

**A study of DEF6 granule
formation using
biophysical and cellular
methods**

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Abstract

DEF6 is a Rho-Guanine Nucleotide Exchange Factor (GEF) with a poorly characterised role in T-cell receptor signalling and unknown structure¹⁻³. Its importance in T-cell signalling has been highlighted by observations that DEF6-deficient T cells display defective actin polymerisation and polarisation, have a decreased susceptibility to CD3-induced apoptosis⁴, and exhibit aberrant expression of the inflammatory cytokine IL-17². DEF6 is a GEF for Rho-family GTPases including CDC42, RhoA and Rac1¹, which unlike other Rho-GEFs exhibits a reversal of the N-C terminal orientation of the DH and PH domains only found in DEF6 and its homolog in B-cells, SWAP70. Through this role DEF6 has been shown to be involved in the spatiotemporal organisation of T cell signalling through the GTPase CDC42⁵. DEF6-deficient mice have been shown to develop autoimmune diseases and have also been shown to be resistant to the development of other autoimmune diseases, indicating a loss of control of the immune response. The molecular mechanism behind these phenotypes remains to be identified.

Exogenously expressed DEF6 has been shown to form cytoplasmic granules under conditions of arrested translation, similar to processing bodies and stress granules which are involved in translational regulation. Data presented here furthers this work and demonstrates endogenous DEF6 forms granules in activated T-cells. Exogenously expressed DEF6 shows partial co-localisation with markers of P-bodies, and demonstrates slow recovery from FRAP indicating a structural role within granules. The granules move within the cell, and analysis of this movement suggests the granules move via active transport, suggesting a link to the microtubule network. Examination of the individual domains of DEF6 demonstrates that the N-terminus is likely to have a role in the co-localisation of DEF6 with P-bodies, whilst the C-terminal DHL domain drives aggregation of DEF6 into granules. Data here confirms the predicted formation of a coiled-coil structure within the DHL domain.

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List of Abbreviations

AGF	Analytical Gel Filtration
AICD	Activation Induced Cell Death
APC	Antigen Presenting Cell
APS	Ammonium Persulphate
ARE	AU Rich Element
CC	Coiled coil
CD	Circular Dichroism
DH	Dbl Homology
DHL	Dbl Homology Like
DLS	Dynamic Light Scattering
EAE	Experimental Autoimmune Encephalomyelitis
EAU	Experimental Autoimmune Uveitis
ESI-MS	Electrospray Ionisation Mass Spectrometry
FRAP	Fluorescence Recovery After Photobleaching
GAP	GTPase Activating Protein
GEF	Guanine nucleotide Exchange Factor
GST	Glutathione S Transferase
IBP	IRF-binding Protein
IFN γ	Interferon gamma
IPTG	Isopropyl-beta-D-thiogalactopyranoside
IRES	Internal Ribosome Entry Site
IRF4	Interferon Regulatory Factor 4
ITAM	Immunoreceptor Tyrosine based Activation Motif
LAT	Linker of Activated T cells
LB	Luria Bertani
mut	mutant

MHC	Major Histocompatibility Complex
NFAT	Nuclear Factor of Activated T cells
NMR	Nuclear Magnetic Resonance
PCR	Polymerase Chain Reaction
PH	Pleckstrin Homolgy
PI(3,4,5)P ₃	Phosphatidyl Inositol 3,4,5 Phosphate
PI3K	Phosphoinositide 3 Kinase
PKCθ	Protein Kinase C theta
PLCγ	Phospho-Lipase C gamma
RA	Rheumatoid Arthritis
RNP	Ribonucleoprotein
RRM	RNA Recognition Motif
SDS-PAGE	Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis
SEE	<i>Staphylococcus Aureus</i> Enterotoxin E
SLAT	SWAP70-like attenuator of T cells
SLE	Systemic Lupus Erythematosus
SMAC	Supramolecular Activation Cluster
TCR	T Cell Receptor
T _H 1	T Helper 1
TNFα	Tumour Necrosis Factor alpha
UTR	Untranslated Region

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1. Introduction

1 Introduction

1.1 The Immune System

The immune system is made up of organs and cells that protect the body against pathogens via the immune response and comprises of two main systems, the adaptive or acquired immune system and the innate immune system. The innate immune system is non-specific, recognising pathogens by pattern recognition and providing immediate protection against infection. The adaptive immune system on the other hand is highly specialised, with a vast number of specific pathogen receptors available, which expand rapidly when required to provide long lasting specific response.

Lymphocytes are the cells of the adaptive immune system, comprising of T and B lymphocytes. A major feature of the adaptive immune system is its ability to remember pathogens previously encountered. Both T and B cells produce memory cells after activation, which undergo a more rapid clonal expansion after reactivation than the initial immune response.

As part of the immune response, immune cells produce a range of small signalling molecules with immunomodulating effects called cytokines, including interferons and interleukins. Cytokines can broadly be split into two functional groups; type 1 regulates cellular immune responses, whilst type 2 promotes antibody response. Differential exposure to cytokines drives the differentiation of T cells into specific subtypes, and these in turn secrete specific sets of cytokines to promote varying effects. Dysregulation of cytokine production leads to aberrant immune response, and is a hallmark of several autoimmune diseases.

DEF6, also known as IRF-4 binding protein (IBP) or SWAP70-like attenuator of T cells (SLAT) is a relatively unstudied 631 amino acid protein expressed predominantly in T cells⁶. It is related to the protein SWAP70, which is expressed in B cells involved in B cell activation and immunoglobulin class switching. The main functions of DEF6 known to date are as a Rho-family guanine nucleotide

exchange factor (Rho-GEF) of Rho family GTPases, including CDC42, RhoA and Rac1¹, and as a binder of the transcription factor Interferon Regulatory Factor 4 (IRF-4)^{3,4}. It has been demonstrated that DEF6 has a role in regulating several autoimmune diseases⁷⁻⁹, a role which is currently thought to be due to its regulation of IRF-4 and thus the cytokines it regulates, IL-21 and IL-17¹⁰. The loss of DEF6 in mice leads to changes in the development of thymocyte subsets, and an effect on the differentiation of T_H1 and T_H2 cells leading to resistance to inflammation mediated by these cells types¹¹. Recent data have shown that DEF6 may also have a role in regulation of mRNA expression via granule formation similar to processing (P)-bodies¹².

1.1.1 T cell maturation

T lymphocytes can be divided into several subsets based on both their cytokine production, and expression of the surface glycoproteins CD4 and CD8. T cells originate from the common lymphoid progenitor cells in the bone marrow which migrate to the thymus and expand to produce a large number of immature thymocytes. These immature thymocytes express neither CD4 nor CD8 on their surface, and are termed double negative. As they mature, cells express the T cell receptor complex on their surface which is comprised of the T cell receptor itself, two heterodimeric transmembrane protein chains with constant and variable regions consistent with the immunoglobulin superfamily of proteins, and the accessory proteins CD3 δ/ϵ , CD3 γ/ϵ and CD247 ζ/ζ which are signalling molecules with long cytoplasmic chains for propagating the signal after antigen binds to the variable region of the T cell receptor. Along with this complex both CD4 and CD8, glycoproteins that serve as co-receptors with the T cell receptor, are expressed and the cells are termed double positive. Positive selection then occurs, in which cells that do not interact with major histocompatibility complex (MHC) molecules or react too strongly are removed ensuring an appropriate level of immune response to pathogens. MHC molecules are cell surface

molecules involved in leukocyte recognition via the presentation of fragments of protein. MHC class II molecules are those found on antigen presenting cells, and present antigen that has been taken up and processed by the cell whilst MHC class I molecules are found on all nucleated cells and present to killer T cells which induce apoptosis of the presenting cell. It is at this positive selection stage that the cells $CD4^+/CD8^+$ fate is decided. Cells that react well with MHC class II eventually become $CD4^+$ cells, and those that react well with MHC class I become $CD8^+$ cells. Following this, negative selection removes cells that react with self MHC-peptides ensuring the body does not attack itself¹³.

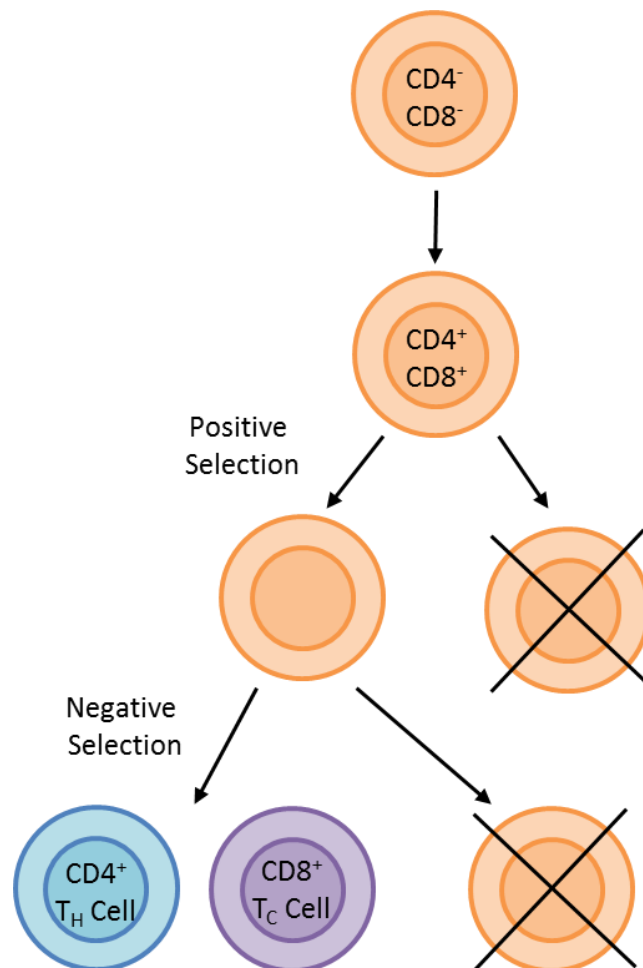


Figure 1 - T cell maturation. Double negative immature thymocytes mature to express both CD4 and CD8 on their cell surface, becoming double positive cells. These then undergo two rounds of selection, positive to select for cells that recognise MHC peptides, and negative selection to remove those that react with self MHC peptides.

A role of DEF6 in T cell development has been reported by two different groups, with two different conclusions. Fanzo *et al*⁴ reported no difference in development in CD4⁺ cells lacking DEF6 and no difference in lymphoid development or size, whilst Becart *et al*¹¹ reported a proportional reduction in number of all types of thymocyte subsets and a reduced thymus size. The differences between these studies is thought to be due to the genetic background of the mice used, an issue discussed in more depth in section 1.2.

1.1.2 T Cell differentiation

T helper cells are a CD4⁺ subset that assists other lymphocytes with the immune response, such as the maturation of B cells¹⁴. Activation by antigen presentation by MHC class II molecules expressed on the surface of professional antigen presenting cells such as dendritic cells, macrophages or B cells results in the differentiation of the T cells into a range of different subtypes that secrete different cytokines¹⁵.

The T helper-1 subtype (T_H1) is activated by IL-12 and produces IFN γ , and is involved in the host response to intracellular bacteria and protozoa by activating macrophages to phagocytose the intracellular bacteria¹⁶. T_H2 cells protect against extracellular threats such as intestinal helminths, are activated by IL-4, and produce the cytokines IL-4, IL-5 and IL-13¹⁶. They stimulate the production of histamine by mast cells¹⁷; and B cell proliferation and antibody class switching¹⁸. Their involvement in histamine production means that T_H2 overreaction leads to allergy^{19,20}. T_H17 cells mediate immunity against fungi and extracellular bacteria, and produce IL-17, which induces the production of proinflammatory cytokines such as TNF-(alpha) and IL-1²¹. Overreaction of T_H17 cells leads to rheumatoid arthritis²². To prevent over production of proinflammatory cytokines by T_H17 cells, they are controlled by the production of IFN γ by T_H1 cells, and IL-5 and IL-13 by T_H2 cells^{23,24}.

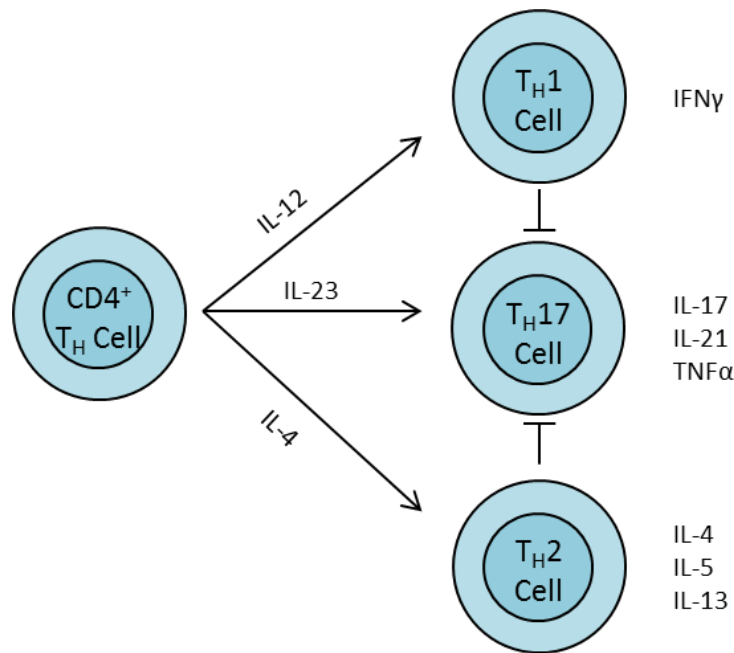


Figure 2 - Upon activation, CD4⁺ T cells differentiate into a range of effector T helper cells that produce different cytokines. T_H1 cells are involved in the host response to intracellular bacteria and protozoa, T_H17 cells mediate the response to fungi and T_H2 cells are involved in the response to extracellular threats.

CD8⁺ T cells are activated by MHC class I molecules, found on the surface of all nucleated cells. They include the cytotoxic T cells, which can destroy cancer cells or cells infected by virus by releasing cytotoxins²⁵.

To confer long term protection to the body, a subset of T cells persists in the body after an infection is cleared. These are called memory T cells and are a key part of the adaptive immune response. Memory T cells are derived from both naïve and effector cells that have encountered antigen. When the body is re-exposed to antigen, the memory T cells rapidly expand the number of effector T cells, providing a much faster response than that upon first stimulation by antigen, termed the secondary response. These cells can express both CD4 and CD8.

Once an infection is cleared, the high number of effector T cells must be removed to prevent excess inflammation and damage to the body. Regulatory T cells (T_{reg} cells) shut down the response by producing immunosuppressive cytokines such as IL-10 and TGF- β . In addition to shutting down the response

after an immune reaction, T_{reg} cells suppress auto reactive T cells that escaped negative selection²⁶.

DEF6 has a role in the response and differentiation of T_H1 and T_H2 cells via its induction of the translocation of the transcription factor NFAT, with mice deficient in DEF6 resistant to induced inflammation normally mediated by these cell types¹¹. DEF6 also has a role in T_H17 differentiation and function via its role in binding IRF4¹⁰. These roles of DEF6 are all linked to its functions after T cell activation by antigen presentation.

1.1.3 Antigen presentation triggers T cell activation

Antigen is material recognised by the body by antibodies, recognition of non-self-antigen in the correct context triggers the adaptive immune response. Whilst all cells can present antigen via major histocompatibility complex molecules, proper triggering of T cells requires the presence of co-stimulatory molecules which are found on the surface of professional antigen presenting cells such as dendritic cells, macrophages and B cells.

In CD4⁺ T cells, recognition of antigen is via presentation by MHC class II to the T cell receptor, and co-stimulation of CD28 by CD80 or CD86. The T cell receptor (TCR) complex comprises of the receptor itself, consisting of a heterodimer of TCR α and TCR β chains; and the CD3 molecules, the CD3 $\epsilon\delta$ heterodimer, the CD3 $\epsilon\gamma$ heterodimer, and the CD3 ζ homodimer²⁷.

As shown in the diagram in Figure 3, upon binding of MHC-antigen complex by the T cell receptor complex and correct co-stimulatory molecules, T cell signalling is activated. Activated CD4 recruits the Src-family tyrosine kinase Lck and together with Fyn these tyrosine kinases phosphorylate the immunoreceptor tyrosine based activation motifs (ITAMS) on the cytoplasmic tails of the CD3 proteins²⁷.

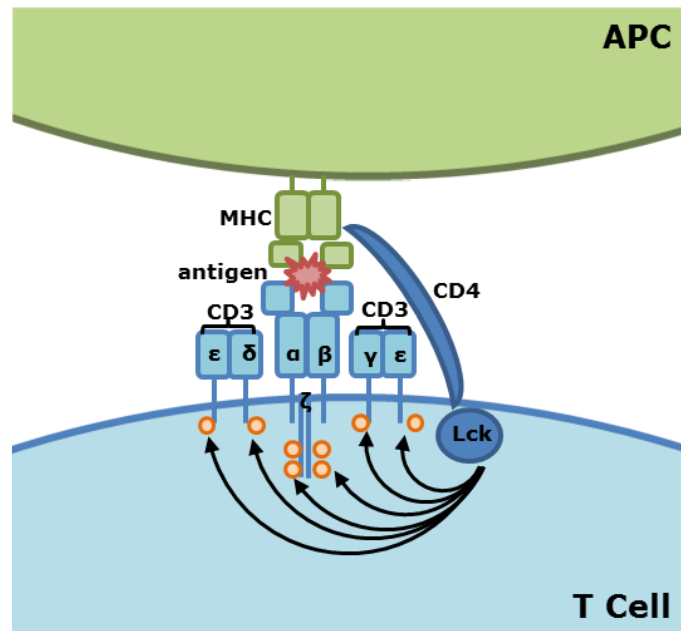


Figure 3 - During the initial activation of the T cell by an antigen presenting cell, the T cell receptor complex is phosphorylated by Lck. The T cell receptor binds to antigen presented by the antigen presenting cell, and CD4 is activated by binding to the MHC on the APC. Activated CD4 recruits the kinase Lck which phosphorylates the cytoplasmic tails of the CD3 proteins.

ZAP-70, a tyrosine kinase, is recruited to the phosphorylated ITAMs on CD3ζ via its SH2 domain, and is phosphorylated by Lck²⁸ as shown in Figure 4. ZAP-70 in turn phosphorylates linker of activated T cells (LAT) which acts as a docking site for several proteins²⁷.

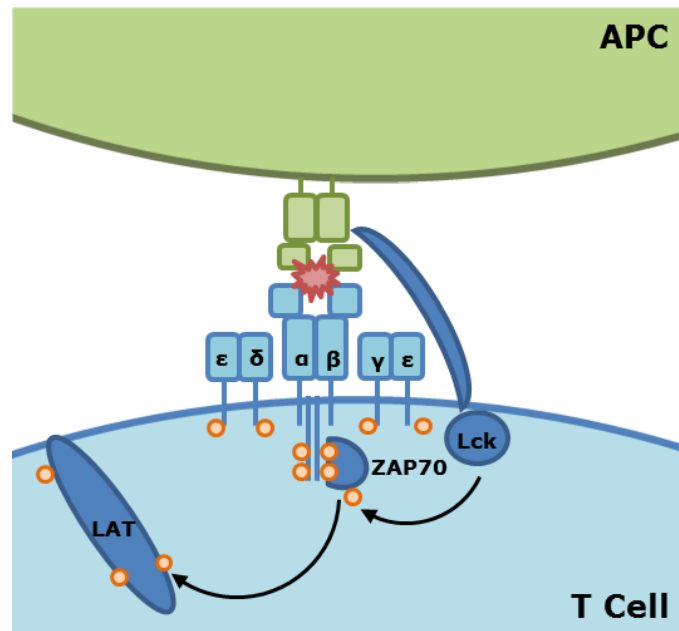


Figure 4 - ZAP70 is recruited to the CD3 subunit, and phosphorylated by Lck, subsequently phosphorylating LAT. The phosphorylation of the ζ chain of the CD3 complex recruits ZAP-70, which is then phosphorylated by Lck. ZAP70 then phosphorylates the adaptor protein LAT which can act as a docking site for further proteins.

At LAT, several proteins are recruited to the membrane including SLP-76, PLC- γ , Vav1 and Itk. This eventually leads to the activation of several transcription factors including AP-1 and NFAT to switch on production of cytokines necessary for the T cell response²⁹. In particular, the Tec-family tyrosine kinases Itk is involved in the activation of PLC- γ via tyrosine phosphorylation, leading to the cleavage of PI(4,5)P₂ in the membrane to form diacyl glycerol (DAG), IP₃ and PI(3,4,5)P₃³⁰⁻³². DAG binds and activates PKC θ leading to the activation of the transcription factors NF- κ B and AP-1²⁷. Meanwhile, IP₃ activates calcium release channels in the endoplasmic reticulum, eventually leading to the translocation of the transcription factor NFAT to the nucleus²⁹. The activation of NFAT is an important step after antigen ligation by the T cell receptor as the NFAT proteins are key regulators of T cell activation and thus production of an effective immune response, and additionally regulate the processes that lead to inactivation and T cell tolerance³³.

