fraction peak is located between 250 – 300 mL. Fractions between C4 - D6 were pooled and concentrated. Fractions are shown on an SDS-PAGE gel (Figure A2). This indicates that the highest protein concentration was found in fraction D3, which contained fractions collected from 190 – 210 mL. These fractions correspond with the highest peak in the chromatogram.

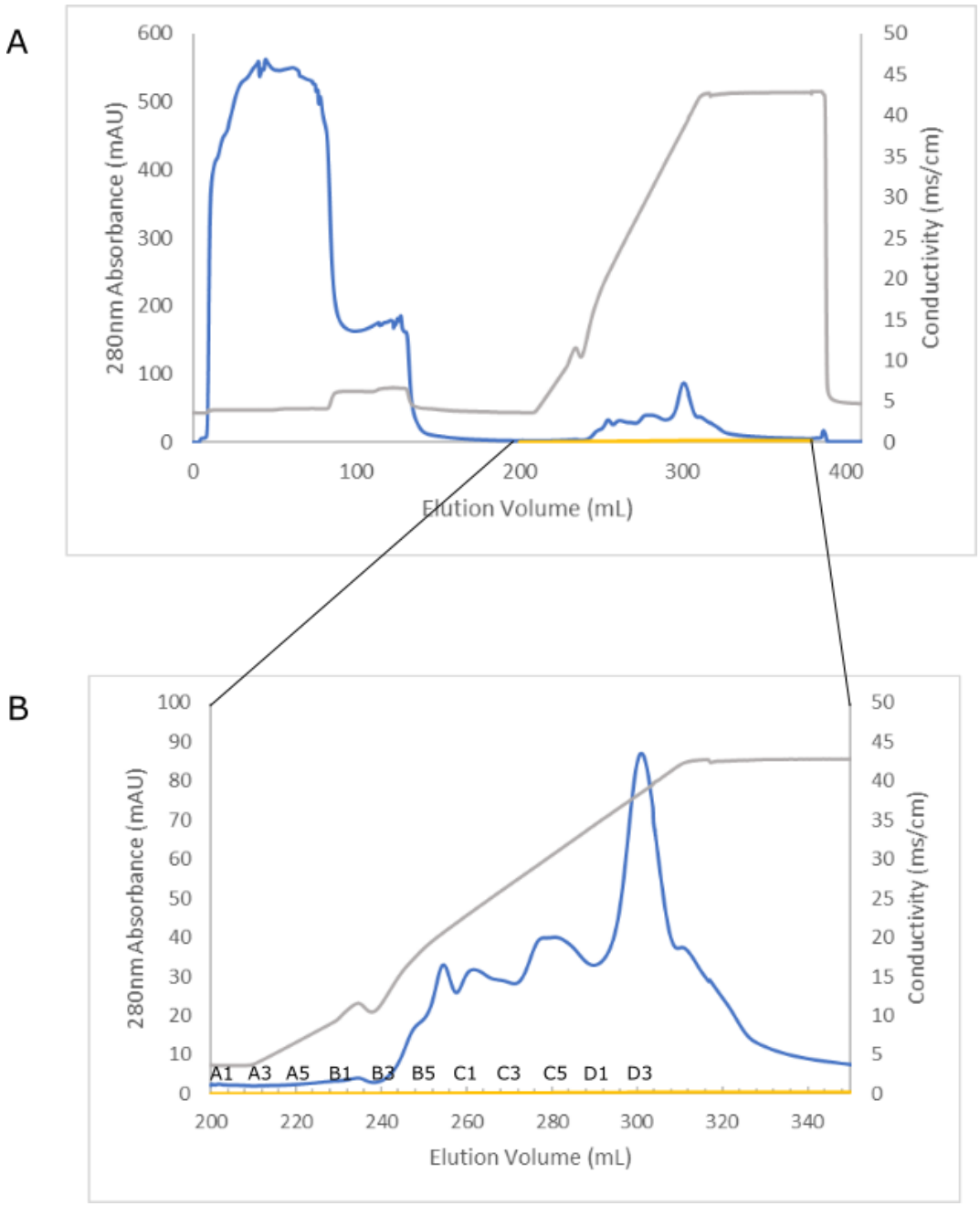

Figure 12. HiTrap S-Sepharose Chromatogram of DnaJ. A. Full trace indicates the UV trace (blue) and the conductivity trace (grey), and fractions for further analysis were highlighted in yellow. B. Detail of chromatogram corresponding to fractions analysed in Figure A2, with numbers for each fraction written along the x axis.

3.3.3 HAP Chromatography

HAP chromatogram is observed in Figure 13. The fraction peak is located between 250 – 300 mL. Fractions between A6 - B5 were pooled and concentrated. The DnaJ concentration was determined with a Nanodrop spectrometer to a final concentration of 1.5 mg/mL. Samples of 300 µL were snap frozen in liquid nitrogen and stored at -80ºC.

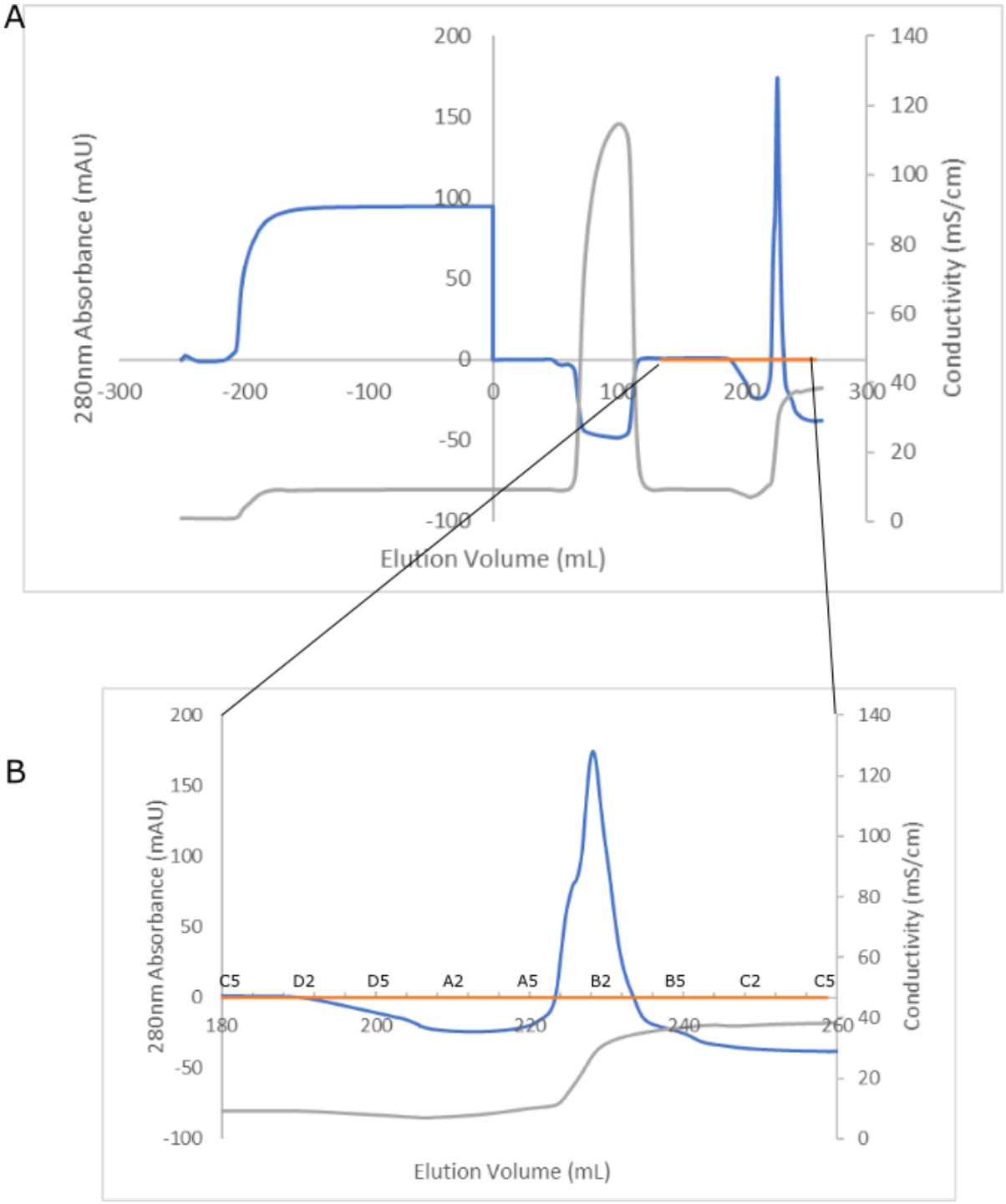

Figure 13. HAP Chromatography of DnaJ. A. Full trace indicates the UV trace (blue) and the conductivity trace (grey), and the fractions for further analysis were highlighted in yellow. B. Detail of chromatogram corresponding to fractions which are written along the x axis.

Now that Hsc70 3T had been purified, the cysteines needed to be labelled with AF488 and AF594 at the right stoichiometry to ensure each protein has both a donor and acceptor on each cysteine. FRET must be identified prior to transduction experiments. Labelled Hsc70 3T was also used in the luciferase assay along with purified DnaJ and unlabelled Hsc70 3T.

3.5 Fluorescent Labelling of Hsc70 3T

Alexa Fluor maleimide dyes 488 and 594 are used to fluorescently label Hsc70 3T. All experiments were observed under argon gas, to ensure no oxygen was present. However, not all cysteines are labelled, as some of the dyes may have oxidised prior to oxidation, or all cysteines may not have been reduced (this is explained further in 3.6.1). Therefore, labelled protein, excess dye, and unlabelled protein must be separated. The labelled sample was loaded onto a HiPrep 26/60 desalting column, and two major peaks were present, as shown in Figure 14. The first peak could be determined to contain labelled protein, due to labelled protein being larger than non-bound/excess dye, and unlabelled protein. A faint yellow colour from the fraction could be detected by eye (indicating the bound AF488). The second peak shows any excess dye which was not bound to the protein, and fractions were very vivid pink from the excess dye. There will always be a second peak, due to AF594 being added in excess of 10-fold to the sample. If these peaks are overlapping, it shows that there will still be excess dye in the labelled sample. Therefore, fractions corresponding to the labelled protein peak were passed through a second desalting on the HiPrep 26/60, resulting in better separation and two peaks (Figure 14).

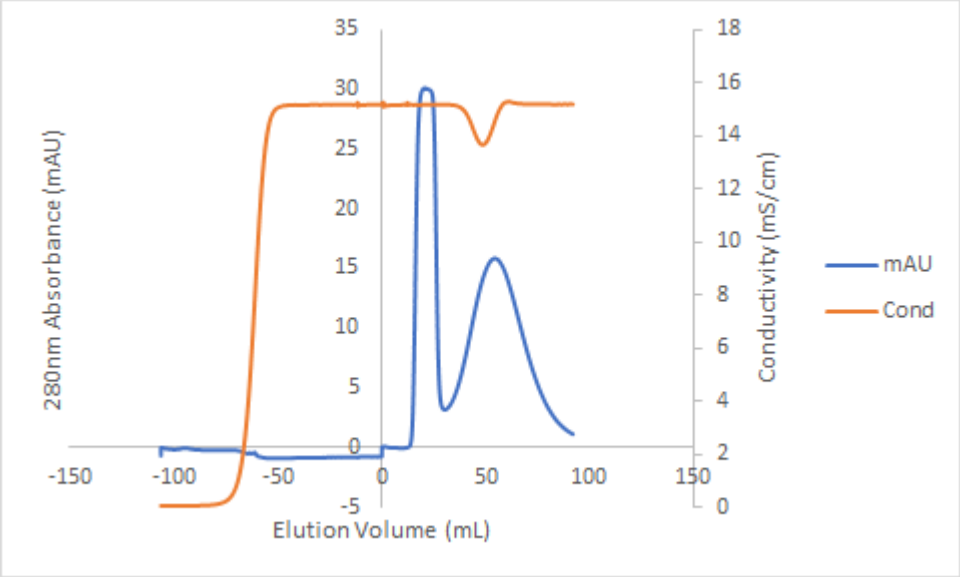

Figure 14. Separation of labelled protein from excess dye with HiPrep 26/60 desalting column. The first peak contains labelled protein, and the second peak indicates excess dyes. An isocratic elution, labelled protein should be eluted in the first peak, and then excess dye and smaller molecules in the second peak. Blue indicates the A_{280} trace which reflects protein and other small molecules, and orange indicates the conductivity. The conductivity trace shows the increase of salt in the desalting buffer with a dip, where the small molecules have been eluted.

3.5.1 Efficiency of dye labelling

Using Equation 2 from section 2.2.7, and data shown in Figure 15, the concentrations of each dye could be estimated. AF594 was added before AF488, as previous experiments done with different concentrations and different orders of addition showed that AF488 had a higher labelling efficiency. Labelling first with AF594 increased AF594 concentration, with a value of 9.4×10^{-7} M. AF488 is estimated to be 6.1×10^{-7} M, and protein concentration is estimated as 5.4×10^{-6} M. This indicates that about one fifth of protein has been labelled with at least one fluorophore. Figure 15 shows that there is a peak around 480 nm, indicating the AF488 labelling, and AF594 labelling is shown as the peak around 590 nm. The absorbance increases around 280 nm due to protein present in the sample. The ratio of the two peaks shows that desalting was effective, due to the height of the AF594 peak. Because 10 x the amount of dye was used for AF594, and the peak present is not 10 x the size of AF488, free dye was removed with the desalting step.

Although the protein concentration was estimated as 5.4 x 10^{-6} M, this is only a rough estimation, due to both the equation being used, and the estimation based on wavelengths. Equation 2 uses the Beer Lambert equation using the data generated from the absorption spectra, however, this protein concentration can be considered to be over-estimated. In Figure 15, the buffer (blue) trace does not contain any protein. However, around 280 nm, the buffer trace increases. This can be attributed both to background scatter. Additionally, extinction coefficients for dyes were based on when dyes were bound to glutathione. These numbers could differ from those of dyes bound to protein.

From the concentrations on dyes generated from the absorbance spectra, the amounts of those dyes can be broken down. For AF594, the amount of labelled protein with AF594 based on the total protein concentration is 18%. Of this, it can be estimated that 2% of protein has 2 cysteines labelled with both AF594, and 84% of the total protein concentration had no dyes attached to cysteines. For AF488, the amount of labelled protein with AF488 attached based on the protein concentration is 9%. Of this, 16.5% would have one AF594 and AF488 on each protein, and 9% of the total labelled protein would have only one AF488 dye. However, this is based entirely on the number calculated from the absorbance spectrometry data, and so it can be estimated that there would be more double labelled protein from the fluorescent labelling experiment.

To prevent competing reactions with reductants, immobilised TCEP was used to reduce cysteines in preparation for labelling. TCEP has reactive thiol groups (Kim et al., 2008), which could bind to the maleimide dyes once added to the sample, rather than the reduced cysteines. Immobilised TCEP was used instead, which is in a slurry and can be separated by centrifugation. By first reducing Hsc70 3T, and then removing the TCEP completely from the reaction, the reduced cysteines of Hsc70 were the most abundant reductive species, therefore increasing the likelihood of AF488/AF594 covalently binding to the reduced cysteines. An additional concern was that not only labelled Hsc70 is being excited, but excess dye which may be present in the sample may also be fluorescing**.** By doing repeated purifications over the HiPrep 26/60 Desalting column, the sample recovered from the second purification had two distinct peaks with little

contamination of excess dye. The contamination from excess dye into the double labelled protein peak could be estimated to be 5%.

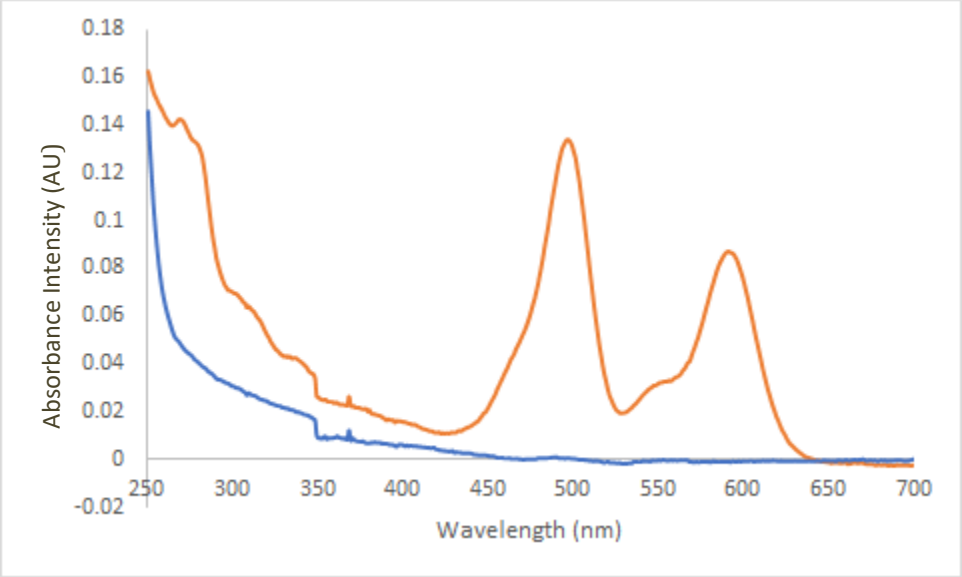

Figure 15. Absorption spectrum of Hsc70 3T labelled with AF488 and AF594. The blue trace indicates fluorescent labelling buffer as the blank, and the orange trace indicates double labelled Hsc70 3T.

3.5.2 Identification of FRET *in vitro*

FRET is a highly sensitive technique to measure distances between protein-protein interactions and is capable of identifying conformational shifts. A fluorescence spectrometer is able to identify the transfer of the energy from AF488 to AF594 when there is a cross over of both excitation and emission spectra at 590 nm. This is a suitable experiment to do prior to transduction into live cells because it is a quick experiment and does not require the growth mammalian cells before knowing if FRET can be observed.