DEF6 has been found to be part of the signalling cascade downstream of T cell receptor activation^{2,11,34}. It has been shown to be phosphorylated by Itk at tyrosines 210 and 222¹², and by Lck at tyrosines 133 and 144^{34,35}. Downstream, DEF6 has been shown to be involved in the activation of NFAT, NF- κ B and AP-1^{11,34}.

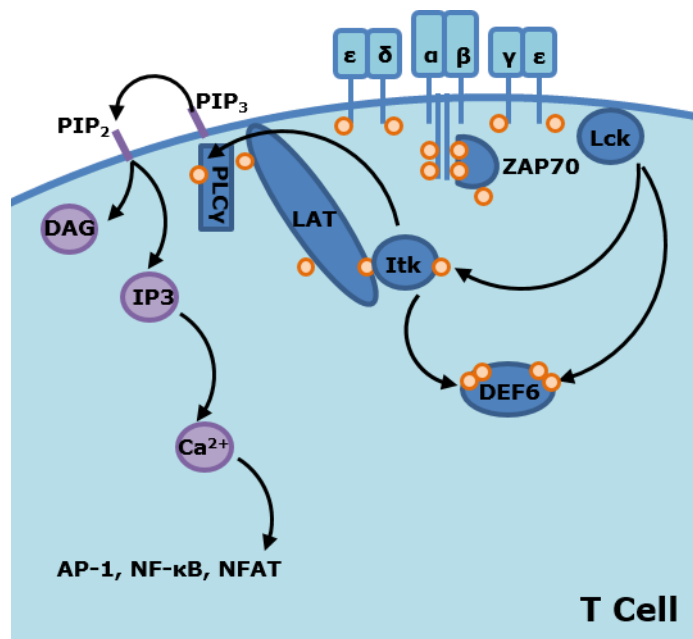


Figure 5 - LAT recruits several proteins to the membrane including PLC and Itk. These lead to the activation of transcription factors and the polymerisation of actin via several pathways including via DEF6 activation.

In addition to the activation of cytokines, an important effect of T cell activation is the remodelling of the actin cytoskeleton^{36,37}. DEF6 is potentially involved in this via its role in enhancing the GTPase activity of CDC42, RhoA and Rac1^{1,5}. These GTPases promote actin polymerisation involved in the reorganization of the cytoskeleton after activation, an event important in polarizing the cell, and organizing the T cell signalling cascade³⁶.

1.1.3.1 The involvement of DEF6 in T cell signalling

DEF6 inactivation leads to suboptimal ERK1/2 activation, decreased Ca²⁺ release from the endoplasmic reticulum and reduced nuclear translocation of NFAT¹¹,

similar to inactivation of Itk. These effects ultimately lead to defects in the development and function of T_H1 and T_H2 cells.

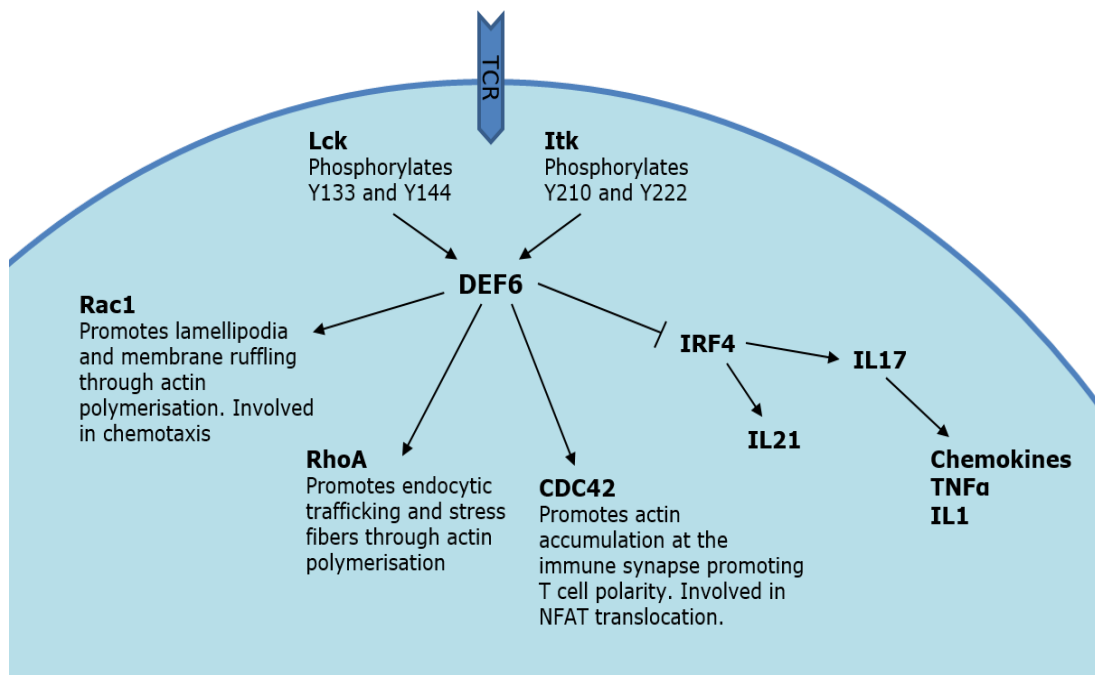


Figure 6 - A diagram summarising DEF6 interactions downstream of T cell receptor signalling. DEF6 is a substrate for the TEC kinase Itk and Src-family kinase Lck. It also interacts with the Rho-GTPases Rac1, RhoA and CDC42, and the transcription factor IRF4.

The main role of DEF6 after activation is currently thought to be its role as a Rho-GEF, activating the Rho family GTPases CDC42 and Rac1³⁸. DEF6 is involved in control of actin polymerisation through activation of CDC42 and Rac1. DEF6-deficiency prevents TCR mediated actin reorganization which can be rescued by constitutively active Rac1⁴. Actin polymerisation is an important event in the organization of the immune synapse and the polarisation of the T cell, and mice in which DEF6 has been inactivated via retroviral insertion show decreased accumulation of proteins at the immune synapse, indicating DEF6 is required for proper immune synapse maintenance⁴.

1.1.4 The immune synapse forms between a T cell and an antigen presenting cell

Organisation of correct timing and location of T cell signalling components is essential for proper T cell activation. One level of spatio-temporal organization is

the segregation of surface receptors at the T cell – antigen presenting cell interface. This interface between the antigen-presenting cell and the T cell is called an immune synapse, or supramolecular activation cluster (SMAC)³⁹. The immune synapse is a collection of proteins involved in T cell receptor signalling, likely held in a membrane raft by a localised mesh of actin⁴⁰. The synapse can be divided into zones, the central or cSMAC, and the peripheral or pSMAC which encircles the cSMAC as shown in Figure 7. Surrounding these is the distal or dSMAC.

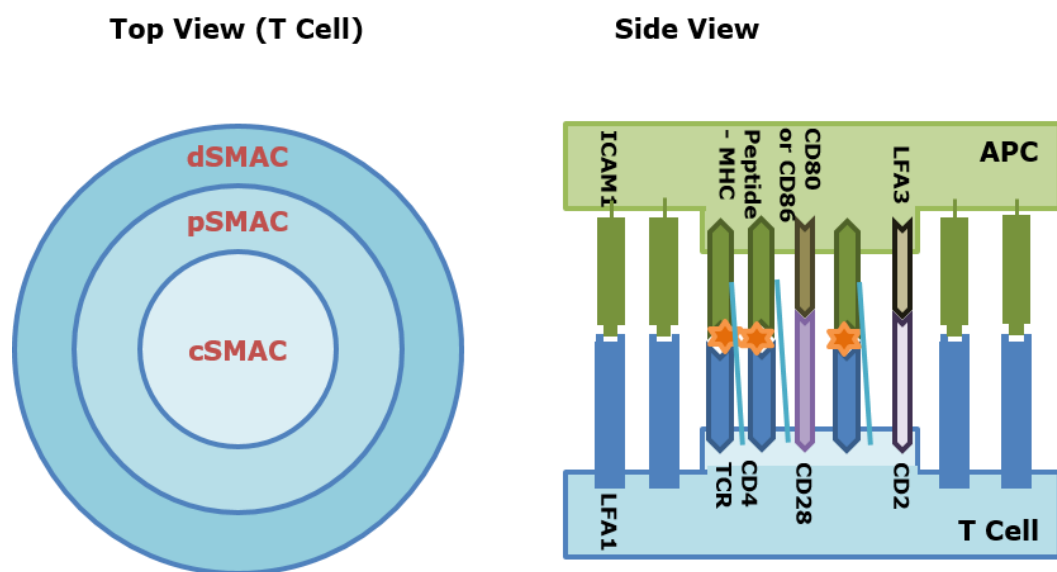


Figure 7 – The immune synapse forms between a T cell and an antigen presenting cell (APC) and is formed of a central region or cSMAC that contains the T cell receptors, a peripheral ring of adhesion molecules called the pSMAC and the distal ring called the dSMAC.

The cSMAC contains the T cell receptor, the co-receptor CD4 or CD8, CD28 which provides a co-stimulatory signal when bound to CD80 or CD86 expressed on the antigen presenting cell thus ensuring that the bound antigen is being presented by a cell and is not an aberrant signal, and CD2 which provides co-stimulation and adhesive properties when bound to LFA3 on the antigen presenting cell⁴¹. Thus the core signalling molecules are clustered centrally at the immune synapse.

The cSMAC is surrounded by the pSMAC which contains integrins, in particular LFA1 which binds to ICAM1 on the antigen presenting cell and stabilises the interaction between the two cells to support robust signalling. Further out, the dSMAC contains proteins with large extracellular domains such as CD45, a protein tyrosine phosphatase which allows for the activation of Lck, and CD44 which is involved in cell-cell adhesion⁴¹.

The immunological synapse forms after cell conjugation, and reaches maturity after initial TCR signalling has started to abate. The formation of a mature immune synapse is thus important not for the triggering of the T cell response to antigen, and is instead important for controlling sensitivity to different antigens allowing adaptive control of the T cell response⁴².

The formation of the immune synapse is driven by actin rearrangement⁴⁰. The rearrangement of surface proteins into the pattern seen in the immune synapse is accompanied by other spatial rearrangement in the T cell including the protrusion of the T cell membrane over the surface of the antigen presenting cell, and the polarisation of the microtubule organisation centre and secretory organelles towards the antigen presenting cell⁴¹. DEF6 has been shown to have a role in the organization of the immune synapse via regulation of actin polymerisation, a role that is in turn regulated by the kinase Itk⁵.

1.1.4.1 The involvement of actin in T cell signalling

In addition to its signalling roles leading to control of transcriptional activation, the Tec family kinase Itk is involved in TCR-induced actin polymerisation^{43,44} and is necessary for maintenance of proper actin localisation at the immune synapse⁴⁵. This is mediated via the localisation of CDC42 to the centre of the immune synapse, a process that is in turn mediated by DEF6⁵. Itk is necessary for the recruitment of DEF6 to the centre of the immune synapse, and it is this central localisation of DEF6 that mediates the localisation of CDC42 and thus proper actin localisation in activated T cells. In addition to its Itk-mediated role

in the localisation of actin at the immune, DEF6 regulates actin accumulation independently of Itk¹.

Ultimately the spatiotemporal organization of T cell signalling promoted by Itk through DEF6 then CDC42 requires control of the location of actin polymerisation. Actin is part of the cytoskeleton, forming filaments of polymerised actin monomers that provide a strong but flexible network to provide structure and enable movement. Rapid polymerisation of large amounts of actin is observed during the formation of the immune synapse upon T cell activation and is related to full activation of the T cell^{40,46}. It both supports strong TCR-MHC interactions via the stabilisation of protein-protein interactions, and has a role in destabilising weak TCR-MHC interactions via the internalisation of TCR signalling complexes⁴⁶. The spatiotemporal regulation of actin polymerisation at the immune synapse is essential for effective function of T cells, as it is involved in several important steps upon T cell activation including preventing further T cell migration, formation of a lamellipodia to increase contact area with the antigen presenting cell, stabilisation of adhesion and reorganisation of T cell receptor micro clusters to form a well segregated immune synapse.

1.1.4.2 Itk has a role in several aspects of T cell signalling

Itk is a Tec family tyrosine kinase highly expressed in T cells⁴⁷ that localises to the immune synapse due to the activation of phosphoinositide 3-kinase (PI3K) which causes the accumulation of phosphatidylinositol (3,4,5)-triphosphate (PIP₃) to which Itk binds⁴⁸. Here Itk is activated by phosphorylation by a Src-family tyrosine kinase, Lck⁴⁹.

Itk has a role in several of the signalling pathways necessary for proper T cell responses upon activation. Phosphorylation of PLC γ by Itk results in the production of the secondary messengers IP₃ and DAG³¹ production of which involves Itk in regulating transcriptional activation, Ca²⁺ flux and release of

cytokines. These processes ultimately regulate T cell development and the resolution of the immune response⁵⁰. IP₃ for example is necessary for the release of Ca²⁺, which allows long term T cell signalling and promotes the formation and maintenance of the immune synapse⁵¹, whilst both prolonged calcium release via IP₃ and DAG are necessary for ERK signalling leading to translocation of NFAT to the nucleus. Without phosphorylation events by Itk controlling the location and timing of these processes, maintenance of robust TCR signalling and proper T cell response would be defective. Itk knockout mice demonstrate defects in T cell activation⁵², defective production of a T_H2 cell response⁵³, decreased susceptibility to activation induced cell death (AICD)³¹, and aberrant production of IL-17A in T_H17 cells⁵⁴. Similarly, inactivation of the Def6 gene leads to resistance to apoptotic cell death⁴ and aberrant IL-17 production¹⁰. In mice lacking DEF6, these defects have been linked to autoimmune diseases including lupus and rheumatoid arthritis.

1.2 DEF6 is implicated in several autoimmune disease states

An effective T cell immune response requires both the correct response to activation such as activation of phagocytic cells or induction of antibody production, and an effective end to the response via the elimination of responding T cells through activation induced cell death (AICD)⁵⁵. Autoimmune diseases result through defects in these processes. In models in mice where DEF6 has been inactivated through retroviral insertion, loss of DEF6 has been shown to lead to a variety of defects in the immune response that paradoxically both lead to, and provide resistance from several autoimmune diseases.

Mice in which the DEF6 gene was inactivated all originated from embryonic stem cells generated by a gene trap strategy by Lexicon Genetics. Different groups have referred to these mice as either DEF6^{trap/trap} or as DEF6^{-/-}. These mice have been bred onto several different background strains of mice; first the Pernis group used the DEF6-deficient cells generated by Lexicon Genetics to generate a

DEF6^{tr/tr} mouse with a mixed 129 and C57BL/6 background⁴. This group further backcrossed these mice onto the C57BL/6 background⁵⁶, who additionally crossed them with SWAP70^{-/-} mice to create a double knockout mouse⁵⁷. The Pernis group further backcrossed the mixed 129 C57BL/6 background mice into both Balb/c mice and C57BL/6 mice¹⁰. The Balb/c DEF6^{tr/tr} mice were then crossed to DO11.10 TCR transgenic mice, whilst the C57BL/6 mice were crossed with IRF4^{-/-} mice to produce a double knockout. The Altman group meanwhile fully backcrossed the mice to C57BL/6^{8,9,11}, and termed them DEF6^{-/-}.

The Pernis group observed Lupus like symptoms and rheumatoid arthritis like symptoms, indicating that DEF6 protects against these autoimmune diseases^{4,10,57}. These symptoms were accompanied by an increase in the production of the cytokines IL-21 and IL-17, which was related to the loss of control of the transcription factor IRF-4 which can be bound by DEF6. An accumulation of memory and effector T cells was also observed and linked to an acquired resistance to apoptotic death due to the loss of DEF6. On the other hand, the Altman group observed results indicating that DEF6 causes autoimmune disease, using mice primed to develop inducible experimental autoimmune encephalomyelitis (EAE)⁸ or experimental autoimmune uveitis (EAU)⁹, which did not develop the symptoms when the DEF6 gene was inactivated. This was accompanied by a decrease in the production of the cytokines IL-2 and IFN γ , and a reduction in the responsiveness of lymphocytes.

These seemingly opposing results potentially point to a lack of DEF6 leading to both hyper and hypo responsiveness in cells and a generalised dysregulation in T cell response, which could lead both to the appearance of spontaneous autoimmune disease, and the resistance to attempts to induce autoimmune disease. However, the Altman group questioned the genetic background of the mice used by the Pernis group⁵⁸, as interactions between 129 and C57BL/6 strains have been shown to induce lupus. This is why the Altman group

backcrossed their mice for several generations, and they did not observe any development of lupus in their C57BL/6 DEF6^{tr/tr} mice¹¹. In response, Pernis indicated it was necessary to use a strain of mice predisposed to developing lupus with autoimmune inducing gene deletions, as development of the disease relies on interactions with strain specific loci^{59,60}. The Pernis group in fact agree with the findings on fully backcrossed C57BL/6 DEF6^{tr/tr} mice in their paper of 2012⁵⁷ in which knocking out SWAP70 in addition to DEF6 is necessary for the development of the lupus like syndrome.

Further work is required to fully tease out the role of DEF6 in autoimmune disease, particularly the way in which it functions in different genetic backgrounds. Differential interactions with components of the TCR signalling pathway due to genetic differences may give a clue to extra interactions DEF6 may be involved in that could lead to disease states, as its role in regulating IRF4 may well not be the only mechanism through which DEF6 regulates the immune response and autoimmunity.

1.2.1 Cells from DEF6-deficient mice show a variety of impairments due to loss of DEF6 expression.

In studies from the Altman lab, a reduction in size and cellularity of the thymus is seen in DEF6 deficient mice with proportional reductions in the number of all thymic subsets¹¹. Cells from mice deficient in DEF6 are noted to be inferior in their response to stimulation from immunising antigens than cells from WT mice, with lower rates of proliferation and additionally secreting lower levels of IFN- γ and IL-17^{8,9}. This lower secretion of IFN- γ and IL-17 indicates an impairment in the function or differentiation of T_H1 and T_H2 cells^{8,11}. However, studies from the Pernis lab demonstrated no difference in size of lymphoid organs in DEF6 deficient mice versus WT mice, and no difference in lymphoid development. They did however confirm the decreased ability of T cells from DEF6 deficient mice to produce IFN- γ and other cytokines⁴. DEF6 is required for T_H17

differentiation, with impaired T_H17 differentiation in T cells from DEF6 deficient mice⁸. Poor cellular responses seen in DEF6-deficient mice is not due to an increase in the activity of T_{reg} cells^{8,9}. The proportion of FoxP3 positive cells is the same in both cells from DEF6 deficient mice and from WT mice^{8,9}. Dendritic cells from DEF6 deficient mice are unaffected, and resemble cells from WT mice in their ability to present antigen^{8,9}.

TCR induced actin polymerisation is defective in DEF6 deficient mice, which impairs the formation of the immunological synapse⁴. Initial recruitment of ZAP-70 and LAT is the same as in cells from WT mice, but there is a decreased accumulation at later time points pointing to a requirement for DEF6 to properly maintain the immunological synapse⁴. This is due to impaired actin polymerisation upon stimulation which is normally mediated via activation of Rac2⁴.

T cells from DEF6 deficient mice are resistant to death, with an increased accumulation of effector/memory T cells in ageing mice deficient in DEF6⁴. This is despite the decreased proliferative response of these cells, suggesting that the accumulation is due to a defect in apoptosis. This effect is due to defective apoptosis via a cell autonomous pathway, as Fas and FasL expression on T cells was not effected by a lack of DEF6 expression⁴.

1.2.2 Potential mechanisms for the contribution of DEF6 to autoimmunity

The work by the Pernis group indicates that DEF6 regulates the production of IL-17 and IL-21 via regulation of IRF-4^{10,57}. Whilst these results were called into question by the Altman who reported a decrease in the production of these cytokines in cells lacking DEF6, the work by the Pernis group include mechanistic studies separate from the mouse studies, removing the issue of mouse background from the question^{10,57}.

The regulation of the production of IL-17 and IL-21 is important due to their role in regulating the inflammatory responses in tissue⁶¹. IL-17 stimulates the recruitment of inflammatory cells by promoting the production of chemokines⁵⁶ and induces the formation of proinflammatory cytokines such as TNF- α and IL-1¹⁰, enhancing inflammation. Meanwhile IL-21 is produced by TH17 cells and promotes further TH17 differentiation in a positive feedback loop as it drives the production of IL-17⁶², and fuels the homeostatic expansion of auto reactive T cells⁵⁶. Deregulation of their production has been implicated in autoimmunity¹⁰ in particular in rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and psoriasis in which elevated levels of IL-17 and IL-21 are found⁶¹. IRF-4 is a transcription factor that regulates the production of IL-17 and IL-21. DEF6 binds IRF-4 controlling its activity in T cells, thus inhibiting and controlling the production of IL-17 and IL-21 by preventing IRF-4 from activating their promoters¹⁰. DEF6-deficiency leads to aberrant production of IL-17 and IL-21 due to enhanced targeting of their promoters by IRF-4.

1.3 The structure of DEF6 and its relation to its known functions

Currently, no structure is known for DEF6 or for any of its constituent domains. DEF6 has been divided into 3 domains based on limited homology to other proteins, particularly SWAP70^{1,3}. These are the N-terminal domain, the Pleckstrin homology (PH) domain and the Dbl-homology like (DHL) domain.

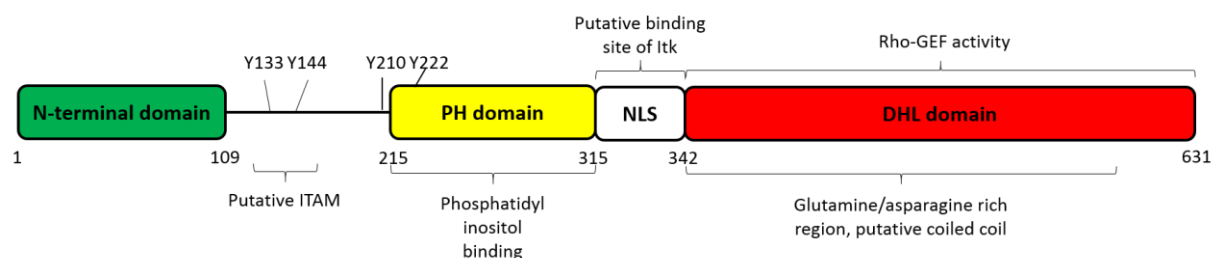


Figure 8 - DEF6 is made up of 3 main domains, the N terminal domain about which little is known, a PH domain, and at its C-terminus a DHL domain. The organisation of the domains with the PH domain N-terminal to the DH domain is highly unusual.

At the N-terminus is a region from 1-109, the function of which is unknown. It has been termed an EF-hand¹ although no evidence exists suggesting an ability bind calcium. Here it is referred to simply as the N-terminal domain.

Between the N-terminal domain and the PH domain is a stretch of 114 amino acids with no known function but with 42% sequence similarity to SWAP70. This region does however contain a putative ITAM at which it is phosphorylated by Lck at tyrosine 133 and tyrosine 144³⁴.

The final domains are a Pleckstrin homology (PH) domain and a Dbl homology like (DHL) domain. These are domains commonly found in Rho family GTPases, although DEF6 exhibits an unusual swap in the position of the domains, with the PH domain N terminal to the DHL domain rather than a DH-PH organisation usually found in Rho family GEFs. PH domains exhibit binding of phosphoinositides^{35,63}, which has been demonstrated in DEF6, whilst the DHL domain provides DEF6 with its Rho-GEF activity¹.

The overall 3D structure of DEF6 is unknown, but Gupta *et al.* have suggested an open and closed model for DEF6 based on that for Vav1³⁵, which is activated upon release of an intramolecular inhibitory interaction by phosphorylation⁶⁴ (turner 2002). They proposed that DEF6 exists in a closed inactive form until an interaction between the C-terminus (amino acids 313-631) and the N terminal 215 amino acids is relieved by phosphorylation. Their evidence for this showed that DEF6 was unable to activate the Rho GTPases Rac1 and CDC42 unless either the N terminal 215 amino acids were removed, or the DHL domain (313-631) was present alone. They also proposed that full activation may also require binding of phosphoinositides at the cell membrane similar to the full activation of Vav1.

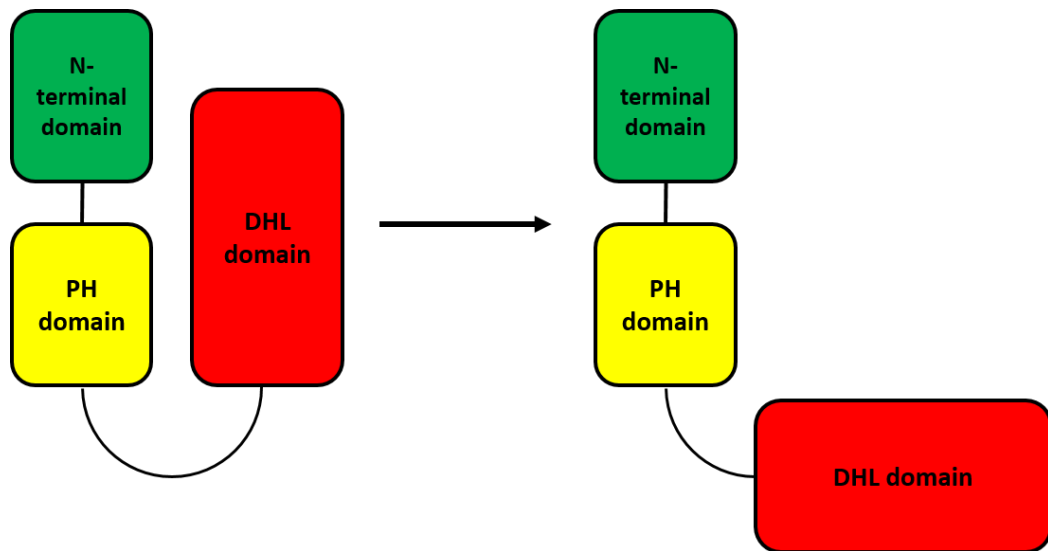


Figure 9 - It has been suggested that DEF6 may have an open and a closed form, with different interactions possible with each. One model has the DHL domain forming interactions at some other point on DEF6 to produce the closed form, which when broken allow the DHL domain to move free and open up the structure.

1.3.1 The PH domain of DEF6

Consistent with its role as a Rho-GEF, DEF6 contains a pleckstrin homology (PH) domain and a Dbl homology like (DHL) domain¹. These two domains are found in all Rho-GEF proteins as indicated in Figure 10, however in all but DEF6 and SWAP70, the PH domain is C terminal to the DH domain, whereas this organisation is switched in SWAP70 and DEF6. The reason for this organisational swap is not yet known.

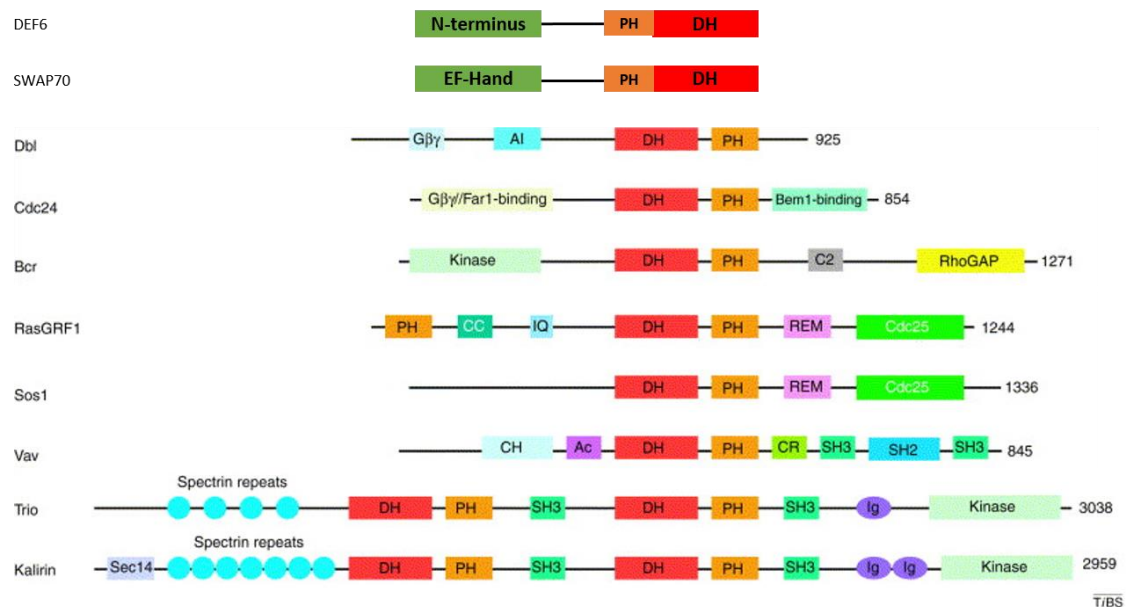


Figure 10 - Rho family GEFs may contain a wide variety of domains including kinase domains, spectrin repeats and SH3 domains which may or may not be present in any individual family member. Common to every member of the family however, are the DH and PH domains. These are normally organised with the DH domain N terminal to the PH domain, except for in DEF6 shown at the top, and in its homolog SWAP70. Diagram adapted from Zheng *et al*⁶⁵.

PH domain are protein domains that share a structural but little sequential similarity, leading to the PH domain fold being labelled as a superfold. The PH domain fold consists of a β sandwich comprised of 7 β strands, and an α helix that caps the sandwich. PH domains are found in a wide range of proteins, and can exhibit a wide range of roles including binding ligands such as phosphatidylinositol lipids, inositol phosphates and poly-proline peptides. There is also evidence for the involvement of PH domains in protein-protein interactions.

The PH domain of DEF6 encompasses amino acids 210-315, and has been shown to be phosphorylated at tyrosines 210 and 222 by the tyrosine kinase Itk¹². It has also been shown to bind $PI(3,4,5)P_3$ via cell pulldowns using $PI(3,4,5)P_3$ beads.

1.3.1.1 The regulation of DEF6 activity by tyrosine kinases

DEF6 is regulated by phosphorylation at several points by tyrosine kinases. Its phosphorylation by the tyrosine kinase Lck leads to recruitment to the immune synapse^{34,35}. This phosphorylation was thought to take place at tyrosines 210³⁵,

but has more recently been shown to take place at tyrosines 133 and 144³⁴. Phosphorylation by Lck has been shown to be necessary for localisation of DEF6 to the immune synapse, and for activation of NFAT³⁴. It has also been shown that Lck phosphorylation of DEF6 allows binding of DEF6 to PI(3,4,5)P₃³⁵, although this interaction is not necessary for the immune synapse localisation³⁴.

In addition to the phosphorylation at tyrosines 133 and 144 for Lck, Itk phosphorylates the DEF6 PH domain at tyrosines 210 and 222¹². Active Itk has been shown to be necessary for organising the central localisation of DEF6 at the immune synapse⁵. Both the central localisation of DEF6 at the immune synapse as regulated by active Itk and the phosphorylation of Y210 and Y222 by Itk is ultimately dependent on Lck activity, as phosphorylation by Itk requires its activation via phosphorylation of tyrosine 511 of Itk by Lck⁴⁹. Phosphorylation of DEF6 by Itk has also been suggested to play a role in the aggregation of exogenous DEF6 into cytoplasmic foci, similar to those involved in the regulation of mRNA translation¹². A mutant of DEF6 that mimics phosphorylation by Itk (Y210E Y222E) has been shown to aggregate into foci that co-localise with a marker for P-bodies, structures involved in the degradation of mRNA^{12,66}.

The interactions that Itk requires for phosphorylation have been studied for its autophosphorylation of an SH2 domain with its own structure, and its phosphorylation of phospholipase C γ ⁶⁷. Itk has been shown to require a binding site separate to its target tyrosine to facilitate phosphorylation. This site has been found for the phosphorylation of the SH2 domain of Itk and for the phosphorylation of phospholipase C γ , and is a contiguous patch of positively charged amino acids⁶⁷. The corresponding negatively charged binding site on Itk has been found that interacts with the binding patch on phospholipase C γ ⁶⁸. The nuclear localisation signal C-terminal to the PH domain of DEF6 (amino acids 315-342) has been identified as a possible binding site for Itk for the

phosphorylation of the PH domain due to its high number of positively charged amino acids.

1.3.2 The DHL domain of DEF6

Whilst the DH domain (amino acids 342-631) of DEF6 has been shown to contain the GEF activity, it demonstrates very little similarity to other DH domains so is referred to as the DH-like (DHL) domain. Its sequence is unusual for a DH domain in containing a higher than normal number of glutamine and asparagine residues, which are normally indicative of amyloid forming proteins⁶⁹. Whilst these regions are normally thought to form extended β -sheet structures, glutamine/asparagine rich regions have recently been shown to be able to form extended coiled coil structures⁷⁰, and the DHL domain of DEF6 has been predicted to have a high probability of forming a coiled coil structure¹². This ability to form a coiled coil structure has been identified as one potential mechanism for driving granule formation¹².

Thus the DHL domain of DEF6, in addition to being C-terminal to the PH domain and limited sequence homology, is not predicted to form the normal Rho-GEF DH domain fold.

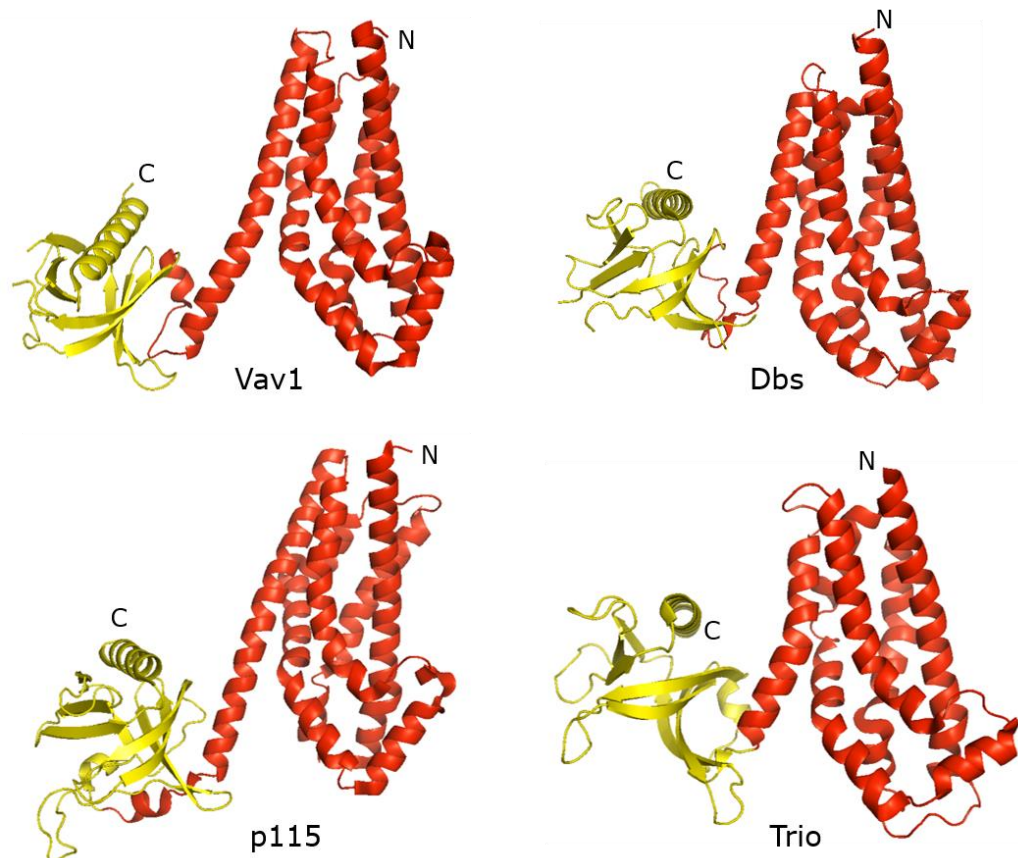


Figure 11 - Structures of the DH and PH domains of Vav1, Dbs, p115 and Trio showing the “chaise longue” structure of the N-terminal DH domain (red) and C-terminal PH domain (yellow). Structures produced using Pymol from the data held in the PDB. Structures used were 3BJI (Vav1), 1RJ2 (Dbs), 3ODO (p115) and 2N28 (Trio).

The Rho-GEF DH domain fold normally consists of helices arranged in a bundle with 6 main axes to form a “chaise longue” shape as seen in Figure 11. They function by inducing changes to the shape of the nucleotide binding pocket of the GTPase upon binding, leading to the dissociation of the bound GDP to be replaced by cellular GTP.

1.3.2.1 Rho Family GTPases and GEFs

DEF6 is a Rho family GEF, and as such acts on Rho family GTPases¹. GTPases act as molecular switches; they are inactive when bound to GDP and active when bound to GTP. GEFs activate GTPases, catalysing the release of GDP from the GTPase, which is replaced by GTP due to the high cellular concentration of GTP⁷¹. The GTPase activity of GTPases is relatively low, allowing it to be regulated by GTPase activating proteins (GAPs) which enhance the intrinsic

GTPase activity⁷¹. These regulators occur in signal transduction pathways that influence changes in cell survival and gene expression which includes the pathways downstream of the immunological synapse, for example the GEFs Vav1 and DEF6.

Rho family GTPases are activated by the Dbl family of GEFs. As noted previously, these all contain a DH and a PH domain, with the PH domain located C terminal to the DH domain. Outside these domains there very few shared domains between family members.

1.3.2.2 The role of Rho-GEFs in immune system

Several Rho GTPases are involved in T cell signalling pathways, including CDC42 and Rac1. These regulate polarity and chemotaxis in immune cells via their role in the control of actin polymerisation⁷², they also regulate T cell development and function⁷³. There are two major GEFs involved in T cell signalling upon activation, Vav1 and DEF6. Vav1 is well studied and is a conventional GEF, whilst DEF6 is relatively uncharacterised, and contains the unconventional domain swap.

Vav1 is a Rho family GEF with conventional domain organisation from the Vav family involved in the regulation of T Cell activation which activates Rac1, RhoA and Cdc42⁷⁴. Vav family proteins are activated by tyrosine phosphorylation upon TCR signalling. Vav1 is required for several processes in T cells including calcium flux, polarisation of the T cell and activation of the ERK MAP kinase pathway and NF- κ B signalling⁷⁵. T cell

1.3.3 SWAP70 is the most closely related protein to DEF6

DEF6 is closely related to SWAP70, a Rac specific GEF ⁷⁶ highly expressed in B cells, but not in T cells⁷⁷. It is strongly induced in B cells that have been induced to class switch⁷⁸, and is part of a larger multiprotein SWAP complex that catalyses DNA recombination between immunoglobulin heavy chain gene switch

region substrates⁷⁷. SWAP70 shares the PH-DH domain swap present in DEF6, and its DH domain also shares very little homology with other DH domains.

SWAP70 is described as having a putative N-terminal EF hand, similar to that once thought to exist in DEF6. Its PH domain is similar in sequence to that of DEF6, and has been shown to bind phosphatidyl inositol lipids and to be necessary for membrane localisation of SWAP70. The C-terminus of SWAP70 has limited homology with other DH domains, and has confirmed GEF activity⁷⁹. The C-terminus additionally contains a tripartite coiled coil region, and an actin binding region⁸⁰. A structure is known for the PH domain of SWAP70, and indicates the normal expected structure of 7 sheets and a capping helix⁸¹. This structure will be used in this work to model the structure of the PH domain of DEF6.

Some mutational analysis has been done on SWAP70, the results from which may be relevant to the structure/function analysis of DEF6. Three sets of mutations have been made in the PH domain, which affect the ability of SWAP70 to bind phosphatidyl inositol lipids and the plasma membrane⁸². These are in areas that may be conserved on the PH domain of DEF6, and are discussed in section 4. A similar mutation has been shown to abolish phosphatidyl inositol binding in the PH domain of DEF6, highlighting the similarities between this domain in these two proteins⁶³. Removing 60 amino acids from the C-terminus of SWAP70 has been shown to affect its ability to target to loose actin filament arrays⁸².

Whilst DEF6 is predominantly expressed in T cells, it is also found in B cells, and mediates control of IRF-4 along with SWAP70 in a non-redundant manner. Rather, SWAP70 regulates plasma cell differentiation whilst DEF6 regulates IL-21 responsiveness of antigen engaged B cells⁵⁷. Thus, DEF6 regulates the early stages of activation, and SWAP70 is required for control of B cell differentiation.

Invertebrates have only one *def6/swap70* gene suggesting a single ancestral gene before vertebrate/invertebrate speciation⁸³. The present *swap70* gene is closer to the ancestral *def6/swap70* than *def6*, and is the only known DH domain-containing GEF found in plants⁸⁴ where it regulates the plant immune response⁸⁵.