The absorbance spectrum shows a higher wavelength peak at around 515 nm. This is the donor wavelength. When ATP is added, the intensity drops. Around 620 nm, there is a change, showing an increase in intensity for the Hsc70 3T with 10 mM ATP sample. This shows a successful energy transfer between the acceptor and donor dyes, indicating FRET transfer, and the protein has been double labelled.

This was one of the few successful labelings, as one had to not only identify successful energy transfer between both fluorophores, but additionally had to ensure that there was equal labelling of both dyes, as shown in 3.6.1. In some instances quenching was observed, where a suitable peak around 515 nm for the ATP/ADP samples were found. This was a preferred observation, where the ADP presence peak was slightly higher than the ATP presence peak. However the second peak (the ATP peak around 610 nm) which indicates FRET transfer did not occur (Figure 16). AF594 was directly excited at 590 nm in the fluorescence spectrometer to confirm the presence of the dye. AF594 did respond to the excitation, and so the dye could be determined to be responsive. Two reasonings could be for this reaction of quenching: the first, that cross talk is not occurring between both domains. An easy way to determine that the linker between the two domains has been cleaved is to load a SDS- PAGE gel, and if two bands were located rather than one at the 66 kDa band, then this would confirm the lack of cross talk between both domains. Another hypothesis which could have led to decreased fluorescence around 610 nm could be that excitation is not being released as fluorescence but is instead being released as heat. However, this is not the case, because direct excitation was found (Figure A1).

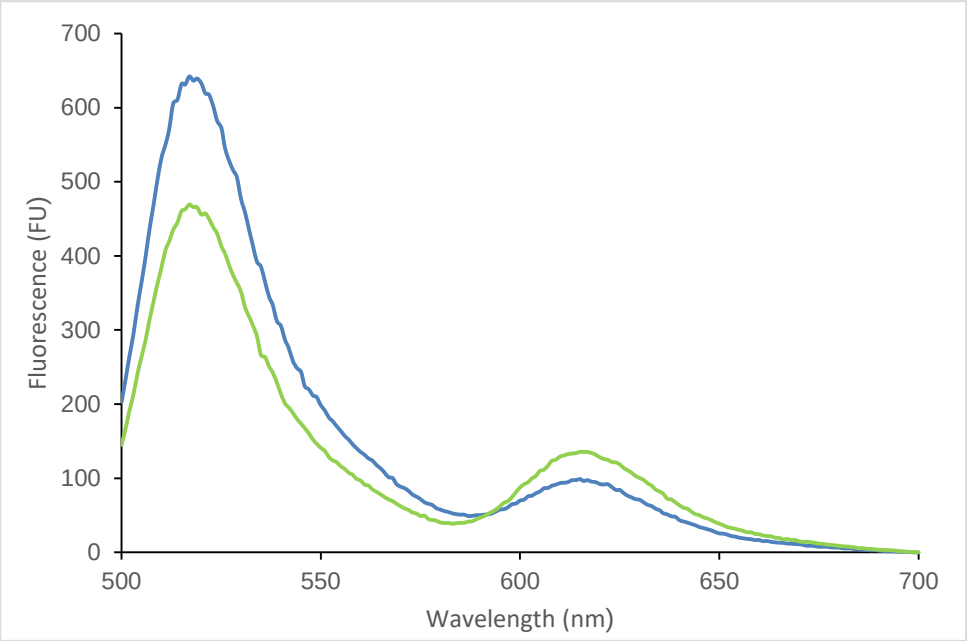

Figure 16. Fluorescence spectra of AF-labelled Hsc70 3T in presence of nucleotides. Identification of FRET *in vitro*. The blue trace is double labelled Hsc70 3T with 10 mM ADP added to the cuvette. The green trace is double labelled Hsc70 3T with 10 mM ATP. Both samples were identical concentrations of double labelled Hsc70 3T. Both samples have been excited at 480 nm and measuring with a 5 nm slit width.

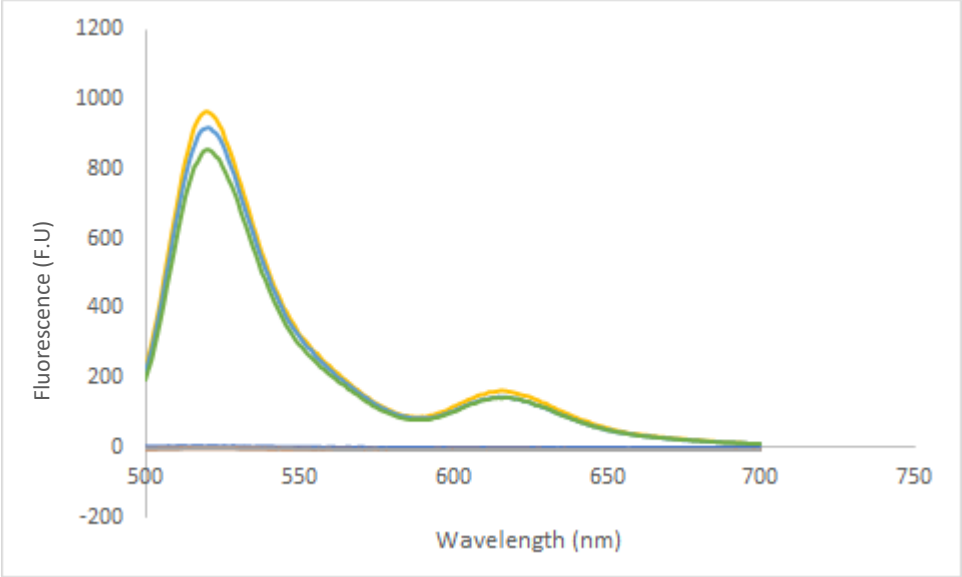

Figure 17. Fluorescence spectra of AF-labelled Hsc70 3T in presence of nucleotides. Demonstration of lack of nucleotide response and FRET *in vitro* measured on a fluorescence spectrometer. The blue trace is double labelled Hsc70 3T with 10 mM ADP added to the cuvette. The green trace is labelled Hsc70 3T with 10 mM ATP. All samples have been directly excited at 480 nm and measured with a 5 nm slit width. ATP bound emission does not rise over the ADP bound trace around 590 nm, and a peak around 620 nm, which would be expected in response to nucleotide.

3.6 Luciferase Refolding Assay

The activity of the protein was determined before cells were transduced. This was done by using a luciferase refolding assay, which uses the recovery by refolded luciferase of the ability to generate light, thus detecting interactions with refolding chaperones. Native Hsc70 accelerates the rate of refolding, however at high concentrations of Hsc70, unfolded substrates remain bound to the protein and folding is slowed after 20 minutes. Hsc70 3T was tested both with and without fluorophore (AF488 and AF594) labels at final concentrations of 1.2, 2.4 and 4.8 µM (Figure 18). A reaction containing only DnaJ and no Hsc70 3T (labelled or unlabelled), was used as an additional control. DnaJ is included in the assay as a cochaperone of Hsc70. The controls showed expected results, with the negative sample refolding slowly, and DnaJ enhancing this reaction. Compared to previous students' work, the kinetics of the double labelled Hsc70 reaction had decreased relative luminescence units (RLU). The rate of control reactions was similar to

previous students' results. Despite the decreased rate of refolding, it does show that luciferase is being bound by Hsc70 3T, and it appears that the Alexa Fluor dyes decreases refolding activity of Hsc70 3T. From these data we can conclude that there is binding activity of Hsc70 3T, but release of unfolded substrate may be impaired.

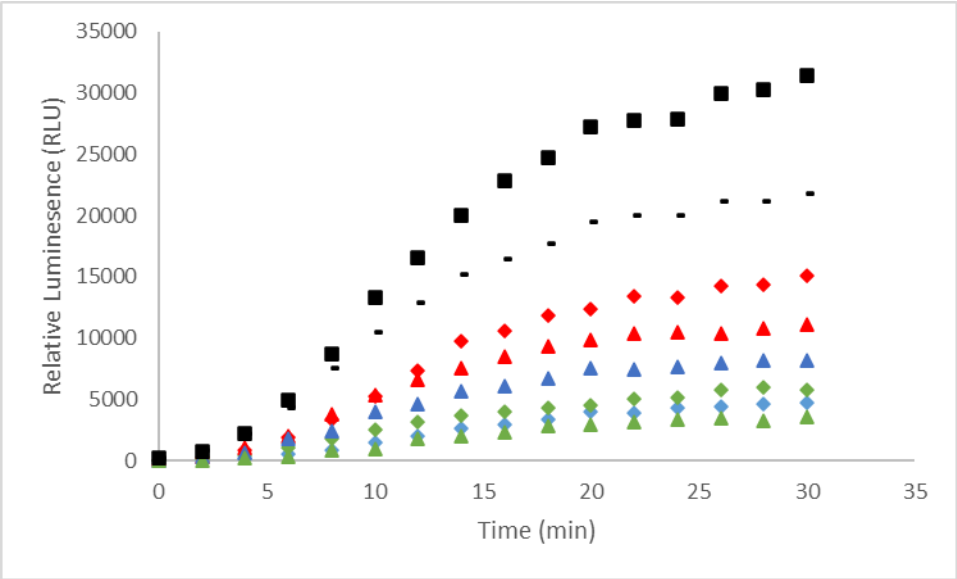

Figure 18. Luciferase Refolding assay of Hsc70 3T, with AF488 and AF594 dyes attached. Blank sample has no chaperones (DnaJ or Hsc70) included. Labelled Hsc70 (diamonds) refers to double labelled with AF488 and AF594, and unlabelled Hsc70 showed as triangles. Numbers refer to the final concentration of Hsc70 3T. Red shapes indicates 1.2 µM, blue 2.4 µM, and green 4.8 µM protein concentrations.