Whilst deficiency in DEF6 leads to a variety of autoimmune diseases, and leads to early death of mice, SWAP70 knockout mice develop normally and are fertile and healthy⁸⁶. However, some defects are seen upon induced inflammation, with reduced migration of B cells into the lymph nodes. This is due to defective polarisation and formation of actin-rich structures leading to defective regulation of integrin mediated adhesion⁸⁷.

1.4 mRNA translation can be regulated via cytoplasmic foci

DEF6 has been shown to form cytoplasmic foci that co-localise in granules with the protein DCP1¹², which is a marker for a class of mRNP granule called processing bodies.

mRNA is associated with messenger ribonucleoproteins (mRNPs) from transcription to degradation. Under certain conditions, these mRNA and mRNPs can aggregate to form non-membrane bound cytoplasmic bodies that have a range of roles, from storage of the mRNA, to degradation via inclusion of proteins involved in mRNA degradation in the granule providing an important mechanism of post-transcriptional gene control⁸⁸⁻⁹⁰.

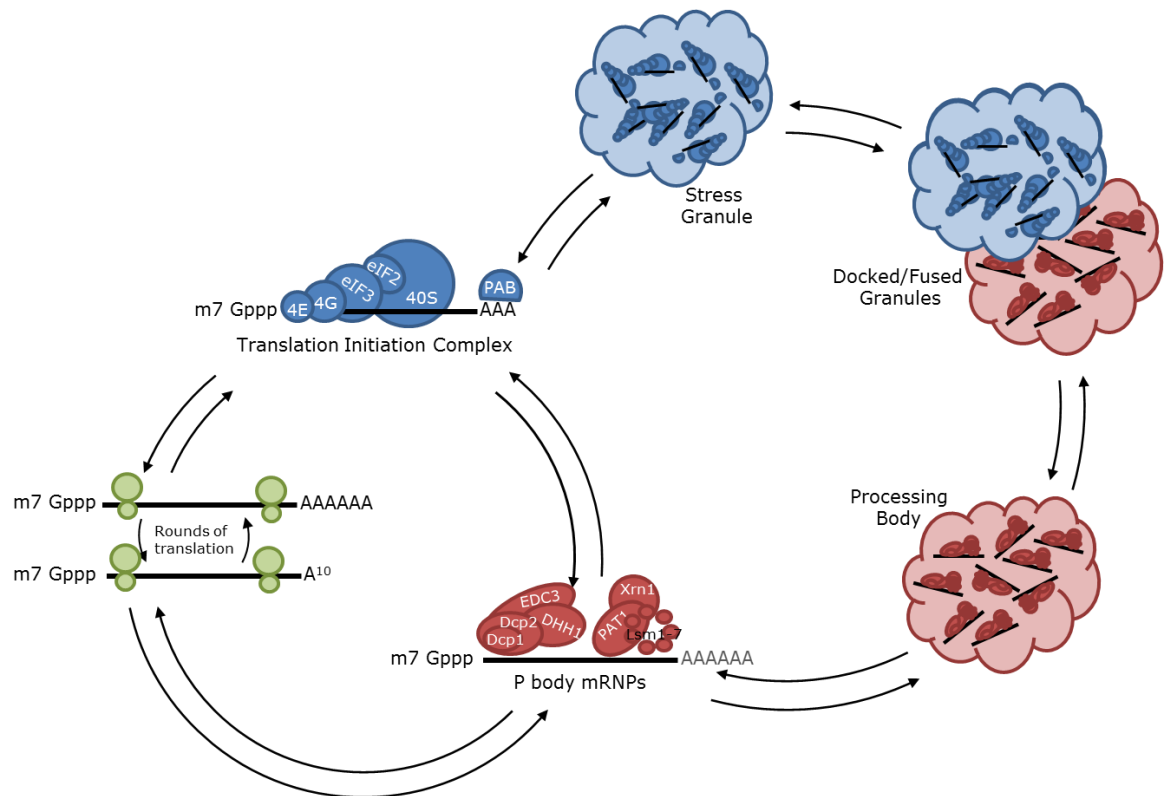


Figure 12 - The mRNA cycle illustrating the link between actively translating mRNA on polysomes, stalled initiation complexes and stress granules and P-bodies. mRNA is assembled into a translation initiation complex with mRNPs prior to entering translation. Stalling of translation leads to stalled initiation complexes which can aggregate to form stress granules⁹¹. mRNP aggregates can also form with protein involved in mRNA degradation and processing to form processing bodies (P-bodies)⁶⁶. Both types of aggregate are thought to be able to release mRNA back into the translation pool, and to be able to dissolve⁹². The two types of granules are also able to interact, potential swapping components and mRNA between them⁹³.

There is a wide range of cytoplasmic mRNP granules the best characterised of which are the stress granule and the processing body (P-body)^{66,89,94}. Broadly, stress granules contain components of stalled translational machinery and initiation complexes, whilst P-bodies contain proteins involved in the degradation of mRNA. These structures can be found constitutively in cells, but their formation is increased upon conditions that cause global arrest of translation, such as glucose starvation or heat shock⁹⁵. It is thought that these structures allow for rapid changes to the pool of translating mRNA, preventing proteins not necessary under stress conditions from being translated and allowing up-regulation of the translation of necessary proteins. Stress granules can release up to 50% their stored mRNA back into the cytoplasm^{93,96,97}, allowing translation

to restart on these mRNAs. In contrast, P-bodies are believed to act as sites of RNA degradation of their stored mRNA, suggesting targeting of mRNAs to different. The ability to return mRNA to the translating pool after storage provides a way to temporarily pause and then restart translation in response to rapid changes in conditions⁹⁸. It has also been suggested that mRNA can be passed between the two granules, and in some cases it appears that different types of granules may be able to merge⁹³.

The role of these granules appears to be linked to the regulation of protein expression under stress conditions that promote global arrest of translation. Supporting this, certain mRNAs have been shown to be excluded from granules including that encoding heatshock protein 70, allowing its translation during stress when it is needed despite the sequestration of other transcripts. This is possible via an internal ribosome entry site (IRES) and is not dependent on 5' cap recognition. The granules could contribute to the stress response either by sequestration of housekeeping transcripts, thus conserving energy during stress, or they may sequester more specific transcripts to actively remodel the transcriptome⁹⁹. Other potential methods of regulation via sequestration of mRNA into granules include their asymmetric segregation into daughter cells by polarisation of the granules to one end of the cell as is the case in germ granules found in germ cells¹⁰⁰.

The DEF6 granules have been shown to co-localise with P-bodies, indicating a role for DEF6 in regulation of translation. This study will further investigate the interaction of DEF6 with these regulatory structures, including the identification of other proteins found in DEF6 granules, and the dynamics of DEF6 protein exchange between the protein and the cytoplasm.

1.4.1 Structural features of proteins involved in granule formation

Proteins found in P-bodies and stress granules contain a wide range of domains, but most are related to RNA binding due to the usual roles of the proteins found

in these structures. RNA recognition motifs (RRMs) are present in many of the proteins, and the binding of RNA is often necessary for the recruitment of a protein to P-bodies or stress granules. This suggests that many proteins end up recruited to granules due to binding of RNA present in the granules, or due to binding of RNA that then gets recruited to them rather than via specific recruitment of the protein itself. Explanation of the mechanisms by which domains that drive the interactions between proteins into these granules have depended largely on the discovery that many of the proteins involved in P-body or stress granule aggregation contain glutamine and asparagine-rich regions, hallmarks of prion-like proteins^{101,102}. This suggests a prion-like aggregation method for condensation of stress granules. Prion-like proteins tend to aggregate via amyloid formation of insoluble fibrils with cross beta sheet quaternary structure. Further to the anti-parallel packing of prion-like units, the capping complex associated protein EDC3 requires an Sm domain for its localisation to P-bodies. An Sm domain is able to form oligomers via antiparallel interactions between the edge beta strands of each subunit^{101,103}. Amyloid like packing is however unlikely to be the mechanism for packing with DEF6, which although it contains a Q/N rich region, has been predicted to be highly helical, with a predicted coiled coil region within its Q/N rich region¹². This project aims to investigate this predicted coiled coil forming region by the use of both purified protein in biophysical studies and GFP tagged proteins in cellular studies to further understand the mechanism of the involvement of DEF6 in the formation of cytoplasmic granules.

1.4.2 There is potential for a role in the immune system for RNP granules and post-transcriptional regulation of protein expression.

Post-transcriptional regulation of gene expression has been identified to control many key 'decisions' of innate and adaptive immune responses^{104,105}.

Dysfunctional mRNA decay may lie at the core of the pathogenesis of autoimmune disease¹⁰⁶⁻¹¹⁰.

There is evidence linking P-bodies and stress granules to the control of translation of mRNA in T cells, for example many cytokine mRNAs contain AU-rich elements (AREs) in their 3' untranslated regions (3'UTRs)⁹⁰. These elements mediate rapid degradation of the mRNA to inhibit gene expression at the post-transcriptional level and mRNAs containing these elements are associated with P-bodies. Additionally, during CD4⁺ T cell differentiation the integrated stress response modulates protein synthesis by integrating stress signals leading to the phosphorylation of eIF2 α , a process that blocks translation and induces stress granule formation. Stress granules sequester TRAF2 during the stress response, thus inhibiting TNF signalling and activation of NF κ B. Furthermore, many stress granule components are more prominent in primed T cells¹¹¹. The formation of DEF6 into foci that interact with P-bodies and its expression predominantly in T cells may suggest a role for DEF6 in the regulation of mRNA translation in addition to its previously discovered roles, and this could contribute to the regulation of the T cell immune response.

Although a trigger is needed to initiate the autoimmune response, the severity of the disease is determined by the duration and intensity of the immune response by the T cells including the release of cytokines. This response is controlled by gene expression at multiple levels¹¹², potentially including control via regulation of mRNA translation via P-bodies and stress granules. The involvement of DEF6 in these granules suggests both a novel role separate to its GEF activity and binding of IRF4 and additionally a potential new avenue for regulation of autoimmune disease through the regulation of translation in DEF6-associated granules. To further investigate this potential link between DEF6 granule formation and its role in autoimmune disease, this study will examine the

localisation of endogenous DEF6 in recently activated T cells, in particular its ability to form granules in an endogenous and relevant system.

1.5 Aims and objectives

The aims of this project are to develop an understanding of the formation of granules by DEF6 at a molecular level, and determine whether granule formation is relevant in its role in immune regulation. Experiments furthering the work of Hey *et al*¹², in investigating the co-localisation of DEF6 with known components of P-bodies and stress granules will serve to further understand the composition of DEF6 granules. The dynamics of DEF6 movement both in and out of the granules and of the granules themselves will build a picture of what the role of DEF6 is in the granules, and allow comparisons with the properties of better characterised cytoplasmic granules, such as P-bodies and stress granules, to be made. Further to this, the precise involvement of the different domains of DEF6 in granules formation will be investigated to gain a fuller picture of their roles, and bring us nearer to an understanding of the mechanisms of DEF6 activity. To this end the phosphorylation of the PH domain will be investigated, in particular whether the nuclear localisation signal is necessary or phosphorylation by Itk. The dissection of the domains and their ability to form foci on their own will also be probed to elucidate which domains are able to drive the formation of the DEF6 foci, and how.

The localisation of DEF6 to the immune synapse which has previously been demonstrated using antibody coated beads will be investigated using Raji B cells as a better mimic of a physiologically relevant system (Chapter 3). This system will also be used to investigate the formation of DEF6 granules in T cell from endogenous DEF6, and explore the formation of other mRNA regulatory granules in activated T cells.

Due to the proposed link between DEF6 granule formation and the phosphorylation of the PH domain by Itk, this phosphorylation event will be

investigated (Chapter 40). The binding patch for Itk recognition will be explored using purified overexpressed protein.

The details of how DEF6 forms granules will be studied by determining the specifics of which domain drives its aggregation into formation, and by calculating the movement of DEF6 in and out of granules and of the granules themselves to compare with other granule forming proteins (Chapter 5). This work will be continued in chapter 6 in which the details of the aggregation domain and the involvement of the predicted coiled-coil forming glutamine/asparagine rich region will be investigated to determine the mechanism of DEF6 granule aggregation using both cellular and biophysical techniques.

2.Materials and Methods

2 Materials and Methods

2.1 Nucleic Acid Techniques

2.1.1 Plasmid extraction

E.coli containing the plasmid of interest was grown overnight in 5ml LB containing 100µg/mL ampicillin or 30µg/mL kanamycin. The culture was spun down at 3000g and the DNA extracted using a Geneflow Q-Step Plasmid Purification Kit following the manufacturer's instructions.

2.1.2 Sequencing

DNA was sent to the University of Nottingham DNA Sequencing Facility for sequencing.

2.1.3 Restriction endonuclease digestion

DNA was digested using restriction enzymes (Promega) in the recommended reaction buffer. Reactions contained:

- 1x reaction buffer
- 1.5µg DNA
- 10 units of each restriction enzyme
- H₂O to 20µL

Reactions were incubated at 37°C for 1 hour.

2.1.4 Agarose gel electrophoresis

Agarose gel was prepared by dissolving agarose (Melford) in TAE buffer. Gels were cast in an 8cm Horizon 58 horizontal gel electrophoresis apparatus. 5x TAE loading buffer was added to the sample. Samples were loaded into gel alongside a suitable DNA ladder (100bp ladder, 2-Log DNA ladder or PCR marker (NEB)). The gel was run at 100mA for 1 hour. The gel was incubated in ethidium bromide and visualised under UV light. Pictures were taken using Aida Image Analyser.

Solutions

TAE buffer: 40mM Tris, 0.1%(v/v) glacial acetic acid, 1mM EDTA pH 8.0

5x TAE loading buffer: 10x TAE buffer, 50%(v/v) glycerol, bromophenol blue

2.1.5 DNA extraction and purification from agarose gel

DNA ran on an agarose gel was viewed on a UV transilluminator and the relevant band excised from the gel. DNA was extracted using the QIAquick Gel Extraction Kit from Qiagen, following manufacturer's instructions.

2.1.6 DNA ligation

Linearised plasmid and DNA fragments were ligated using the overhangs created using appropriate restriction digests (2.1.3) followed by separation by electrophoresis (2.1.4) and extraction (2.1.5). Ligation reactions were set up as follows:

- 2µL 10x T4 DNA ligase buffer (Promega)
- 100ng plasmid
- 50ng fragment
- 1µL T4 DNA ligase (Promega)
- H₂O to 10µL total volume

The reaction was incubated overnight at 15°C. 5µL of the reaction was used to transform chemically competent cells.

2.2 Polymerase Chain Reaction

2.2.1 Nucleic acid amplification by PCR

PCR reactions were set up as follows:

- 10ng DNA template
- 0.5µM final concentration each primer
- 200µM dNTP
- 3u Pfu DNA polymerase (Promega)
- Pfu 10 x buffer (Promega)

PCR was carried out under the following thermal cycling conditions using a SensoQuest labcycler:

Step	Temperature	Duration	Cycles
Initial denaturation	95°C	2 minutes	1
Denaturation	95°C	1 minute	28
Annealing	60°C	30 seconds	
Extension	72°C	2 minutes	
Final extension	72°C	5 minutes	1

2.2.2 Site-directed mutagenesis

Two positive PCR reactions were set up as follows:

10ng DNA template
 125ng each primer
 200µM dNTP
 3u PfuUltra HF DNA polymerase (Agilent)
 PfuUltra HF 10 x buffer (Agilent)

H₂O to total volume of 49.6µL

An additional reaction with no template was set up as a negative control, with H₂O increased to account for the reduction in volume.

PCR was carried out under the following thermal cycling conditions using a SensoQuest labcycler:

Step	Temperature	Duration	Cycles
Initial denaturation	95°C	1 minute	1
Denaturation	95°C	50 seconds	18
Annealing	60°C	50 seconds	
Extension	68°C	7.5 minutes	
Final extension	72°C	7 minutes	1

0.5µL *Dpn1* restriction endonuclease (20U/µL) (NEB) was added to one of the two positive PCR reactions containing template, mixed gently and incubated at 37°C for 1 hour. The 50µL PCR reaction was quenched by heating at 60°C for 20 minutes. 5µL was used to transform chemically-competent NEB-5α *E.coli* according to manufacturer's protocol and spread on plates.

2.2.3 Site-directed deletion

Deletions were made using the Q5 Site Directed Mutagenesis Kit (NEB).

Reactions were set up as follows:

12.5µL Q5 High Fidelity 2x master mix

0.5µM final concentration each primer

25 ng DNA template

H₂O to total volume of 25 µL

PCR was carried out under the following thermal cycling conditions using a SensoQuest Labcycler:

Step	Temperature	Duration	Cycles
Initial denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	25
Annealing	72°C	30 seconds	
Extension	72°C	30seconds/kb	
Final extension	72°C	2 minutes	1

1µL of the PCR product was added to 5µL KLD reaction buffer, 1µL 10x KLD enzyme mix and 3µL H₂O and incubated at room temperature for 5 minutes. 5µL was used to transform chemically-competent NEB-5α *E.coli* according to manufacturer's protocol and spread on plates.

2.2.4 Primers Used

Primer name and function	Sequence
Q371P_A374P (CCm1) Encoding the sequence EAPRQPER amino acids 369-376	5'- GGA GGC GCC GCG GCA GCC CGA GCG G -3'
I428P_L431P (CCm2) Encoding the sequence QRQRPKEPEEM amino acids 424-434	5'- CAG CGG CAG CGC CCC AAG GAG CCG GAG GAG ATG CA -3'
L470P_L473P (CCm3) Encoding the sequence EEEEKPKQPMQLKE amino acids 465-478	5'- GAG GAG GAA GAG AAG CCG AAG CAG CCG ATG CAG CTG AAG GAG -3'
L512P_V515P (CCm4) Encoding the sequence QQQPEEPRQN amino acids 509-518	5'- CCA GCA GCA GCC GGA GGA GCC GCG GCA GAA CC -3'
Δtrigger Deletion of amino acids 442-455, LQLEVKARRDEESV.	Forward: CGAATCGCTCAGACCAGACTG Reverse: GGCCTCCTGCAACCGCTG
109 STOP Replacing G109 with a stop codon	5' – CTA TCG GGC AGA TAG CAA CTA GAA CAGTAT GCT CTC CAA T – 3'
PH domain start	CGGATCCGCTGCAGGCCGAGGGGAAGACGTCC
PH domain + NLS	GAATTCCTCTTACTTGGCCGCCCGGCG
PH domain - NLS	GAATTCTTAGGCCTGCAGCCGGATCGC
CC Start	GGATCCCTGCAGGCCGAG
CC end	GAATTCCTTACAGCCGGTTC

2.3 Bacterial Culture

2.3.1 Strains

The *E.coli* strain C41 was used for expression of protein for purification. It is derived from the BL21 strain, and contains DE3, a λ prophage which contains both lacI and the T7 RNA polymerase gene. It can be grown and expresses protein at low temperatures, allowing for a reduction in the rate of expression of protein which often increases solubility.

For production of large amounts of DNA for eukaryotic transfections, the *E.coli* strain TG1 was used.

2.3.2 Making calcium competent *E.Coli*

The *E.coli* strain was grown overnight at 37°C in an incubator shaker in 10mL Luria Bertani (LB) broth. The next day the overnight culture was diluted to 1/100 in 10ml LB and grown for ~90 minutes at 37°C until an OD of 0.6 was achieved as measured using a Biophotometer (Eppendorf) at 595nm. Once this was reached, the cells were pelleted at 3000g for 10 minutes using a bench top centrifuge. The cells were re-suspended in 5mL ice cold 0.1M CaCl₂, then re-pelleted as before. The cells were then re-suspended in 0.5mL ice cold 0.1M CaCl₂.

Solutions

LB broth: 1% (w/v) bactopectone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl

2.3.3 Bacterial transformation

50-100ng DNA was added to 100µL cells and incubated on ice for 30 minutes. The cells were then heat shocked at 42°C for 90 seconds, then returned to ice for 30 minutes. The cells were added to 1mL of LB broth in a universal and incubated for an hour at 37°C in the incubator shaker. LB-agar ampicillin plates were then spread with the cells.

Solutions

LB-agar: 1.5% (w/v) Agar dissolved in LB broth

2.4 Expression and purification of recombinant protein

2.4.1 Induction and expression of recombinant protein

The *E.coli* strain containing the plasmid for the relevant protein was inoculated overnight in 20ml LB broth at 37°C in an incubator shaker. The next day 1L LB broth was inoculated with the overnight culture to a dilution of 1/50 of the overnight culture. The culture was grown for approximately ~90 minutes at 37°C until an OD of 0.3 was achieved as measured using a Biophotometer (Eppendorf) at 595nm. The flasks were moved to an incubator shaker at 20°C and grown until an OD of 0.4 was achieved and the culture had cooled to 20°C. The culture was induced to start producing protein by the addition of IPTG to a final concentration of 0.5mM to the flask, which was returned to the 20°C incubator shaker for 48 hours.

2.4.2 Harvesting of protein

The culture was then pelleted by centrifugation at 3000g for 10 minutes, and transferred to a 50mL universal using lysis buffer to re-suspend the pellet. This was then spun at 3000g for 10 minutes, and the pellet stored at -80°C.

Solutions

Lysis Buffer: 20mM potassium phosphate pH 7.4, 150mM NaCl

2.4.3 Purification of protein

The pellet from 2.4.2 was thawed and re-suspended in 8mL lysis buffer, protease inhibitor and DNase. The re-suspended pellet was sonicated 30s on 30s off 5x at 4°C to lyse cells. Lysate was pelleted by centrifugation at 30,000g for 30 minutes, and then the supernatant re-pelleted at 35,000g for 30 minutes.

Solutions

Lysis Buffer: 20mM potassium phosphate pH 7.4, 150mM NaCl

2.4.4 His tag purification

Recombinant protein with a His₆ tag was purified using cobalt beads. After sonication and clearing spins, the soluble fraction was incubated with beads for 30 minutes with end-over-end mixing. The column was drained, and washed with 1 column volume of lysis buffer. Imidazole was used to elute protein at a range of concentrations.

2.4.5 GST tag purification

Recombinant protein with a GST tag was purified using glutathione sepharose beads. After sonication and clearing centrifugation, the soluble fractions was incubated with beads for 30 minutes at 4°C end over end. The column was drained, and washed with 1 column volume of lysis buffer. Thrombin was diluted in 1mL lysis buffer and added to the column and incubated for 48hr at 4°C end over end. Protein was collected from the column, and an extra 1.5mL of lysis buffer used to flush through.

2.4.6 Desalting

A HiTrap Desalting column (GE) attached to an Akta Prime was used to desalt the protein. The column was washed with degassed H₂O to remove ethanol. 2M NaCl was run through the column to remove any traces of other protein. For certain purifications the buffer was switched from H₂O to 25mM ammonium acetate (PH domain protein was desalted in ammonium acetate, coiled coil purified protein was desalted in H₂O). When the salt reading evened out, the protein was applied to the column using a 5ml loop. The column was run at 0.5ml/min and 7mL fractions collected. The samples corresponding to absorbance peaks on the trace were lyophilised, with salty fractions separated for further desalting.

2.4.7 Lyophilisation

Samples were lyophilised by freeze-drying on a Micromodulyo (Thermo) for two days. Samples desalted in ammonium acetate were re-suspended in H₂O and the process repeated. Samples were then stored at -20°C.

2.5 Protein Techniques

2.5.1 SDS-PAGE

SDS-PAGE was used to visualise protein presence and purity. A biorad kit was used to make the gels, using 15% resolving polyacrylamide gel and stacking gel. Samples were mixed with SDS loading buffer and heated for 5 minutes at 90°C before being loaded onto the gel. Samples were run at 35mA per 2 gels for .75mm gels and 70mA per 2 gels for 1.5mm gels for 1 hour. Protein markers were run along with the samples to aid band recognition. After being run, the gel was placed in a tray and stained with coomassie stain for 1 hour. The coomassie stain was then removed and the gel de-stained. The gel was imaged using Gene Genius Bio Imaging System and GeneSnap software from SynGene.

Solutions

15% resolving gel: 15% (v/v) Protogel acrylamide mix, 0.375M Tris (pH8.8), 0.1% (w/v) SDS, 0.1% (w/v) APS, 1% (v/v) TEMED

Stacking gel: 5% (v/v) Acrylamide mix, 0.125M Tris (p6.8), 0.1% (w/v) SDS, 0.1% (w/v) APS, 1% (v/v) TEMED

5x SDS loading buffer: 0.35M SDS, 50% (v/v) glycerol, 0.5M Tris (pH6.8), 20% (v/v) β-mercaptoethanol, pinch bromophenol blue to colour.

SDS running buffer: 14.4 g/L glycine, 3g/L Tris, 1g/L SDS

Coomassie Stain: 50% (v/v) Methanol, 0.25% (w/v) Coomassie blue R, 10% (v/v) acetic acid

Coomassie Destain: 30%(v/v) Methanol, 10%(v/v) Acetic Acid

2.5.2 Western blotting and immunodetection

PVDF membrane was incubated in 100% methanol for 20 seconds, then washed in H₂O for 5 minutes with agitation, then in transfer buffer for 5 minutes with agitation. Proteins were transferred to the membrane from a resolved SDS-PAGE gel using a 20cm x 20cm semi dry blotter (Geneflow) for 1 hour at a constant current of 480mA. The membrane was then removed and incubated with blocking solution overnight. The membrane was then incubated with primary antibody diluted in blocking solution at the appropriate dilution (see section 2.10) for 1 hour at room temperature with agitation. The membrane was washed in PBST 3x with agitation then incubated with a horseradish peroxidase conjugated secondary antibody in incubating solution for 40 minutes at room temperature with agitation (see section 2.10). The membrane was washed in PBST 3x with agitation. The membrane was blotted dry and placed on saran wrap and incubated with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) for 2 minutes at room temperature. Excess chemiluminescent substrate was removed and the membrane wrapped in saran wrap and placed in an autoradiography cassette. In a darkroom X-ray film (Kodak) was exposed to the membrane for 30 seconds – 1 hour depending on signal strength. The membrane was then hand developed in PQ Universal Paper Developer and fixed in 2000RT Fixer (both Ilford UK) before being rinsed in H₂O and left to dry.

Solutions

PBS: 8g/L NaCl, 0.2g/L KCl, 1.44g/L Na₂HPO₄, 0.24g KH₂PO₄

Transfer Buffer: 25mM Tris, 192mM glycine, 10% (v/v) methanol

Blocking solution: 5% (w/v) marvel milk powder, 0.01% (v/v) Tween20 in PBS

Incubating solution: 3% (w/v) marvel milk powder in PBS

PBST: 0.01% (v/v) Tween20 in PBS

2.5.3 Cyanogen Bromide Coupling

Cyanogen bromide-activated sepharose beads (Sigma) were rehydrated in a suction funnel with gentle suction with 200mL cold 1mM HCl. The resin was washed with 10 column volumes distilled H₂O followed by 5mL/g dry gel coupling buffer. Following this, the resin was immediately added to a solution of 5-10mg of the protein of interest per 1mL gel dissolved in coupling buffer. Protein and resin were mixed end over end overnight at 4°C or 2 hours at room temperature. The resin was pelleted by centrifugation at 1000g and the supernatant discarded. The resin was washed with 10mL coupling buffer for 5 minutes then centrifuged at 1000g and the supernatant discarded. Unreacted groups on the resin were blocked with 1M Tris pH8.0 for 2 hours at room temperature. The resin was washed with coupling buffer, then with acetate buffer for a total of 5 times each with 5 minutes end over end mixing between washes, and supernatant removed after centrifugation at 1000g. Resin was stored at 4°C in storage buffer.

Solutions

Coupling Buffer: 0.1M NaHCO₃, 0.5M NaCl. pH 8.5

Acetate Buffer: 0.1M sodium acetate buffer pH4.0, 0.5M NaCl

2.5.4 Pull down assay

Suspension cells were harvested by centrifugation at 600g, washed with PBS then re-centrifuged and re-suspended in lysis buffer, whilst adherent cells were washed with PBS then suspended with lysis buffer with 5 minutes agitation. Cells were sonicated 3 times for 10 seconds then pelleted by centrifugation at 14,000g for 10 minutes. 20µL resin from 2.5.3 was washed with wash buffer and centrifuged at 600g for 1 minute and the supernatant removed. Cell lysate supernatant was added to the resin, and incubated for 1hour at room

temperature end over end. After incubation the resin was washed for 5 minutes with wash buffer then centrifuged at 600g for 1 minute and the supernatant discarded. Washes were repeated 3 times. The resin was re-suspended in 1x SDS loading buffer and heated to 95°C for 5 minutes before separation on an SDS-PAGE gel and western blotting.

Solutions

Lysis Buffer: 150mM NaCl, 100mM Tris pH7.5, 0.2% NP40, 0.5mM PMSF

Wash Buffer: 150mM NaCl, 100mM Tris pH7.5

2.5.5 Immunoprecipitation

Suspension cells were harvested by centrifugation at 600g, washed with PBS then re-centrifuged and re-suspended in lysis buffer, whilst adherent cells were washed with PBS then suspended with lysis buffer with 5 minutes agitation. Cells were sonicated 3 times for 10 seconds then centrifuged at 14,000g for 10 minutes. Primary antibody at an appropriate dilution was added to the cleared lysate, and incubated over night at 4°C end over end. 20µL protein A/G sepharose (GE healthcare) was added and incubated end over end at room temperature for 2 hours. After incubation the resin was washed for 5 minutes with wash buffer then centrifuged at 600g for 1 minute and the supernatant discarded. Washes were repeated 3 times. The resin was re-suspended in 1x SDS loading buffer and heated to 95°C for 5 minutes before separation on an SDS-PAGE gel and western blotting.

Solutions

Lysis Buffer: 150mM NaCl, 100mM Tris pH7.5, 0.2% NP40, 0.5mM PMSF

Wash Buffer: 150mM NaCl, 100mM Tris pH7.5

2.5.6 Kinase assay

A 50µL reaction was assembled containing 1 x kinase assay buffer, 5U recombinant active Itk (SignalChem), 1µg substrate protein and 6µCi of γ-

[32P]ATP. The reaction was incubated for 15 minutes at 30°C. Samples were resolved by SDS-PAGE, and the gel dried. Dried gels were analysed for incorporation of radioactivity by using them to expose X-ray film which was then developed.

Solutions

Kinase Assay Buffer: 150mM NaCl, 50mM Hepes pH 7.5, 8mM MgCl₂, 8mM MnCl₂, 100μM Na₃VO₄, 1μM ATP

2.6 Biophysical Techniques

2.6.1 Electrospray Ionisation Mass Spectrometry (ESI-MS)

Mass Spectrometry experiments were carried out using electrospray ionisation and a quadrupole time of flight (qTOF) on a SYNAPT High Definition Mass Spectrometry (HDMS) system (Waters).

Acquisition and processing of data was done with Mass Lynx software. Lyophilised samples were weighed and re-suspended in 25 mM ammonium acetate to a concentration of 10 μM. 100 μL was injected at 5 μL/min.

2.6.2 Circular Dichroism (CD)

CD spectra were recorded on a Pi-Star-180 Spectrophotometer (Applied Photophysics).

Protein spectra were recorded using a 300μL cuvette with a 1mm path length with a protein concentration of 0.1mg/mL. Proteins were dissolved in CD buffer.

CD spectra were recorded between the wavelengths 250nm to 180nm with data points recorded at 0.5nm steps. The bandwidth was 1nm and adaptive sampling was enabled. CD spectra were reported as the average of 10 scans, with appropriate buffer blanks recorded in the same fashion for subtraction from the experimental data. CD data was collected using Pi-Star software and analysed using Excel (Microsoft). After subtracting the buffer control from each spectra,

the machine units of ellipticity were converted to mean residue ellipticity to correct for concentration and size of protein using the following equation:

$$\Theta_{MRW} = (MRW \times \Theta) / (10 \times d \times c)$$

Where

$$MRW = M / (N - 1)$$

M = molecular mass of the polypeptide chain (Da)

N = number of amino acids in the chain

Θ = observed ellipticity (degrees)

d = cell pathlength (cm)

c = sample concentration (g/ml)

Solutions

CD buffer: 20mM potassium phosphate buffer pH7.4

2.6.3 Nuclear Magnetic Resonance (NMR)

NMR experiments were carried out on an Avance III 800 MHz spectrometer (Bruker) with a QCI CRYOPROBE at 298 K.

For each experiment the 90° pulse and transmitter frequencies were set manually. One dimensional proton NMR experiments were done with a spectral width of 15.0 ppm, using excitation sculpting water suppression. Spectral acquisition and processing was carried out using Topspin 3.1.b.53 software.

Solutions

NMS buffer: 20mM potassium phosphate buffer pH7.4, 150mM NaCl

2.6.4 Analytical Gel Filtration (AGF)

Analytical Gel Filtration experiments were carried out on a Superdex 200 HR 10/30 column in AGF buffer.

A Superdex 200 HR 10/30 column (GE) attached to an Akta Prime was used to separate the protein sample by hydrodynamic radius. The column was washed with degassed H₂O to remove ethanol. 10µM of protein was run in AGF buffer. The column was run at 0.5mL/min and the elution of protein followed via the UV signal at 280nm.

Solutions

AGF buffer: 20mM potassium phosphate buffer pH7.4, 150mM NaCl

2.6.5 Dynamic Light Scattering (DLS)

DLS readings were obtained from Malvern Zetasizer Nano S.

1mg/mL protein was loaded into the Zetasizer and measured using the standard settings.

Solutions

DLS buffer: 20mM potassium phosphate buffer pH7.4, 150mM NaCl

2.7 Cell Culture Techniques

2.7.1 Maintenance and passaging of adherent cells

Cos7 and NIH-3T3 cells were maintained in a monolayer culture in Dulbecco's Modified Eagle Medium (DMEM) in a humidified atmosphere at 37°C and 5% CO₂. Adherent cells were passaged when they reached a confluency of ~80%. The cells were first washed with PBS, then 1mL trypsin was added. This was removed, and the cells incubated for 5 minutes at 37°C. When the cells had visibly rounded up and detached from the flask surface, they were gently washed from the surface using DMEM. An appropriate volume was then added to fresh pre-warmed DMEM in a new flask. A 1:4 split would typically be ready for passage again in 2 days.

2.7.2 Maintenance and passaging of suspension cells

Jurkat and Raji cells were maintained in a suspension culture in Roswell Park Memorial Institute (RPMI) 1640 medium in a humidified atmosphere at 37°C and

5% CO₂. Cultures were maintained at 5-8x10⁵cells/mL. Once this density had been reached, cells were split typically at a 1:8 dilution into fresh pre-warmed RPMI 1640 medium and returned to the incubator.

2.7.3 Transient transfection

To grow them to 50-70% confluent on the day of transfection as required, cells were plated onto 6well plates at a density of 3x10⁵ cells per mL, with 2mL in each well. To calculate cell density, cells were washed with PBS, then 1ml trypsin added but not removed. The cells were incubated for 5 minutes at 37°C. When the cells had rounded up, they were washed from the flask surface using DMEM, and placed in a 50ml falcon tube. The cells were spun at 1200rpm for 5 minutes, and the DMEM removed. The cells were then resuspended in ~8mL DMEM, and ~10µL placed on the grid of a haemocytometer and the cover slip added. Cells were counted using the standard procedure, and then diluted to the correct density before being plated. Cells were transfected 24 hours after plating with DNA constructs using PromoFectin Transfection Reagent, following the manufacturer's instructions.

2.8 Microscopy

2.8.1 Slide Preparation

For cells expressing fluorescent-tagged protein not requiring additional antibody labelling, oven baked 22mm x 22mm glass coverslips were placed in the wells of 6well dishes before cells were added. 24 hours after transfection medium was removed from cells and the cells were washed twice with ice cold PBS. Arsenite treated cells had arsenite to a final concentration of 1µM added to wells for 1 hour prior to fixing. The cells were then fixed with chilled 4% w/v PFA or 100% methanol at room temperature for 10 minutes. Cells were washed 3x in PBS before being mounted on slides using DAPI containing mountant (Vectashield). Slides were sealed with nail varnish for storage at 4°C.

Solutions

PFA: 4% (w/v) paraformaldehyde, 50% H₂O, 5mM NaOH. Heat to 60°C to dissolve. 50% 2x TBS

2.8.2 Immunofluorescence

For immunofluorescence, oven baked 22mm x 22mm glass coverslips were placed in the wells of 6well dishes before cells were added. 24 hours after transfection media was removed from cells and the cells were washed twice with ice cold PBS. The cells were then fixed with chilled 4% PFA at room temperature for 10 minutes. Cells were washed twice with ice cold PBS. Cells were permeabilised with either 0.2% w/v TritonX. Alternatively cells were both fixed and permeabilised with ice cold 100% methanol for 10 minutes for preservation of granule structure. Cells were washed twice with PBS. Cells were blocked for 1 hour in blocking solution at room temperature. The cells were incubated with the primary antibody diluted in blocking solution to the appropriate concentration (see section 2.10) and phalloidin (Invitrogen) if required at 1/1000 for 1 hour at room temperature then washed three times in PBS. Cells were incubated with Alexafluor dye conjugated secondary antibody (Molecular Probes, Invitrogen, see section 2.10) diluted to 1:500 in blocking solution and incubated for an hour at room temperature. Cells were washed 3x in PBS before being mounted on slides using DAPI containing mountant (Vectashield). Slides were sealed with nail varnish for storage at 4°C.

Solutions

Blocking Solution: 1% (w/v) BSA in PBS

2.8.3 B cell T cell assay

5x10⁶ Raji B cells were collected and resuspended in 1mL RPMI medium with 10% w/v FBS. Cells were incubated with Calcein AM (blue or green, Life Technologies) at a final concentration of 1µg/mL for 1 hour at 37°C. Cells were washed twice with PBS, before being preloaded with superantigen (*S.aureus* Toxin A and Toxin B, Sigma) to a final concentration of 1µg/mL each and

incubated for 1 hour at 37°C. Cells were washed twice and re-suspended in 250µL RPMI. 5×10^6 JURKAT T cells were collected and re-suspended in 250µL RPMI and combined with the Raji cells. Cells were incubated together for 1 hour at 37°C. Cells were diluted to 1/10, and 200µL allowed to settle onto poly-L-lysine coated coverslips (BD Biocoat) for 10 minutes. Cells were fixed and processed as for other immunofluorescence.

2.8.4 Widefield Microscopy

Widefield fluorescence microscopy was performed using an AxioVert S100 (Zeiss) microscope. The slides were visualised using an AxioCam MRm (Zeiss) camera and AxioVision software typically using a 32x Accroplan (Zeiss) objective.

2.8.5 Confocal Microscopy

Confocal fluorescent microscopy was performed using a LSM710 (Zeiss) microscope with argon 488 laser using a 63x (1.4NA) oil objective (Apocromat). Slides were visualised using Zen software.

2.8.6 Fluorescence Recovery After Photobleaching (FRAP)

Cells were plated onto glass bottomed plates prior to transfection. Plates were allowed to equilibrate for 10 minutes before FRAP experiments. Granules were selected using a circular selection tool, covering the entirety of the selected granule. Granules were photobleached using 100% laser power with 30 iterations, 4 prebleached scans, 146 post bleached scans and 2 seconds between scans. Images were obtained at 512x512 resolution with 4 averages. Fluorescence recovery was measured using ImageJ as outlined in section 2.9.5.

2.9 Computational Techniques

2.9.1 I-TASSER

Sequences were submitted to I-TASSER for structural prediction based on multiple-threading alignments and iterative assembly simulations¹¹³.

2.9.2 **SWISS-MODEL**

Sequences were submitted to SWISS-MODEL for structural prediction based on homology modelling¹¹⁴. The sequence of the PH domain of DEF6 was submitted with the structure for the PH domain of SWAP70 to produce a model of the interactions surrounding tyrosine 222.

2.9.3 **COILS**

Sequences were submitted to both the COILS server¹¹⁵ and to the Paircoil2 server¹¹⁶ for prediction of coiled coil regions.

2.9.4 **PyMOL**

PyMOL was used for the visualization of generated structures and production of images for results¹¹⁷.

2.9.5 **ImageJ**

ImageJ was used to visualise images from microscopy, and to measure band density in gel images.

The ImageJ StackReg plugin was used to correct for cell movement during live imaging. The plug in aligns stacks of images, removing changes in granule position due to changes in the cell position. For FRAP experiments, cells first had their movement corrected as outlined above, then a circular region was selected around the bleached granule, and the signal over time measured. The signal from both a non-bleached granule, and the background signal outside the cell were measured, and the bleaching over time corrected for using these measurements.

For tracking granules the Mosaic Particle Tracker 2D/3D plugin was used. This is a point-tracking tool that automatically detects and tracks particles. The parameters used were as follow

Radius

5

Cutoff	0
Percentile	0.5%
Link Range	2
Displacement	5

This provides coordinates at each time point for numbered particles. This data was opened in Excel and the total distance travelled, displacement, velocity, mean squared displacement and area visited was calculated for randomly selected trajectories longer than 5 frames (10s).

To measure the size of granules, images were thresholded and granules present above the threshold measured for total area. Z-stacks were searched to find the widest point for each granule to measure. Granule diameter was calculated from the area.