3.7 Cell Culture

Transduction of Hsc70 into cells was verified by both live and fixed cell microscopy. Using fixed specimens, previous work from other students was repeated with ABCA1, to identify location of Hsc70 relative to ABCA1. Rachel Moir had done this previously with single labelled Hsc70, and in HEK293. HepG2 was transfected with a plasmid for expression of GFP-ABCA1 and transduced with double labelled Hsc70, and imaged. HepG2 were able to be transfected using the same protocols as HEK293, although timing in between plating and transfection were longer, due to the growth rate of HepG2 being slower than HEK293. Unlike HEK293, which are able to grow on the coverslips without any treatment beforehand, HepG2 died when plated onto

coverslips which had not been treated. However, treatment with either PLL or PLO (both yielded the same results) made HepG2 adhere better to the coverslips and grow normally.

3.7.1 Live Microscopy

Using live cell images, HEK293 work from Rachel Moir with single labelled Hsc70 was extended to double labelled Hsc70, and a preliminary characterisation of heat shock. A Grienier 96 well plate was used to test five different conditions; serum free Opti-MEM media swapped for full DMEM media 15 minutes prior to imaging, wells with no labelled proteins transduced, normal PULSin/protein concentrations (1.2 µL to 0.3 µg), and half the protein/PULSin concentration (0.6 µL to 0.15 µg). Additionally, filtering the protein prior to addition was investigated to decrease clumping on cell surfaces.

Two concentrations of PULSin and protein were used to determine which concentration would be better for future experiments (Figure 19 and 20). The reasoning for using the lower concentration was to determine if cells would survive longer in the Cytation imager, allowing longer time courses to be investigated. It was found that a PULSin/protein concentration of 0.3 µg protein to 1.2 µL PULSin was the optimal amount for fluorescence detection in 200 µL wells. In wells with 0.15 µg protein to 0.6 µL PULSin, limited AF594 fluorescence was observed in cells and little to no AF488 fluorescence was detected. The cell longevity in wells with half the concentration of PULSin did not appear to be affected. For both PULSin/protein concentrations, AF488 fluorescence was detected later on in imaging, after 150 minutes of transduction, while AF594 was detected much earlier. AF488 fluorescence may have been present prior to this time but could have not been at the threshold for detection by the Cytation. Brightfield images showed minimal cell death at the end of the experiment (3hr). Therefore, future experiments can be investigated with longer time periods to see more AF488 fluorescence and eventual cell death.

Wells with the standard PULSin/protein concentration both were imaged in the Opti-MEM media and were also swapped with full media shortly before imaging. Brightfield images show that HEK293 cells were thriving more in the full media, as the healthy morphology is retained, compared to Opti-MEM media, where cells appear to be more spherical. However, this comes at

a cost, as the phenol red in the full media contributes to background signal when detecting fluorescence. Fluorescence images appeared to be duller compared to Opti-MEM wells. Therefore, it was concluded that although cells appear to be healthier during imaging, fluorescence detection is hindered.

Rachel Moir had previously found clumping of protein on the cell wall in previous live microscopy. In order to minimise this, protein was filtered with a spin filter column prior to addition to PULSin to decrease aggregation which may occur. A decrease of AF488 fluorescence was observed in filtered samples (Figure A3). Because double labelled Hsc70 had already been desalted twice prior to transduction, the removal of excess dye during filtering is not a likely explanation for the reduced fluorescence but is possible. In order to test for this, the FRET *in vitro* experiment could be done again, and can be compared to the results of protein before filtration.

Clumping did not appear to occur in unfiltered samples (Figure 19). Around 180 minutes, both AF594 and AF488 is shown on the cell wall, however at 210 minutes, more fluorescence has appeared within the cell, rather than accumulating on the cell wall. Therefore, desalting after labelling suffices for removing aggregated protein prior to transduction.

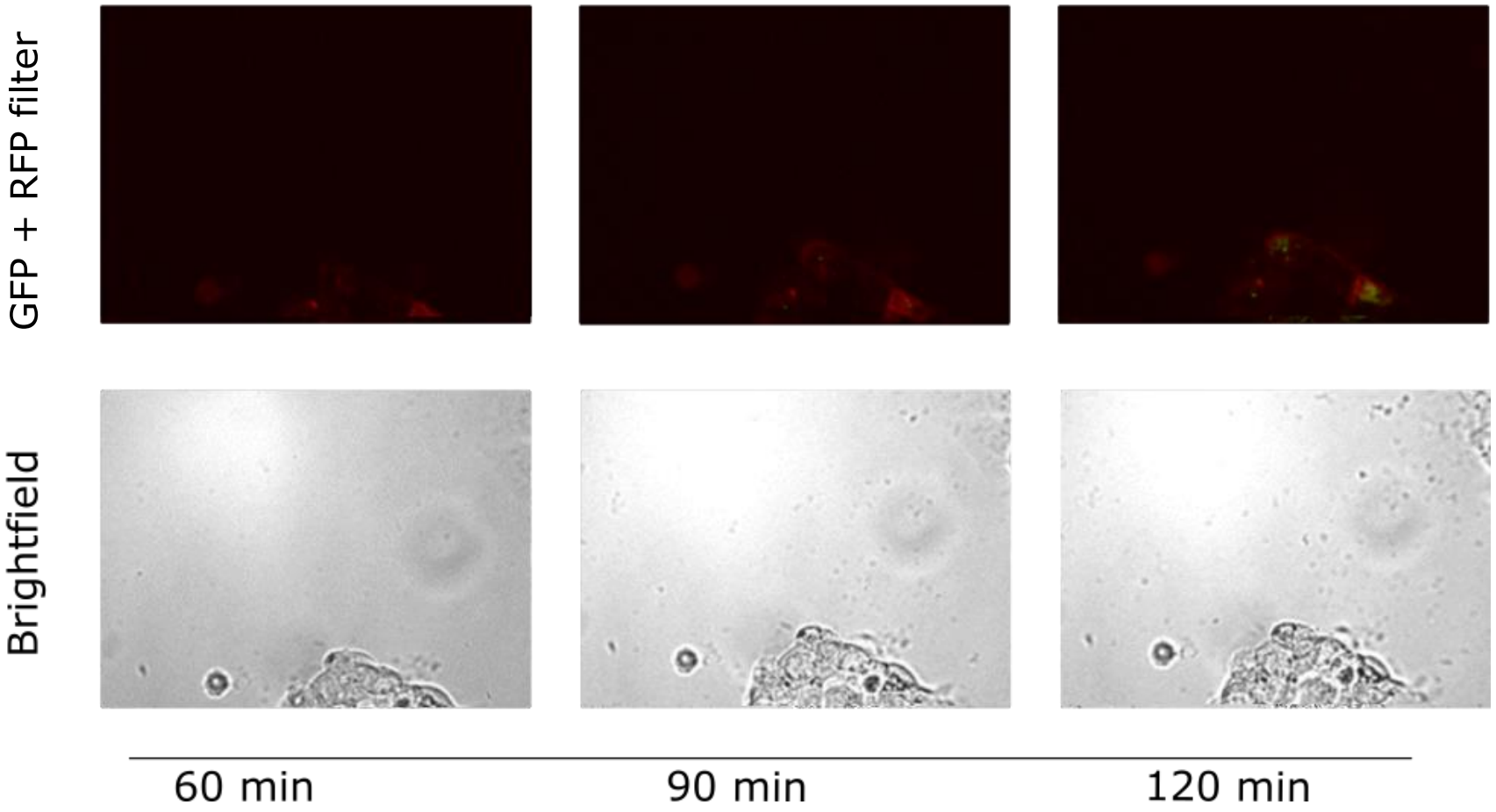

Figure19a. Transduction of AF488 and AF594 labelled Hsc70 3T into HEK293 mammalian cells. Time course of period after transduction with 0.3 µg protein with PULSin. Time points indicated at the side. Scale bar indicates 100 µm.