2.10 Antibodies

Table 1 - Primary Antibodies

Protein	Company	Dilution for Western blot	Dilution for Immunofluorescence
DDX6	AbCam	1:4000	1:500
DEF6	AbCam	1:10,000	1:500
EIF4E	SantaCruz	1:1000	1:250
Itk	AbCam	5:1000	
PABP	SantaCruz		1:250
Xrn1	AbCam	1:5000	1:200

Table 2 - Secondary Antibodies

Protein	Company	Dilution for Western blot	Dilution for Immunofluorescence
HRP-goat anti-rabbit	Cell Signalling Technologies	1:2000	
HRP-goat anti-mouse	Cell Signalling Technologies	1:2000	
Alexafluor 594 goat anti-mouse	Invitrogen		1:500
Alexafluor 568 goat anti-rabbit	Life Technologies		1:500
Alexafluor 488 goat anti-mouse	Invitrogen		1:500
Alexafluor 488 goat anti-rabbit	Life Technologies		1:500

3. Results:

Characterisation of granules formed by DEF6

3 Granule Characterisation

3.1 Introduction

The cellular localisation of DEF6 has been demonstrated by several groups in both cells normally that do not express endogenous DEF6 expressing exogenous GFP-DEF6 constructs, and both endogenously expressed DEF6 and exogenous GFP-DEF6 in Jurkat T cells. Early work indicated an association with actin seen in both fibroblasts expressing exogenous GFP-DEF6¹, and endogenous DEF6 in Jurkat cells where DEF6 localised to immune synapses which are rich in actin³⁵. Later work discovered the ability of full length GFP-DEF6 to form granules when exogenously expressed in Cos7 cells, a property that was enhanced in a DEF6 mutant that mimicked Itk phosphorylation of DEF6¹².

When studying the localisation of the GFP-DEF6 protein in NIH 3T3 fibroblasts cells, Mavrikis *et al.* demonstrated that DEF6 was diffusely distributed throughout the cytoplasm, but found predominantly in the perinuclear region¹. It was noted that the transfection with GFP-DEF6 increased the number of filopodia, a reaction indicative of cdc42 activation. This effect was increased upon treatment of the cells with H₂O₂, which leads to an increase PIP₂ levels at the membrane which has previously been shown to lead to the activation of Rho family GTPases^{118,119}. These membrane ruffles and filopodia were enriched in DEF6, which co-localised with actin in these structures. A direct interaction between a sequence in the N-terminus of DEF6 and F-actin was demonstrated using a co-sedimentation assay.

3.1.1 DEF6 can localise to immune synapses

The translocation of DEF6 to the immune synapse has been demonstrated using both antigen coated beads¹² and antigen presenting cells pre-loaded with super antigen³⁵. To confirm its localisation to the immune synapse, protein kinase C Θ was used to mark the immune synapse.

In T cells Gupta *et al.* demonstrated that endogenous DEF6 localises to the immune synapse, a structure enriched in actin³⁵. This was demonstrated in T cells activated by presentation of *Staphylococcus* enterotoxin E (SEE) superantigen by Raji B cells. SEE acts as a polyclonal activator of T cells, and is presented to the T cells by the Raji B cells thus producing a T cell – antigen presenting cell conjugate with an immune synapse and activated T cell. The redistribution of DEF6 to the immune synapse was found to be dependent on both phosphorylation of DEF6 by the kinase Lck, and the activity of PI3K.

3.1.2 DEF6 is able to form granules similar to mRNP granules

The appearance of DEF6 as cytoplasmic granules was first reported by Hey *et al.*, in Cos7 cells lacking endogenous DEF6 transfected with the GFP-Y210E/Y222E double mutant of DEF6 that mimicked phosphorylation by Itk¹². In addition, DEF6 was further shown to localise into granules upon treatment with arsenite, which causes arrest of mRNA translation suggesting some link with events that regulate translation within cells. The formation of granules by the overexpressed GFP tagged Y210E/Y222E mutant was further demonstrated in JURKAT cells stimulated by anti-CD3 and anti-CD28 coated beads. GFP-DEF6 was confirmed as localising to the synapse formed with the beads, a localisation which could be disrupted by treatment with phosphatase which led to the redistribution of DEF6 from the synapse to granules.

The DEF6 granules have been shown to co-localise with DCP1, a marker for P-bodies indicating an interaction between DEF6 granules and P-bodies¹². P-bodies are cytoplasmic foci that contain proteins involved in the degradation of mRNA and are upregulated under cellular conditions that cause the arrest of translation, including treatment of the cells with arsenite. There is a link between P-bodies and stress granules, which store stalled translation machinery and initiation complexes. The difference in component proteins between P-bodies and stress granules allows for identification of granule type based on protein

markers. DCP1 is one of these markers, and co-localisation of DEF6 with DCP1 suggests an interaction between DEF6 granules and P-bodies. The interaction of DEF6 with these mRNA regulating foci suggests the possibility of a role for DEF6 in mRNA regulation via interactions with P-bodies. The presence of P-bodies in T cells could provide a level of control over the translation of proteins at different stages of T cell activation, a role which DEF6 may have a part in via its interactions with these mRNA regulating foci.

This chapter focuses on further investigation of the localisation of DEF6 both endogenously at immune synapse, and when expressed ectopically in Cos7 cells, and the localisation of proteins which are known to localise to either P-bodies or stress granules.

3.1.2.1 P-body associated proteins

P-bodies are multi-protein complexes that including proteins involved in the degradation of mRNA. There are several proteins found only in P-bodies and not stress granules allowing for differentiation between the two forms of granules, and thus allowing for the identification of the function of a granule⁹⁵. One protein only found in P-bodies is DCP1, a protein involved in the decapping of mRNA. Also used to define P-bodies are Xrn1, an exoribonuclease involved in the 5' to 3' degradation of mRNA and DDX6 (previously known as Rck/p54) which functions in mRNA degradation and suppression of translation, which can also be found in low levels in stress granules but is found at much higher levels in P-bodies⁹⁵. The granules formed by GFP-DEF6 have previously been shown by Hey *et al.* to co-localise with overexpressed DCP1 in both Cos7 cells overexpressing DEF6, and in Jurkats overexpressing the Y210E Y222E construct of DEF6¹².

3.1.2.2 Stress granule associated proteins

The markers specific for stress granules include G3BP-1, a sequence specific endoribonuclease and DNA-unwinding enzyme found only in stress granules. Another marker sometimes used to identify stress granules is eIF4E, an initiation

factor which forms part of the pre-initiation complex and directs the mRNA cap to ribosomes. Whilst eIF4E can be found in P-bodies, it can be used as a marker for stress granules as it often is not detected as the protein composition of the P-bodies reflects its role in degradation of mRNA rather than the storage of stalled initiation complexes as in stress granules.

3.1.2.3 Induction of translational arrest via arsenite treatment

P-bodies and stress granules are both induced to form or upregulated upon conditions that promote the global arrest of translation. This can be induced by various types of stress including heatshock and oxidative stress. Sodium arsenite is used to promote the formation of P-bodies and stress granules due to its ability to produce redox stress^{95,120}, inducing synthesis of heat shock and other stress related proteins¹²¹. The redox stress is sensed by heme regulated inhibitor kinase (HRI, an eIF2 α kinase) which in turn phosphorylates serine residue 51 of eIF2 α . This phosphorylation event depletes the eIF2/tRNA/GTP ternary complex that is required for initiation of translation, thus leading to translational arrest. This phosphorylation of eIF2 α can also be induced by heat shock and UV radiation which have also been used to promote the formation of P-bodies and stress granules. This prevention of elongation causes the mRNA to run off the polysomes, which converts them into mRNPs. Treatments that disrupt polysomes thus also lead to an increase in P-body size, for example puromycin treatment¹²². On the other hand, stabilization of polysomes via cycloheximide treatment disrupts P-bodies.

3.2 Results

3.2.1 Endogenous DEF6 localises to the immune synapse in activated T cells and forms granules

To confirm the localisation of DEF6 to the immune synapse in T cells, conjugates between Jurkat T cells in which DEF6 is endogenously expressed and superantigen-loaded Raji B cells as the antigen presenting cells were visualised in line with the experiments by Gupta *et al*³⁵. First the Raji cells were loaded with calcein dye, so as to differentiate between B and T cells later in the experiment. The B cells were then loaded with *Staphylococcus* enterotoxins A and B which are superantigens and promote the formation of conjugates between the B and T cells and the activation of the T cells, with an immune synapse forming at their interface. The conjugates were then allowed to settle on poly-L-lysine coated coverslips to aid adhesion, fixed with 4 w/v PFA and permeabilised with 0.2% triton, and stained with antibodies for various proteins. The conjugates were imaged using confocal microscopy, which was used both to allow clearer visualisation of co-localisation on a single plane due to the ability of the technique to eliminate out of focus light, and to allow the production of three-dimensional images via the taking of a stack of images of single focal planes. This allowed visualisation along the immune synapse from a wider range of angles, thus providing a better image of the proteins detected at the immune synapse.

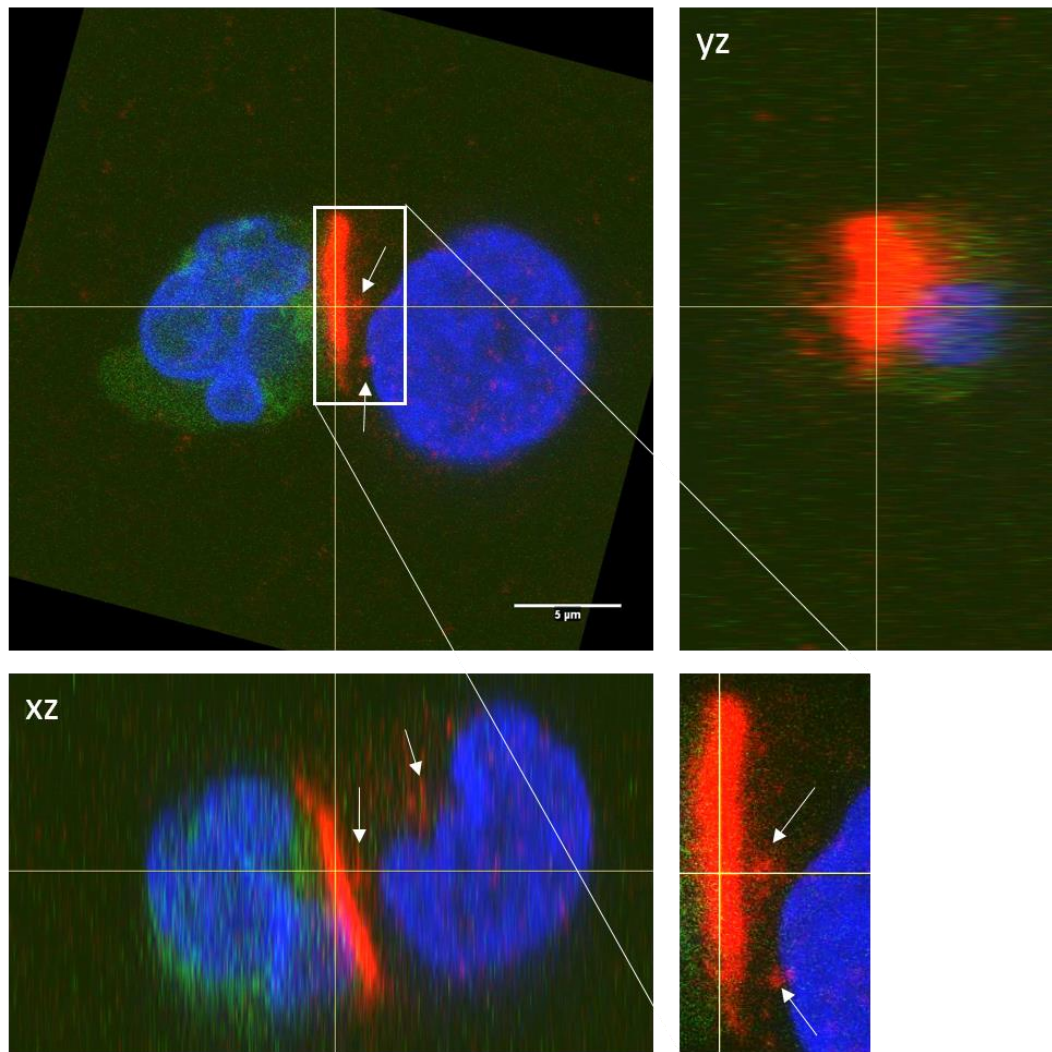


Figure 13- DEF6 (red) localises to the immune synapse formed between T Cells and B Cells and forms foci. DEF6 is highly localised to the interface between the antigen presenting B cell (green) and the T cell which forms the immune synapse. It also forms foci, as indicated by arrows. RAJI B Cells were stained with calcein (green) and loaded with *Staphylococcus aureus* enterotoxins A and B before incubation with Jurkat T cells. Cells were probed for DEF6 (red) using antibody and mounted using DAPI containing mounting media (blue). Z stacks were obtained using a Zeiss LSM710 confocal microscope. Presented is a Z stack with a xz and a yz projection chosen to show the immune synapse. Scale bar is 5μm. The contrast has been enhanced to highlight the DEF6 granules.

In activated T cells, DEF6 localises to the immune synapse as illustrated in Figure 13 and Figure 14. The localisation to the immune synapse in JurKat cells activated with B cells and antigen is much more extensive than that previously seen with Jurkats activated by beads coated with anti CD3 and anti CD28 as seen by Hey *et al*¹². The DEF6 is present across the entire surface of the contact rather than localised to the centre of the immune synapse contact as seen by Singleton *et al*⁵, most likely due to differences in Itk localisation between the T

cell lines used. In Jurkat cells, Itk is constitutively located at the plasma membrane, due to a defect in PTEN, a phosphatase that usually dephosphorylates the phosphatidyl inositol lipids that recruit Itk to the plasma membrane¹²³. DO11.10 cells on the other hand do not exhibit this defect, and thus a normal lower amount of Itk is found at the plasma membrane¹²³.

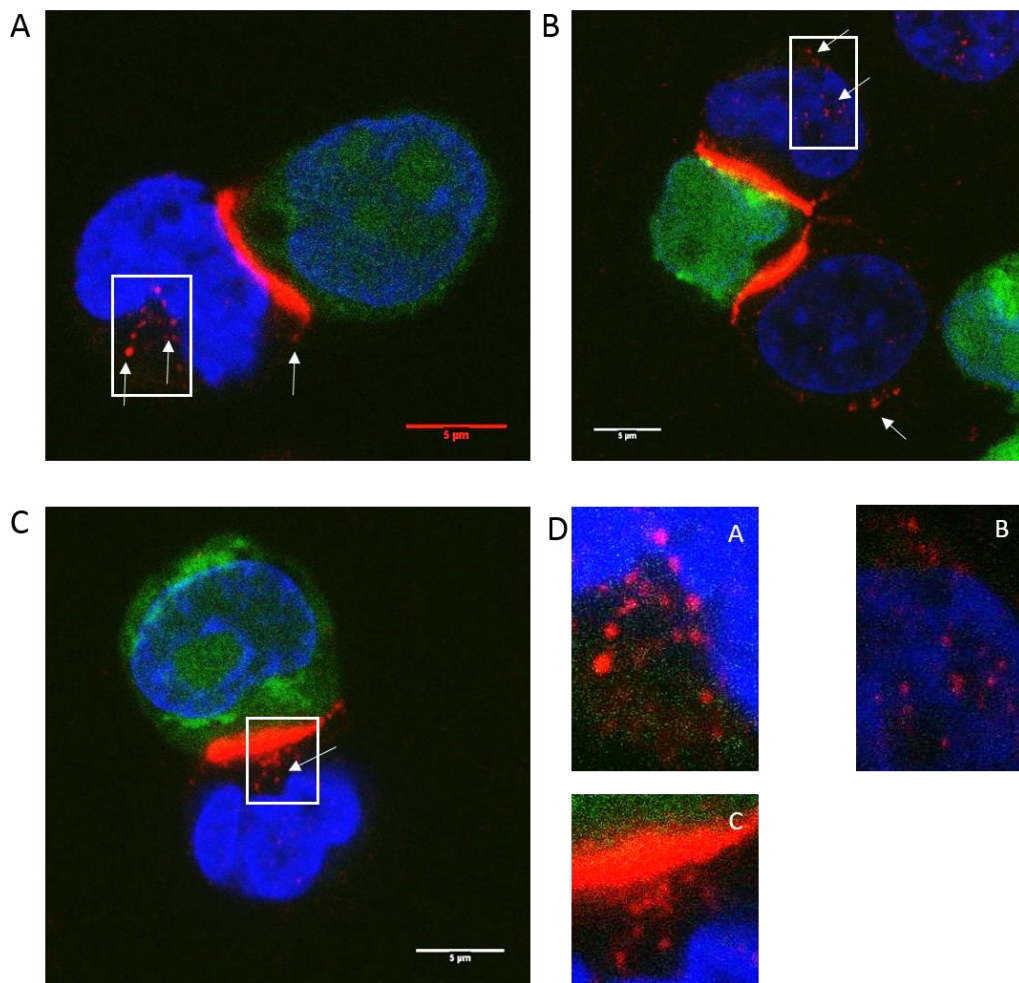


Figure 14 - DEF6 (red) localises to the immune synapse formed between T Cells and B Cells and forms foci. Further images showing DEF6 is highly localised to the interface between the antigen presenting B cell (green) and the T cell which forms the immune synapse, and that it forms foci in the cytoplasm of the T cells as indicated by arrows. Raji B Cells were stained with calcein (green) and loaded with *Staphylococcus aureus* enterotoxins A and B before incubation with Jurkat T cells. Cells were probed for DEF6 (red) using antibody and mounted using DAPI containing mounting media (blue). Z stacks were obtained using a Zeiss LSM710 confocal microscope. Presented are representative Z stacks of 3 T cell B cell conjugates. Panel D highlights the granules from panels A, B and C. Scale bar is 5μm. The contrast has been enhanced to highlight the DEF6 granules.

In addition to DEF6 localisation to the synapse, it is clear in both Figure 13 and Figure 14 that DEF6 is present in foci in activated Jurkats. These have been

highlighted in Figure 14D and have on average a radius of 0.15µm measured as described in the materials and methods (2.9.5). These foci are present in all images, and can be seen at the opposite side of the cell to the synapse, next to the synapse, and to some extent in the nucleus of some cells. The identification of the location of the foci as being within the nucleus rather than above it is possible due to the nature of confocal microscopy eliminating out of focus light and thus any objects not in the same plane. Interestingly, the foci appear to be aligned in a linear fashion which can be seen clearly in Figure 14A, suggesting the possibility of movement along the cytoskeletal network. This differs from the foci seen by Hey *et al*, which were only noted with the overexpressed Y210E/Y222E construct or with pervanadate treatment. It could be that activation of T cells by antigen presenting cells rather than by antigen coated beads accounts for this difference due to the additional signals present with antigen presenting cells.

3.2.1.1 Regulation of mRNA metabolism at the immune synapse

The potential role for DEF6 in regulation of mRNA metabolism as suggested by its association with P-bodies raises the possibility that DEF6 could be regulating mRNA metabolism at the immune synapse. To date, whether translation takes place at the immune synapse of T cells has not been described. To determine whether translation happens at the immune synapse, antibodies against the translation initiation factor eIF4E and the regulator of translation initiation PABP were used to visualise sites at which regulation of translation initiation is happening.