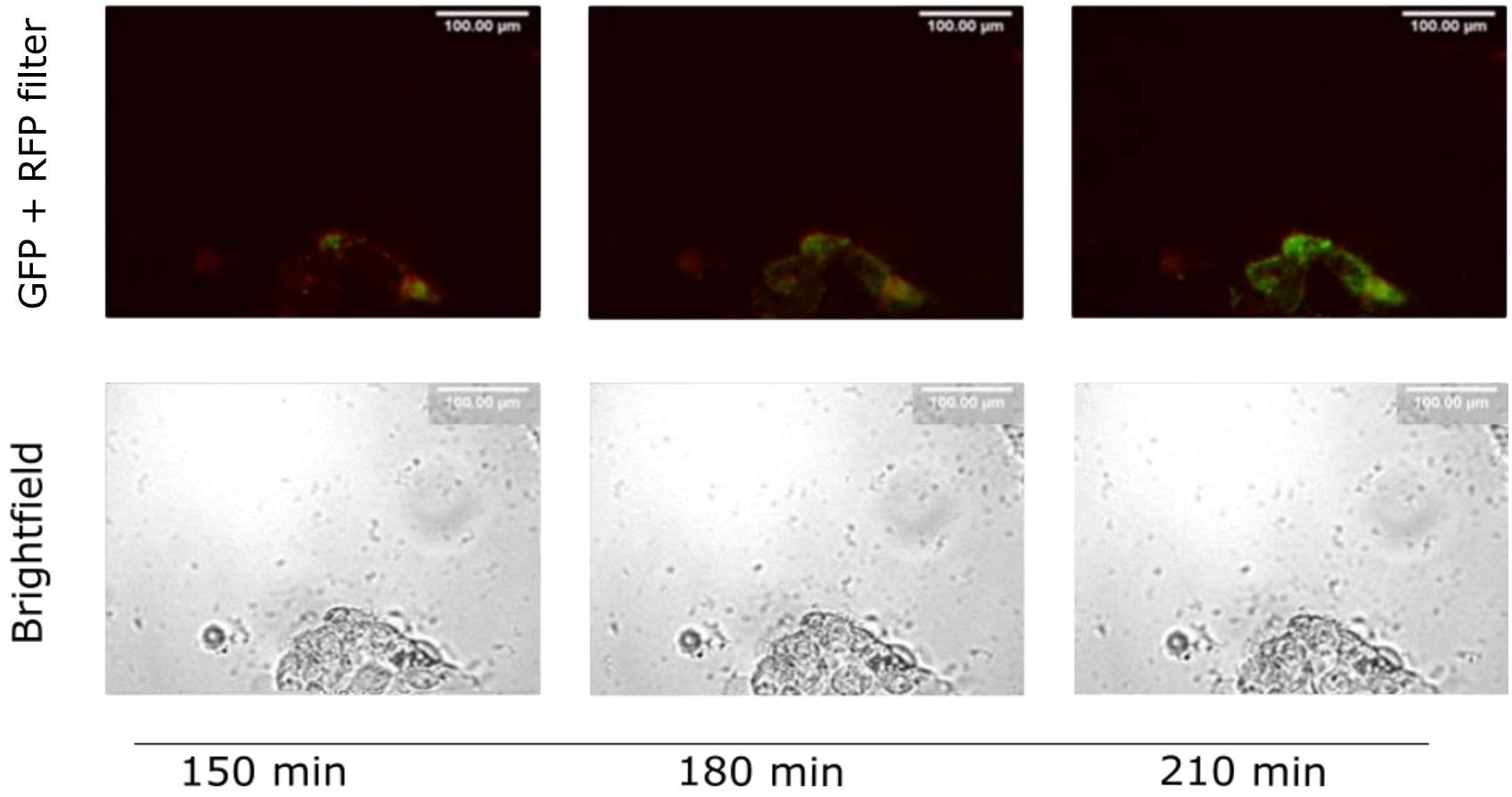

Figure 19b. Transduction of AF488 and AF594 labelled Hsc70 3T into HEK293 mammalian cells. Time course of period after transduction with 0.3 µg protein with PULSin. Time points indicated at the side. Scale bar indicates 100 µm.

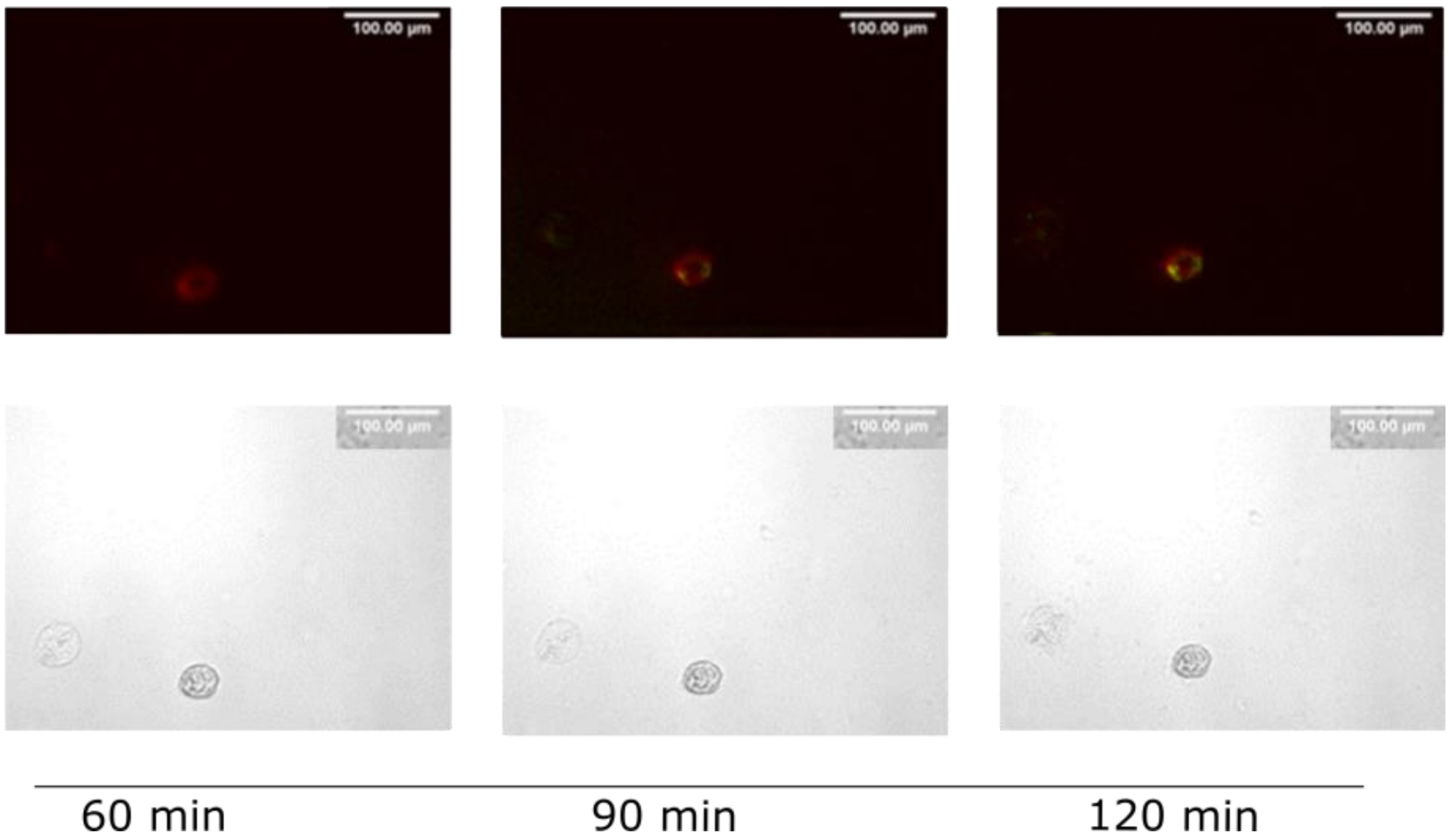

Figure 20a. Transduction of AF488 and AF594 labelled Hsc70 3T into HEK293 mammalian cells. Time course of period after transduction with 0.15 µg protein with PULSin. Time points indicated at the side. Scale bar indicates 100 µm.