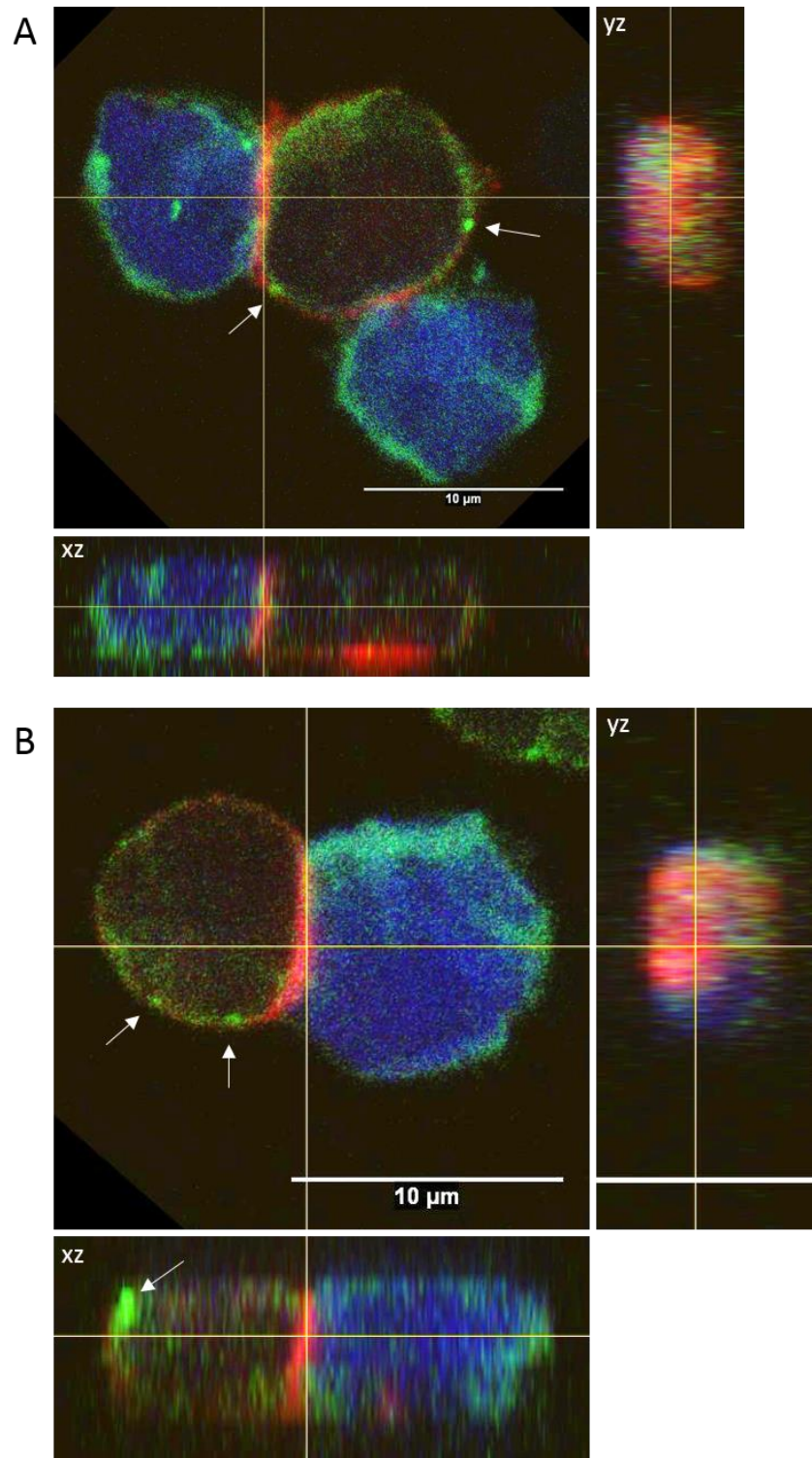


Figure 15 - eIF4E (green) is present at the immune synapse (as marked by DEF6, red) but does not extensively localise to it. eIF4E localiseis present at the interface between the antigen presenting B cell (blue) and the T cell which forms the immune synapse. It also forms foci, as indicated by arrows. Raji B Cells were stained with calcein (blue) and loaded with *Staphylococcus aureus* enterotoxins A and B before incubation with Jurkat T cells. Cells were probed for eIF4E (green) and DEF6 (red) using antibody. Z stacks were obtained using a Zeiss LSM710 confocal microscope. Presented are Z stacks with xz and a yz projections chosen to show the immune synapse. Scale bar is 10μm.

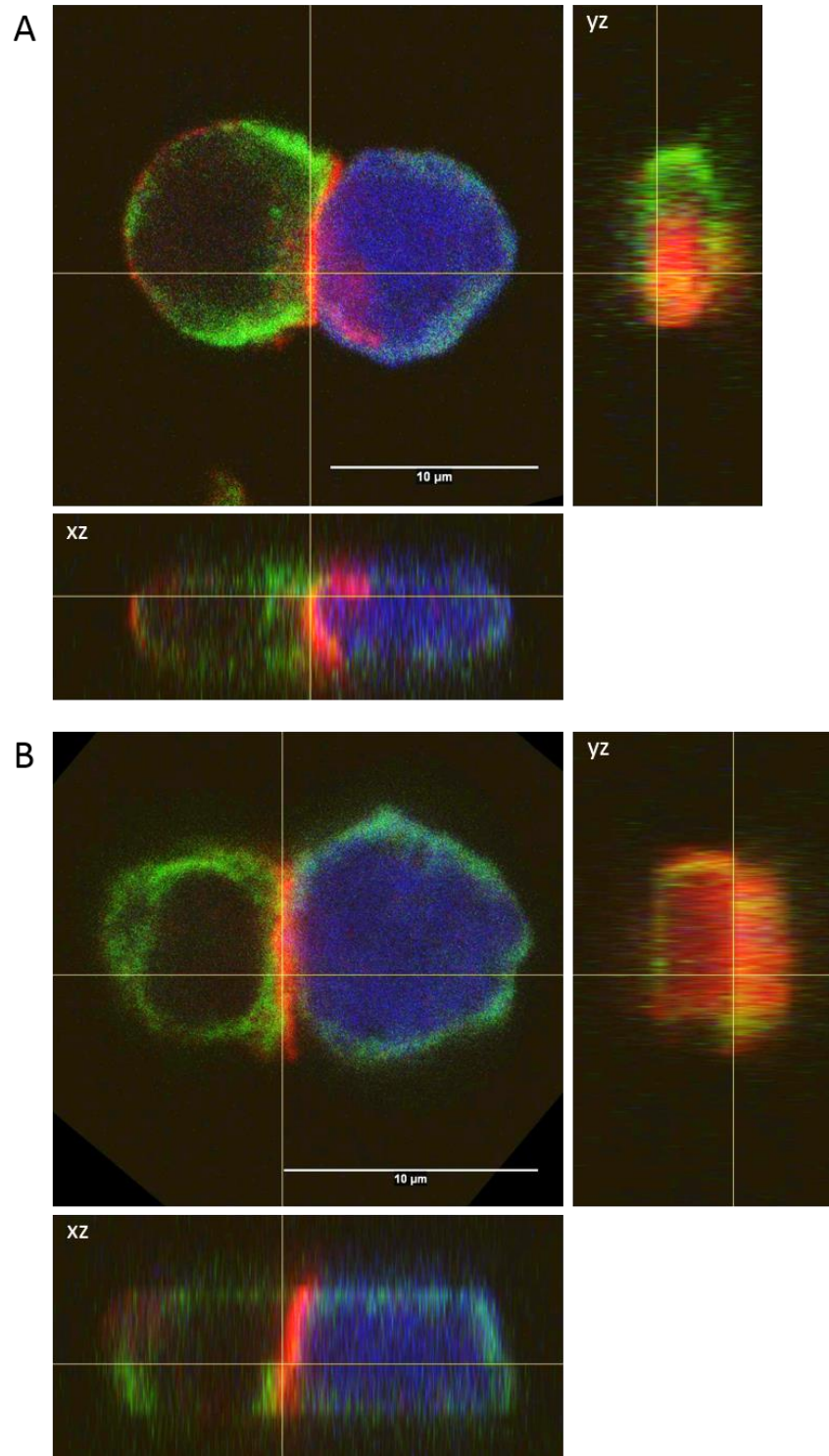


Figure 16 - PABP (green) is present at the immune synapse but does not extensively localise to it although it demonstrates some polarisation towards the synapse. PABP is polarised towards localiseand is present at the interface between the antigen presenting B cell (blue) and the T cell which forms the immune synapse. Raji B Cells were stained with calcein (blue) and loaded with *Staphylococcus aureus* enterotoxins A and B before incubation with Jurkat T cells. Cells were probed for PABP (green) and DEF6 (red) using antibody. Z stacks were obtained using a Zeiss LSM710 confocal microscope. Presented are Z stacks with xz and a yz projections chosen to show the immune synapse. Scale bar is 10µm.

Both PABP and eIF4E are located throughout the cytoplasm of both T and B cells (Figure 15 & Figure 16). Whilst there is no extensive localisation of either protein to the immune synapse to the extent that DEF6 localises to the IS, both proteins are present at the immune synapse indicating that translation could take place at the immune synapse. PABP exhibits polarised localisation to the side of the cell that is forming an immune synapse, but does not extensively localise to the centre synapse itself as DEF6 does (Figure 16). The yz projections indicate that this polarisation appears to form a ring around the immune synapse as marked by DEF6. Whilst DEF6 occurs in the c-SMAC and p-SMAC of the immune synapse, PABP may be located in the d-SMAC (distal SMAC) which is the zone peripheral to the immune synapse, a zone marked by a ring of actin around the immune synapse¹²⁴. The mRNA cap protein eIF4E forms granules in both the T and B cells, some of which are found near the immune synapse (Figure 15). These are most likely to be stress granules. Whilst eIF4E can be found in P-bodies, it is at levels that are usually undetectable⁹⁵. A lack of co-localisation between the eIF4E granules and DEF6 suggests no interaction between DEF6 and the stress granule structures eIF4e is likely to mark.

Whilst these results suggest that there isn't extensive localisation of initiation of translation and regulation of translation happening at the immune synapse, both proteins are present at the immune synapse and show some overlap with the location of DEF6. Additionally, a level of polarisation towards the immune synapse appears to be evident in the case of PABP. Whilst these results do not suggest extensive interaction between DEF6 and either PABP or eIF4E, DEF6 may have a role in regulating the location of translation through the regulation of cellular polarisation after activation.

3.2.2 Analysis of DEF6 granule co-localisation with P-body and stress granule markers

Due to the different roles of P-bodies and stress granules, with P-bodies having a role in degradation of aggregated mRNAs and stress granules storing mRNA and pre-initiation complexes, the presence of proteins specific to either of these foci in DEF6 foci would allow us to suggest a role for DEF6 containing foci. Lists of both fluorescently tagged proteins¹²⁵ and antibodies⁹⁵ suitable for characterisation of P-bodies and stress granules have been compiled by Kedersha *et al.* A combination of overexpressed markers and antibodies against endogenous markers were used to characterise the type of granules DEF6 interacts with.

3.2.2.1 DEF6 co-localises with ectopically expressed DCP1; a marker of P-bodies.

DCP1 is a decapping protein found in P-bodies but not stress granules ectopically-expressed DCP1 has been used by several groups^{12,92,125} to define P-body localisation of proteins. Co-localisation of DEF6 with DCP1 would suggest association of DEF6 with P-bodies. To promote the aggregation of DEF6 into foci, cells were treated with sodium arsenite for 1 hour to induce translational arrest. Arsenite treatment induces the aggregation of GFP-DEF6 but not GFP alone (Appendix section 8.8), demonstrating that aggregation is not due to aggregation of the GFP tag. GFP tagged DEF6 has been used in previous studies, and the work described here showing endogenous DEF6 in Jurkats forming granules has shown that granule formation by DEF6 is not a side effect of its over expression or GFP tag.

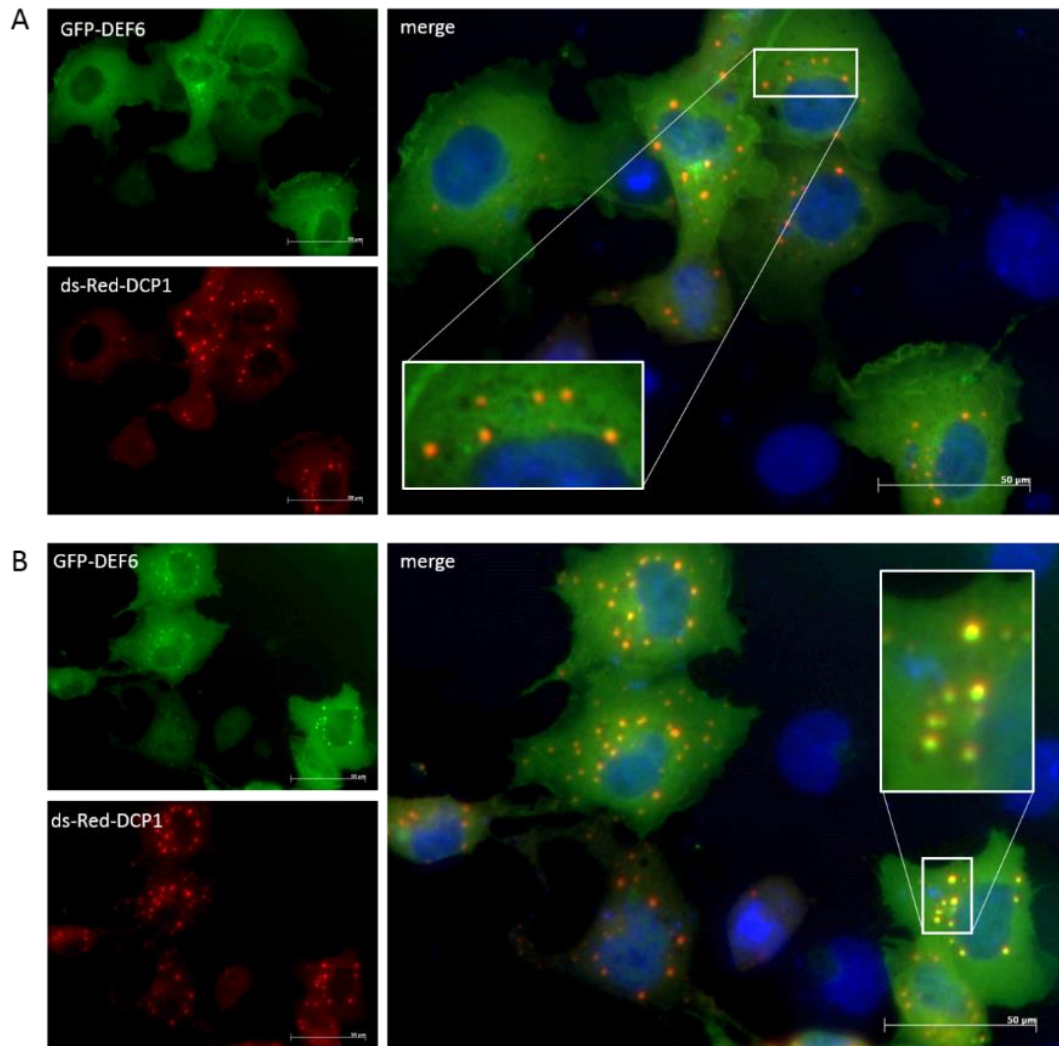


Figure 17 – GFP-DEF6 co-localises with dsRed-DCP1 in granules, indicating DEF6 associates with P-bodies. GFP-WT DEF6 forms granules when treated with arsenite (B) which co-localise with dsRed-DCP1. Cos7 cells were transfected with GFP-DEF6 either left untreated (A) or treated with 1µM arsenite for 1 hour (B). Cells were fixed and stained with DAPI containing mounting media (blue). An AxioVertS100 microscope was used to take widefield images of the fixed cells. Scale bar is 50µm.

Cytoplasmic granules were formed by arsenite-treated full length DEF6 which co-localised with DCP1 in line with the data shown by Hey *et al*¹². This suggests that the DEF6 can associate with P-bodies. The untreated cells show no enrichment of DEF6 in the DCP1 granules, indicating that DCP1 does not recruit DEF6 universally, but that foci-forming DEF6 induced by translational arrest is specifically able to co-localise with DCP1 and associate with P-bodies.

3.2.2.2 **DEF6 does not co-localise with ectopically expressed G3BP-1; a marker of stress granules.**

G3BP1 is a DNA unwinding protein found in stress granules but not P-bodies and has been used by several groups¹²⁵⁻¹²⁷ to define stress granule localisation of proteins. Co-localisation of a protein with G3BP-1 would suggest the association of DEF6 with stress granules in addition to its association with P-bodies indicated in 3.2.2.1.

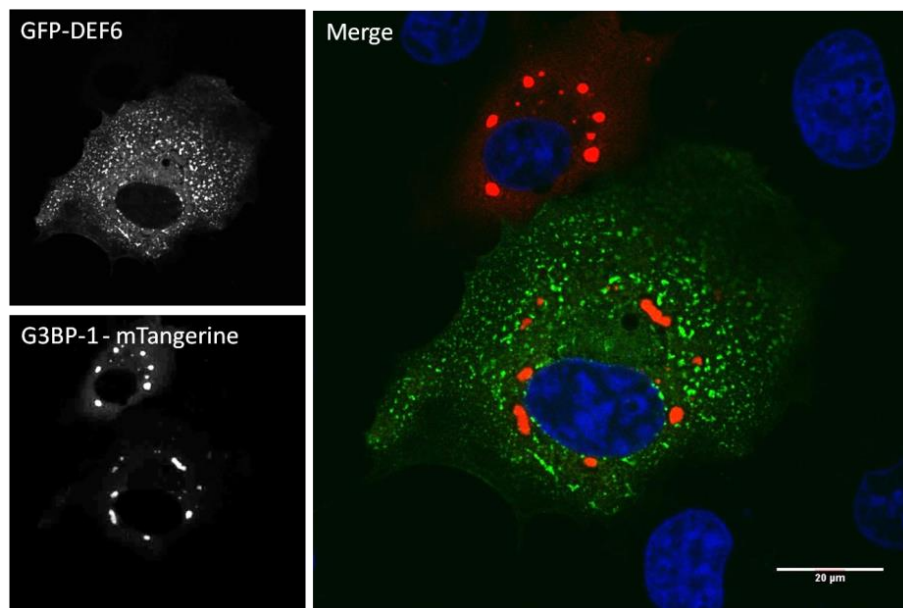


Figure 18 – GFP-DEF6 does not co-localise with G3BP-1-mTangerine, suggesting no association with stress granules. GFP-DEF6 (green) does not co-localise with G3BP-1-mTangerine (red) in arsenite treated Cos7 cells. Cos7 cells were transfected with GFP-DEF6 and G3BP-1-mTangerine and treated with 1μM arsenite for 1 hour. A single Z plane image was obtained using a Zeiss LSM 710 confocal microscope. An AxioVertS100 microscope was used to take widefield images of the fixed cells. Scale bar is 50μm.

DEF6 granules formed by the arsenite treatment of Cos7 cells do not co-localise with G3BP1. The DEF6 granules and stress granules are distinct and separate, suggesting DEF6 does not associate with stress granules. This also supports the data demonstrating specificity in the co-localisation of DEF6 with DCP1, as it demonstrates that the GFP DEF6 construct does not co-aggregate non-specifically with other aggregating proteins.

3.2.2.3 **DEF6 partially localises to endogenous granules**

Antibodies against several P-body and stress granule proteins were used to assess the interaction of DEF6 with endogenous foci. Endogenous foci can behave differently to foci formed via ectopic expression of P-body and stress granule forming proteins. Some vectors have been shown to alter the localisation of the foci, whilst some transfection reagents have been shown to change the length of time for which the foci persist⁹⁵. It is also possible that the interactions can vary between endogenous foci and foci made up of ectopically-expressed protein, as those made up of ectopically-expressed protein may be more homogenous due to the amount of protein present. Xrn1 (an exoribonuclease that forms P-bodies) and DDX6 (a suppressor of translation found in P-bodies) were used due to their common use as markers by Kedersha *et al*⁹⁵.