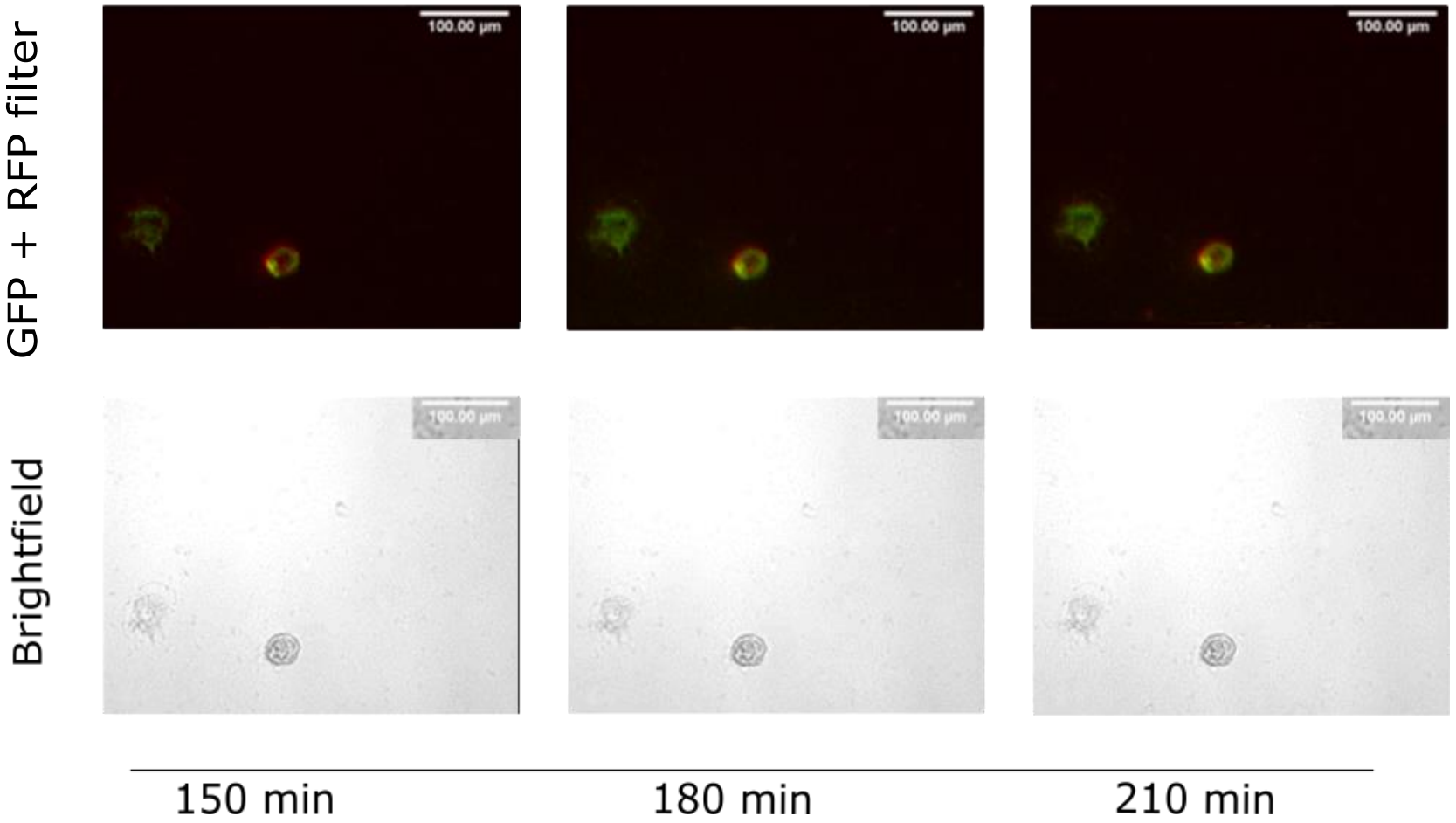

Figure 20b. Transduction of AF488 and AF594 labelled Hsc70 3T into mammalian cells. Time course of period after transduction with 0.15 µg protein with PULSin. Time points indicated at the side. Scale bar indicates 100 µm.

3.7.2 Cell Stress

No certain conclusions could be drawn from the cellular stress experiment in section 2.2.9.7. As per the manufacturer's instructions, 10,000 – 15,000 cells were plated per well in the 96 well Grienier plate. However, cells had to adhere to the bottom of the well before transduction experiments could be carried out. For the live microscopy experiments, 16 hours were left in order for cells to adhere. However, cells were left for 24 hours, and this affected the cells greatly. Cells had grown on top of each other, and cells had grown to be clumped with each other. However, in wells imaged with full media, the morphology was retained, like wells with full media from the experiment in section 2.2.9.10. Instead of the 10, 000 – 15,000 cells per well, it could be estimated that around 20 – 30,000 cells were in each well. This resulted in the concentration of PULSin/protein for 10,000 cells not being enough, and imaging of fluorescence was difficult. However, even though cells are growing on top of each other, cell survival was still stable, and so there is confidence that the protocol can be further optimised. Additionally, full media had been used in the wells where a small amount of fluorescence could be identified. As explained in section 3.9.1, this contributed to larger background fluorescence in the well, and so the small amount of AF594 fluorescence which was identified could not be quantified. No AF488 fluorescence could be identified, however because only cells were imaged for two hours, AF488 fluorescence may be identified if cells had been imaged for a further hour, like which was seen in section 3.9.1.

If this experiment were to be repeated, Opti-MEM media would not be replaced for full media. Cells would be incubated at 44ºC for a longer time period to demonstrate cell stress, for 90 minutes or the full 120 minutes as observed in Bellmann et al., 2010. Additionally, cells would only be allowed to adhere for 16 hours.

Chapter 4: Conclusion

In this project, a higher expression of pMSK003 was achieved, based on Figure 4. With enough purified Hsc70 3T to do different labelling experiments on, it was found that immobilised TCEP ensured that cysteines were suitably reduced, and reductant was removed from the experiment before addition of dyes. It was also found that AF488 bound to cysteines more than AF594, so AF594 was used first and AF488 in excess to ensure more labelling. The excess dye was removed from the labelled protein over a Desalting HiPrep 26/60 column, twice. This resulted in almost equal stoichiometry of the two dyes on the protein and showed nucleotide dependent conformational change through FRET *in vitro.* A protocol to transduce protein into HEK293 was optimised and double labelled Hsc70 3T was successfully transduced into HEK293. Preliminary experiments of heat shock in live cells were investigated (results not shown).

As discussed previously, labelling proved difficult to assess due to the uncertainty in quantitating cysteines available for labelling. An estimate that only 1/7th of cysteines was labelled, suggesting that only a small fraction of protein in the sample had both a donor or acceptor attached. This was estimated by comparing emission spectra of unbound dye to bound dyes. Additionally, protein concentration was not able to be determined as accurately with a A_{280} reading, because the dyes and ATP have an absorbance at 280 nm. To estimate the protein concentration, a series of proteins with known concentrations can be loaded onto the gel alongside the unknown concentration and can be worked out from there. One way to avoid this uncertainty would be to create a GFP fusion protein and put this plasmid directly into HEK293 strains, so the cell line will produce the mutant Hsc70 3T. This way, the PULSin reagent would not be required, and more importantly, labelling will not have to occur. Because GFP and RFP filters are widely available on imaging microscopes, these proteins could be suggested to make the fusion protein. This has been done in Kasaby et al., 2014, where Hsp70-1A was fused with YFP and were then transfected and expressed in HEK293, and FRET was used to track association with another CFP-fusion protein.

Many FRET experiments with labelled Hsc70 resulted in a quenching affect, and the ATP trace would not pass over the ADP trace. In order to understand how fluorescence quenching had occurred during measurements of *in vitro* FRET, it would be better to understand the conformation with the dyes. The models which have been used previously are based on the crystal structure of the DnaK homologue, and there is not a crystal structure of a full length Hsc70 model, only separated subunits. If Hsc70 3T double labelled could be crystallised, a model of how the dyes affect conformation between ATP bound and free states could inform more of a conclusion of quenching and energy transfer.

Due to timing mishaps, more work will need to be done in the cell culture aspect. In this thesis, heat was used to create cellular stress. However, other methods such as ATP depletion uncouplers and changes in pressure can contribute to a stressful environment in the cell. ATP depletion can be created with the aid of mitochondrial uncouplers. Kabakov et al., 2002 used carbonyl cyanide m-chlorophenylhydrazone (CCCP), which depletes ATP levels by uncoupling the proton gradient by reducing the activity of ATP synthase. Immunoprecipitation showed that cells retained a soluble pool of Hsp70 longer than controls that had not undergone ATP depletion, but found that the difference between the insoluble fraction in control compared to ATP depleted cells was not significant. Therefore, they hypothesised that more soluble Hsp70 would be bound in complexes with other proteins which have a higher tendency to aggregate. However, as stated by Kabakov et al., this process is challenging and requires many steps before supplementing cells with the mitochondrial uncoupler. Further analysis than a fluorescence microscope was also required to determine the effects of the drug, making the experiment more complicated. Another way to induce cellular stress was by investigating the effect of hydrostatic pressure and Hsp70 gene expression (Kaarniranta et al., 1998). This is a very interesting study, because they submerged the cells, and found that although Hsp70 levels had increased, there was no activation of the HSF1, or induction of the hsp70 gene. But the mRNA was elevated as well as protein levels of Hsp70.

Double labelled Hsc70 3T was successfully transduced into HEK293 cells with varying conditions. It was found that unfiltered samples, imaged with Opti-MEM in the wells, and at the suggested PULSin concentration by the manufacturer was sufficient to detect fluorescence of AF488 and AF594. Unfortunately, due to the Cytation live cell imager not having a

monochromator, FRET cannot be measured, as fixed paired wavelengths can only be excited (GFP and RFP filters were used). Because of this, extracellular concentrations of Hsc70 3T could not be measured. Like the *in vitro* experiment, 10 µL of ATP (50 µM) would be injected into each well. Ideally, FRET would be observed when 480 nm was excited, and both fluorophores would be shown. This would have been a reliable way to watch the movement of Hsc70 3T into cells, because ideally the extracellular FRET would decrease over time as more Hsc70 moves into the cytoplasm. Confocal imaging is more sensitive and is capable of producing Z-stack data, so localisation would be more pinpointed.

The aim of the project was to establish conditions to observe FRET in mammalian cells. Although FRET detection was not achieved in this project, each step in order to do this was done and could be successfully done, and imagery achieved in an inverted fluorescence microscope. FRET detection should be achievable if confocal microscopes were available, and so an optimised protocol to achieve this has now been made. With the confocal microscope, separate regions of the cell would be able to be viewed, giving a more in depth analysis of localisation and transfection patterns. Cellular stress experiments could also be repeated, as a heat shock protocol was established. Rotenone is a widely used pesticide and has been shown to induce stress in cells. Li et al., 2019 found that Hsp70 were able to reduce apoptosis levels in cells which were treated with rotenone for 1hr, and 3 µg/mL was sufficient enough to invoke a response from varying concentrations of Hsp70. This could be repeated in this experiment and an investigation into if the average conformation changes with the treatment, and to correlate the conformational state with other features.

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Appendix

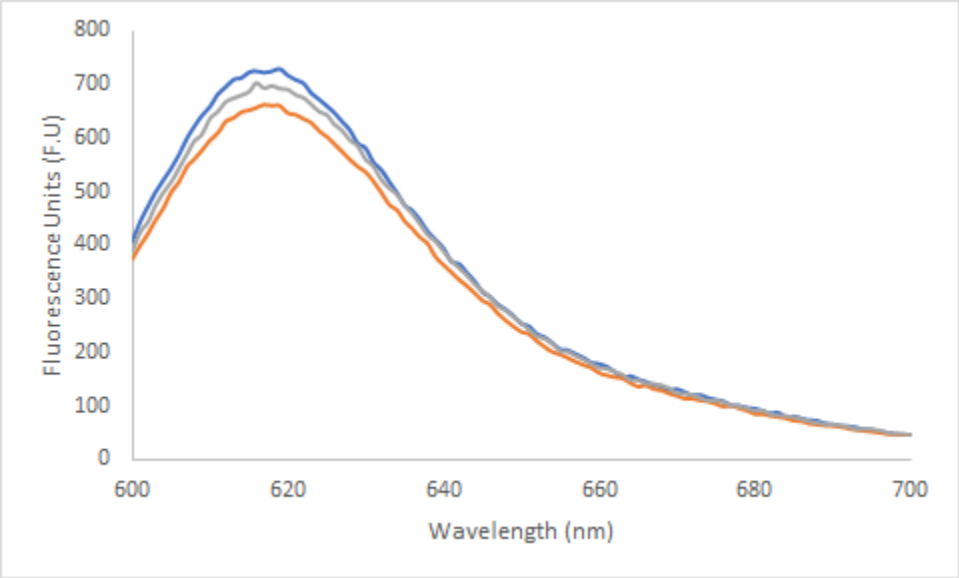

Figure A1. Direct excitation of AF594 *in vitro* measured on a fluorescence spectrometer. The orange trace is double labelled Hsc70 3T with 10 µL ADP added to the cuvette. The grey trace is labelled Hsc70 3T with 10 µL ATP. The blue trace is labelled Hsc70 3T with no nucleotide added, as a control.

All samples have been directly excited at 590 nm and measuring with a 5 nm slit width.

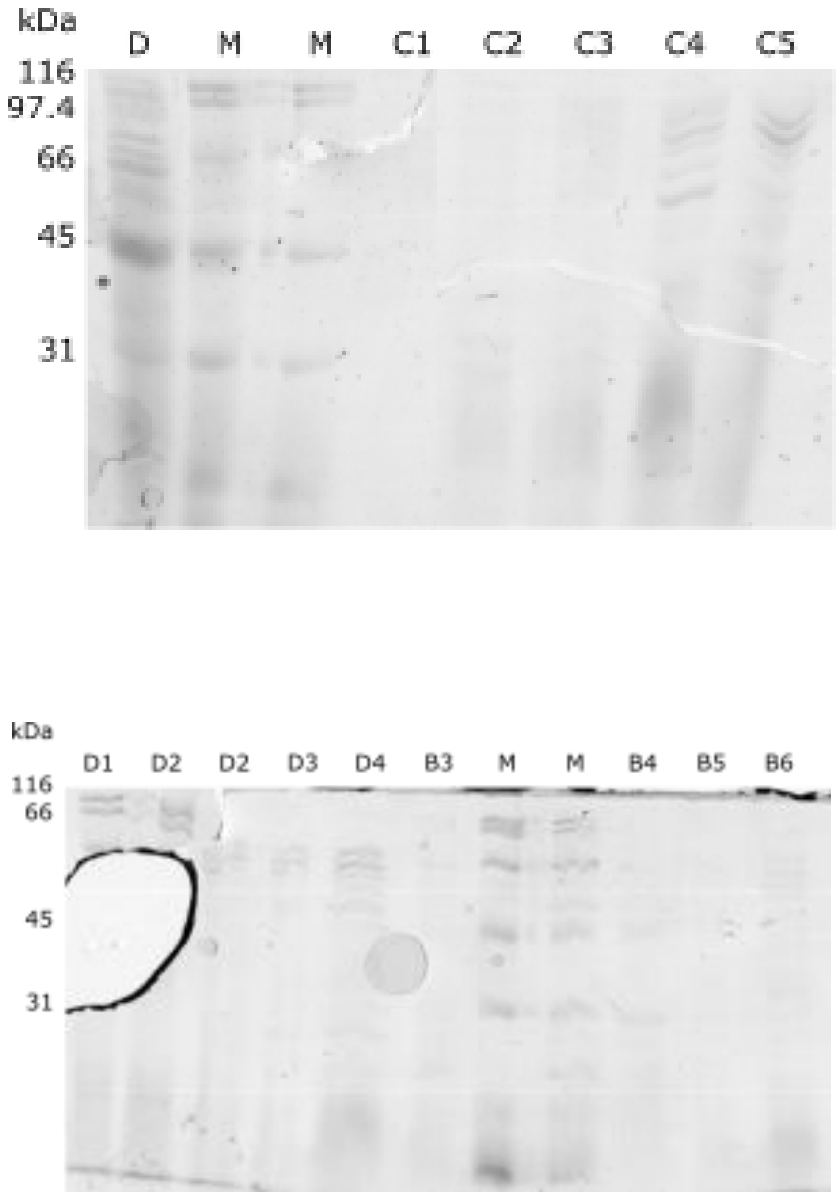

Figure A2. 12% SDS-PAGE of HiTrap S-Sepharose Chromatography purification of DnaJ. 4 µL of fractions eluted between 278 – 310 mL are included in the gel. D: dialysed sample taken before HiTrap S-Sepharose, but after ammonium sulfate precipitation. DnaJ was estimated to be co-purified with Hsp70, which shows why there are bands around 66 kDa. M: SDS-PAGE standards, broad range (BIO-RAD)

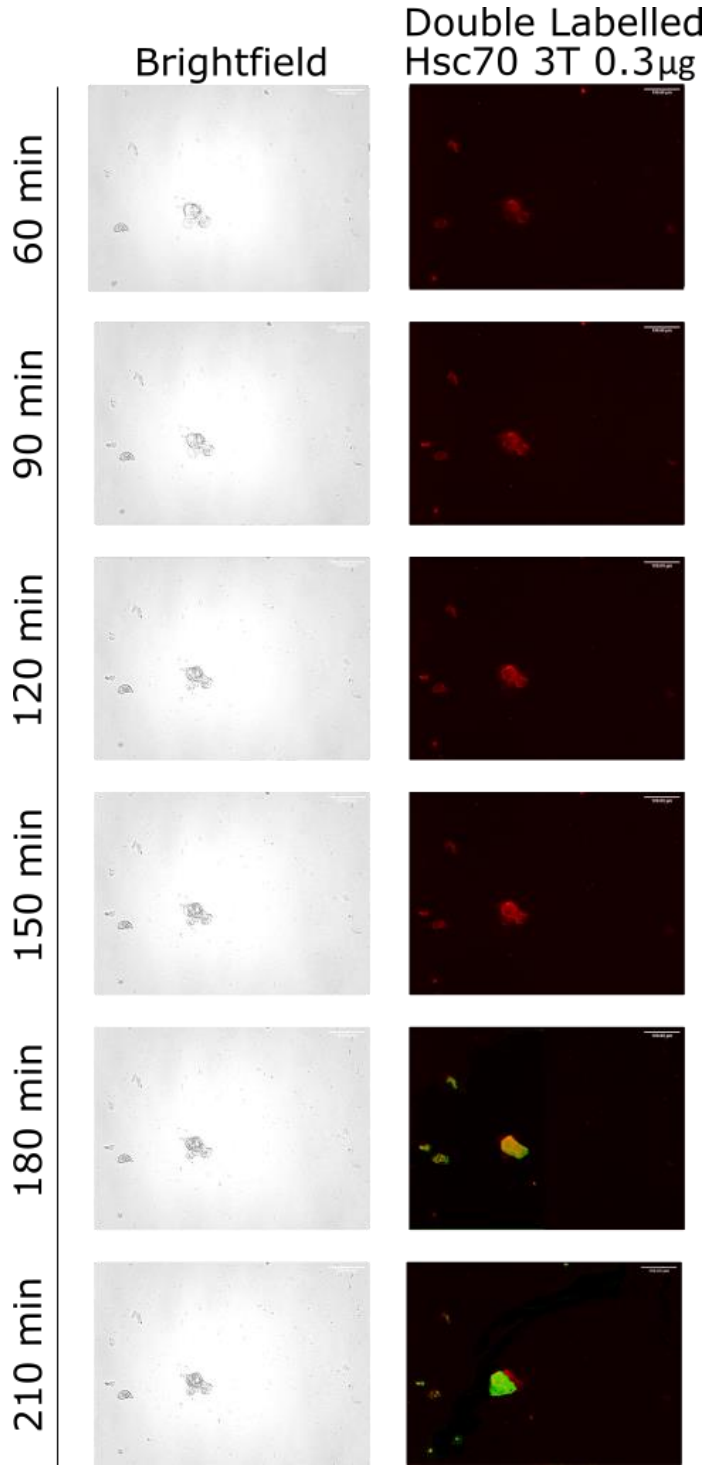

Figure A3. Transduction of AF488 and AF594 labelled Hsc70 3T. Time course of period after transduction with 0.3 µg filtered protein with PULSin. Cells are kept in Opti-MEM media during imaging. Protein amount indicated across the top, and time at the side. Scale bar indicates 100 µm.

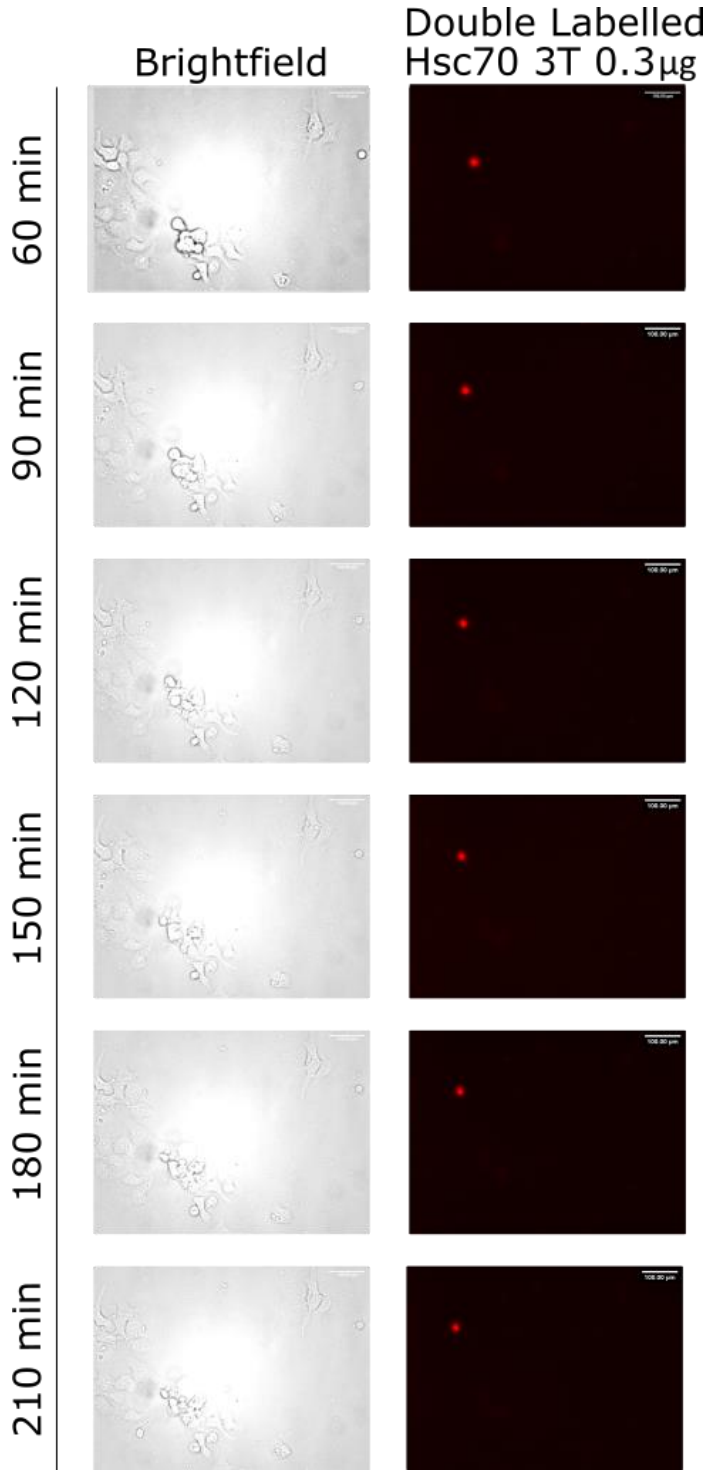

Figure A4. Transduction of AF488 and AF594 labelled Hsc70 3T. Time course of period after transduction with 0.3 µg unfiltered protein with PULSin. Cells are kept in full DMEM during imaging. Protein amount indicated across the top, and time at the side. Scale bar indicates 100 µm.

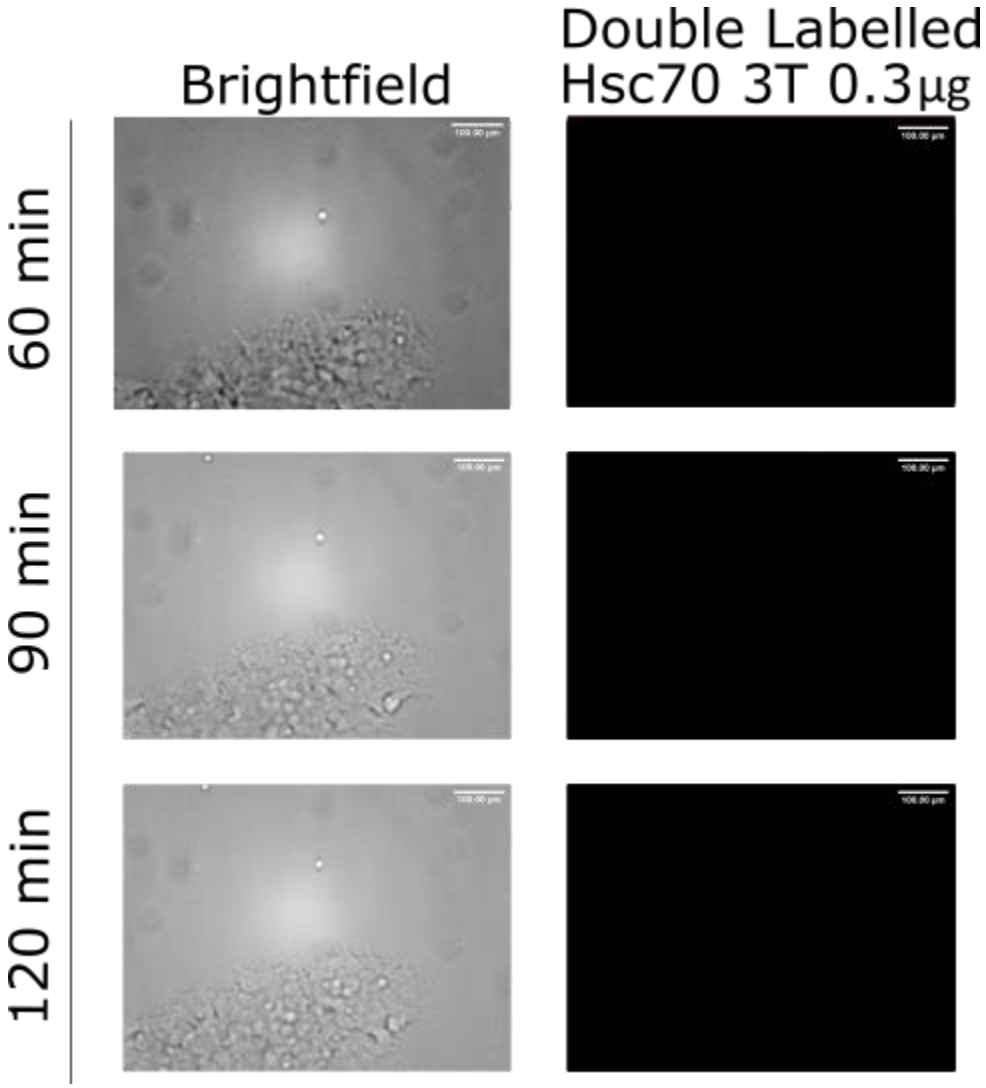

Figure A5. Transduction of AF488 and AF594 labelled Hsc70 3T, heat shocked at 44degreesC at 90 minutes. Time course of period after transduction with 0.3 µg unfiltered protein with PULSin. Cells are kept in full DMEM during imaging. Protein amount indicated across the top, and time at the side. Scale bar indicates 100 µm.