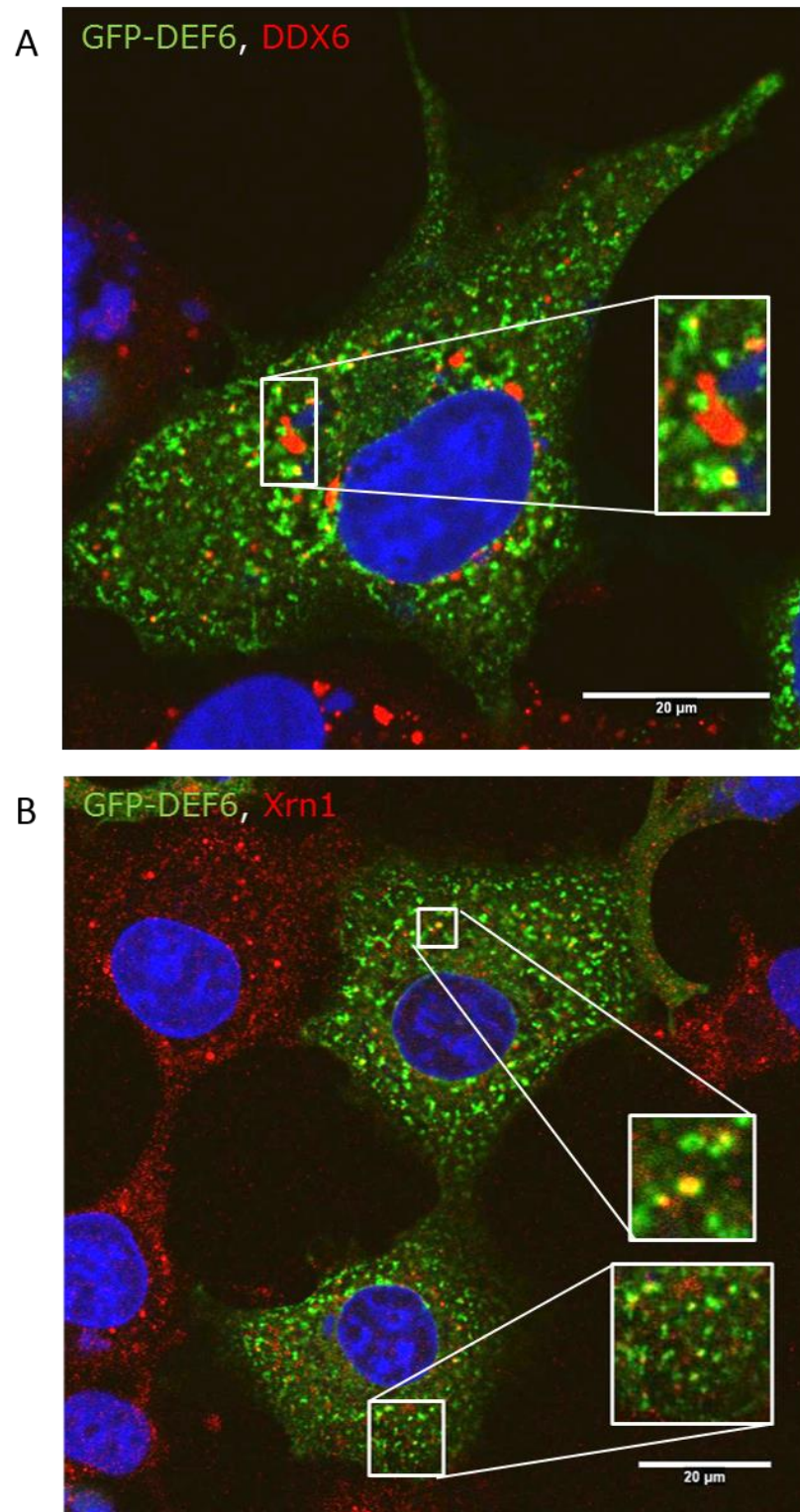


Figure 19 – GFP-DEF6 granules are found in close proximity to and partially co-localise with markers for endogenous P-bodies, DDX6 (A) and Xrn1 (B) Cos7 cells were transfected with GFP-DEF6, treated with 1 μ M arsenite for 1 hour and probed with antibody for either DDX6 (A) or Xrn1 (B). A single Z plane image was obtained using a Zeiss LSM 710 confocal microscope. Scale bar is 20 μ m.

DEF6 appears to partially co-localise with both DDX6 (Figure 19A) and Xrn1 (Figure 19B). This suggests that the DEF6 granules may be separate to both P-

bodies and stress granules, but can interact with P-bodies as shown by the partial co-localisation with some granules. These experiments present a snapshot of a single time point and the partial co-localisation may be explained by DEF6 foci interacting in a dynamic manner, with its interactions changing over time depending on functional requirements within the foci.

The difference between the endogenous P-body markers and the result obtained with ectopically-expressed DCP1 may suggest that DEF6 has a high affinity for DCP1 in P-bodies. The endogenous granules contain lower levels of DCP1 than the overexpressed DCP1 granules, leading to a normal level of only partial co-localisation.

3.2.3 P-body components can be found adjacent to the immune synapse

DEF6 is the first protein to be shown to localise both to granules associated with mRNA metabolism¹² and to the immune synapse in activated T cells³. The work described here has shown that the granules formed by exogenous DEF6¹² also form endogenously. To determine whether other P-body proteins localise to the immune synapse, the co-localisation of DDX6 and Xrn1 which constitutively form granules in Jurkat cells (see appendix sections 8.4 and 8.5) with actin at the immune synapse was assayed using a T cell – B cells to form immune synapses *in vitro*. During T cell activation F actin localises to the immune synapse and DEF6 co-localises extensively with this. The F-actin binding molecule phalloidin was used here to identify the immune synapse when it was not possible to use the DEF6 antibody in combination with antibodies raised in the same species against other markers of the immune synapse.

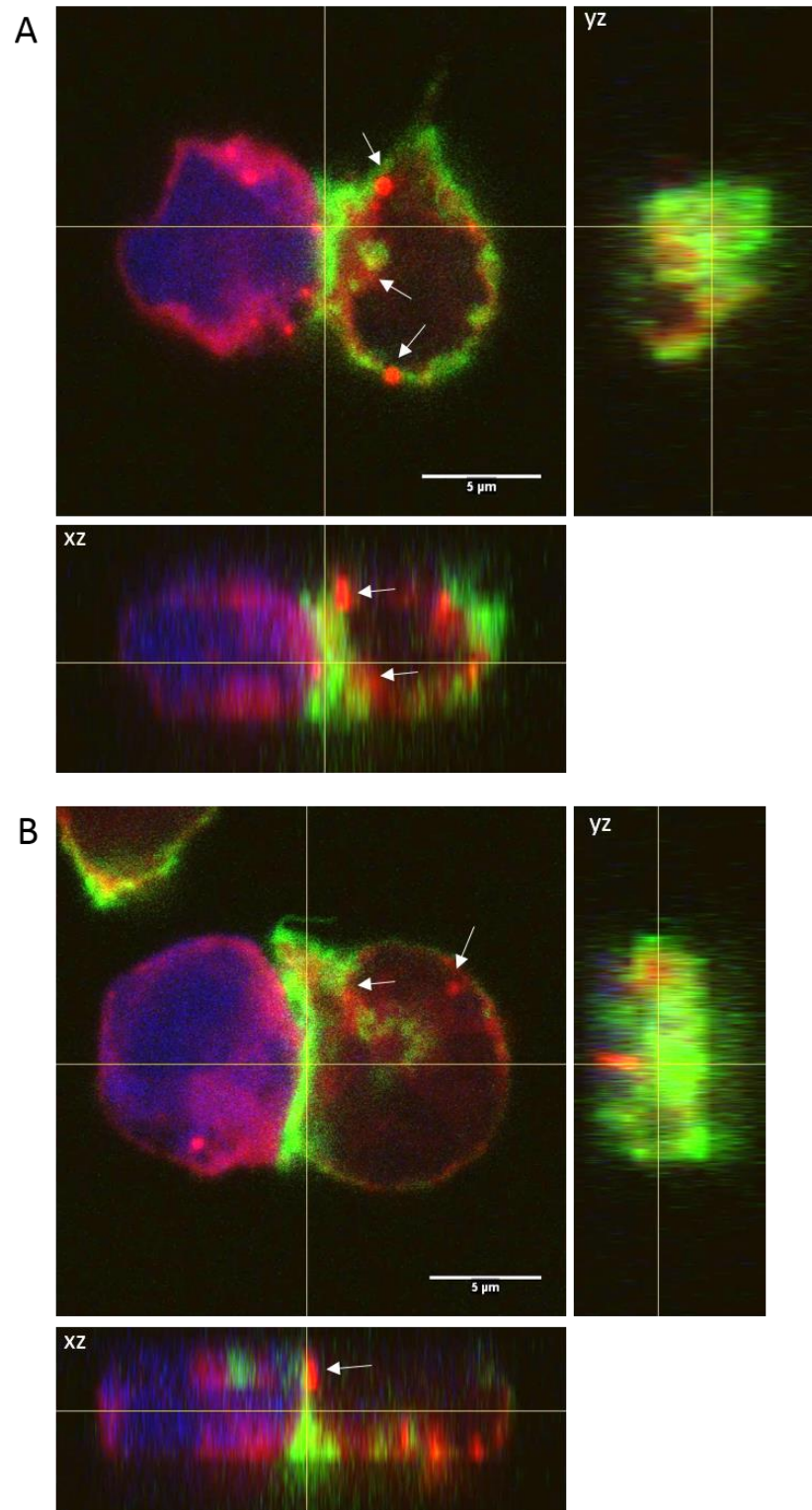


Figure 20 – DDX6 forms granules in activated T cells localise which are present at the immune synapse. DDX6 forms granules indicated by the arrows close to the interface between the antigen presenting B cell (blue) and the T cell which forms the immune synapse. Raji B Cells were stained with calcein (blue) and loaded with *Staphylococcus aureus* enterotoxins A and B before incubation with Jurkat T cells. Cells were probed for DDX6 (red) using antibody and actin using phalloidin (green). Z stacks were obtained using a Zeiss LSM710 confocal microscope. Presented are representative Z stack with xz and yz projections chosen to show the immune synapse. Scale bar is 5μm.

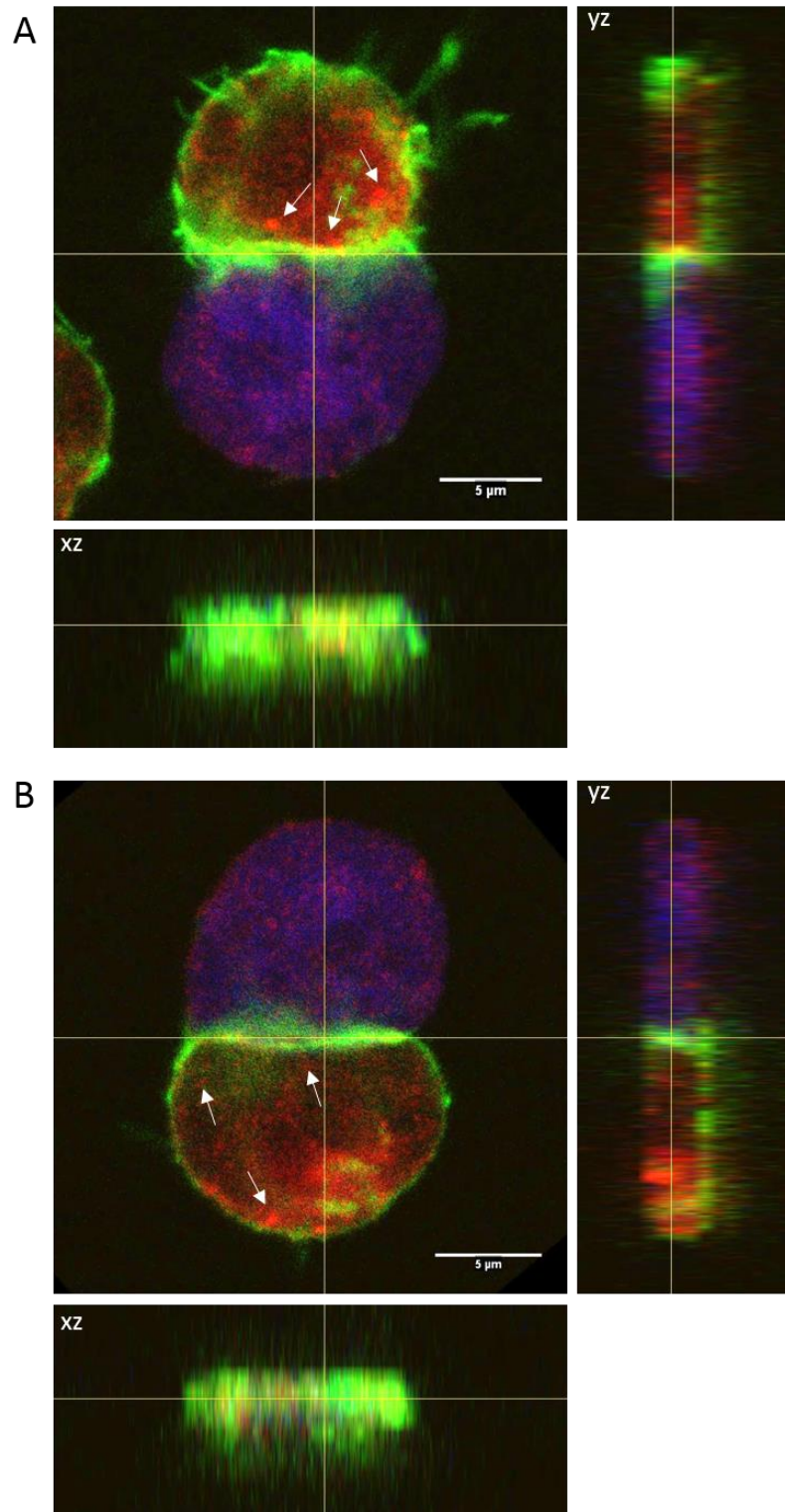


Figure 21 - Xrn1 forms granules in activated T cells localise and is present at the immune synapse. Xrn1 forms granules indicated by the arrows close to the interface between the antigen presenting B cell (blue) and the T cell which forms the immune synapse. Raji B Cells were stained with calcein (blue) and loaded with *Staphylococcus aureus* enterotoxins A and B before incubation with Jurkat T cells. Cells were probed for Xrn1 (red) using antibody and actin using phalloidin (green). Z stacks were obtained using a Zeiss LSM710 confocal microscope. Presented are representative Z stack with xz and yz projections chosen to show the immune synapse. Scale bar is 5µm.

Whilst both Xrn1 and DDX6 are present in granules in Jurkats activated by the presentation of SEE by Raji B cells, the proteins do not additionally localise as extensively at the immune synapse as DEF6 (Figure 20 & Figure 21). However, both are present and not excluded from the immune synapse, indicating again a potential for mRNA regulation at the immune synapse. Both the Xrn1 and DDX6 granules are also larger than those formed by DEF6 with an average radius of 0.25µm (measured as described in 2.9.5) compared to the DEF6 granules which had an average radius of 0.15µm, although Xrn1 also demonstrates a large number of very small granules as can be seen in Figure 21 which were not measured to give the average radius as they did not come in above the threshold. The granules can be found adjacent to the immune synapse, and in the case of Xrn1 there does appear to be low levels of protein localisation at the synapse in Figure 21A, as seen in the yz plane. Due to current availability of antibody it is not possible to study co-localisation of endogenous DEF6 and these markers of P-bodies simultaneously but due to their presence, this allows for the possibility that DEF6 foci may interact with P-bodies in activated T cells. The presence of Xrn1 and DDX6 foci in activated T cells suggests P-bodies potential for translational regulation in activated T cells via P-body formation. Although it has not been possible to demonstrate an interaction between DEF6 and the P-body markers in T cells, the presence of DEF6 granules along with data from Cos7 cells showing some interaction between DEF6 and P-bodies supports interaction between DEF6 and P-bodies in T cells, which suggests a role for DEF6 in T cells in regulating translation.

3.2.4 DEF6 co-localises with actin at cell – cell junctions

DEF6 has been shown to interact directly with actin¹, in addition to its role in actin polymerisation via the Rho GTPase CDC42^{1,5}. In addition to this, preliminary pull down experiments in our lab have suggested that DEF6 may be able to interact with tubulin. DEF6 co-localises with actin at the immune synapse

as can be seen in Figure 22A. It was noticeable in these experiments that DEF6 could also localise to T cell – T cell junctions with actin and was enriched in these regions as well as the B cell – T cell junction of the immune synapse. This co-localisation with actin at cell junctions is also seen in Cos7 cells (Figure 22B).

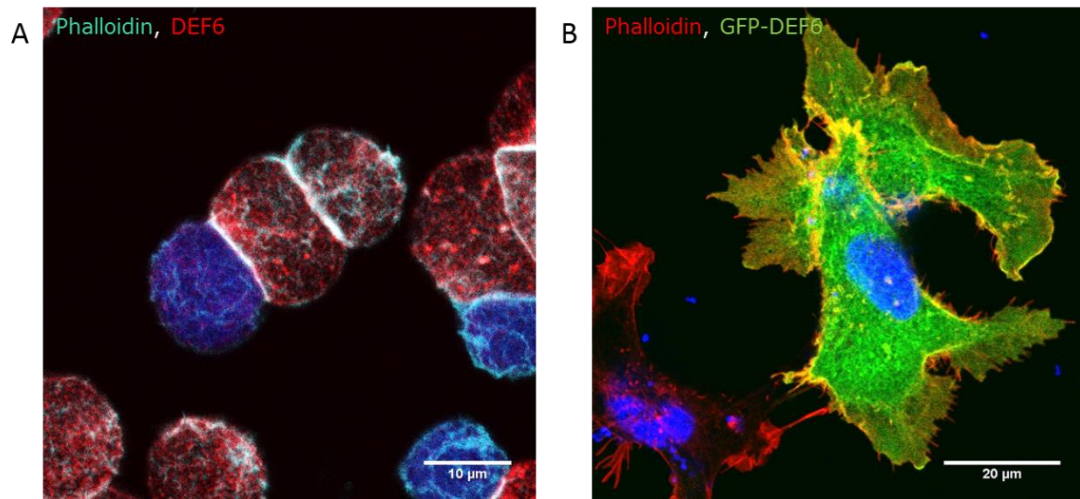


Figure 22 - DEF6 co-localises with actin at cell junctions in both JURKAT cells (A) and Cos7 cells (B). (A) RAJI B Cells were stained with calcein (blue) and loaded with *Staphylococcus aureus* enterotoxins A and B before incubation with JURKAT T cells. Cells were probed for endogenous DEF6 (red) using antibody and actin using phalloidin (cyan). Scale bar is 10µM (B) Cos7 cells were transfected with GFP-DEF6 (green) and probed for actin with phalloidin (red) Scale bar is 20µM. Representative Z stacks were obtained using a Zeiss LSM710 confocal microscope.

Whilst this may suggest that DEF6 localisation to the immune synapse may be modulated by interaction with F actin it is of interest to note that DEF6 is not present in several of the actin rich protrusions seen in the Cos7 cells or as extensively in the membrane ruffles of the Jurkat cells (Figure 22A). It is possible that apart from interacting with and promoting the polymerisation of actin, DEF6 is able to localise to cell-cell adhesion zones via interaction with cell adhesion molecules found in many different types of cell-cell contacts.

3.3 Discussion

3.3.1 DEF6 forms granules and co-localises to the immune synapse in T cells activated by B cell antigen presentation.

The results in section 3.2.1 show that endogenous DEF6 is able to form granules in activated T cells, indicating that the granule-forming properties of DEF6 demonstrated by Hey *et al*¹² are not simply a by-product of the overexpression of DEF6, or due to aggregation of the GFP tag. These granules are mainly cytoplasmic, with many occurring in the space formed by the indentation of the nucleus. However, it does appear that there are some small DEF6 granules within the nucleus. The ability of DEF6 granules to move about the T cell was not determined; P-bodies exhibit movement within the cytoplasm as demonstrated by Aizer *et al*^{92,128}, who showed that whilst most P-bodies are confined to an area of the cytoplasm, quick directed movement can happen along microtubules. It is currently unknown whether the DEF6 granules in Jurkat cells are able to be transported along the cytoskeletal network and whether any movement would be towards or away from the immune synapse. The granules can sometimes be seen in close proximity to the immune synapse and it is possible that granules may be able to either merge with or bud from the pool of DEF6 at the immune synapse.

To assess whether P-bodies were also forming in activated T cells, antibody was used to probe for both Xrn1 (an exoribonuclease that forms P-bodies) and DDX6 (a suppressor of translation found in P-bodies). Unfortunately, it was not possible to probe for both endogenous DEF6 and the P-body markers simultaneously to assess co-localisation due to the lack of antibodies available that were raised in different species that are essential for this type of experiment. Both Xrn1 and DDX6 formed granules structures in the T cells activated by presentation of SEE by Raji B cells. In the cells viewed, some Xrn1 appeared to localise to the immune synapse on the T cell but not the B cell side of the synapse. DDX6 on the other hand did not appear to localise to the

immune synapse. The distribution of Xrn1 was more similar to that of DEF6 than the distribution of DDX6. This discrepancy in localisation is not unexpected, as despite both Xrn1 and DDX6 being used as markers for P-bodies, P-bodies have a heterogeneous protein composition and in some foci levels of DDX6 can be undetectable¹²⁹. Both of these proteins can also be found in stress granules although they are normally found in larger amounts in P-bodies leading to a possibility that in addition to variance in P-body composition, some of the granules observed may be stress granules with higher than normal levels of either DDX6 or Xrn1 but lacking the other.

3.3.2 DEF6 granules co-localise with P-body markers in a heterogeneous manner

Further to the work in identifying the type of granules formed by DEF6 described previously¹², the co-localisation of DEF6 with both ectopically-expressed markers and endogenous markers of P-bodies and stress granules was determined. DEF6 co-localises with DCP1, supporting previous work by Hey *et al.* which suggests that DEF6 associates with P-bodies. DEF6 did not co-localise with G3BP-1, a marker for stress granules, indicating that it does not have a role to play in stress granules. This also decreases the likelihood that the co-localisation of DEF6 with DCP1 is not merely due to co-expression of the proteins or interactions between their fluorescent tags. The picture was less clear with antibody staining for endogenous P-bodies marked by either Xrn1 or DDX6. DEF6 appeared to partially co-localise with some of the granules marked by these proteins, but not extensively. However, these granules are known to be highly heterogeneous¹²⁹, so whilst it may be able to interact with P-bodies, DEF6 may also form granules separate to them. Additionally, the composition of the granules can change over time, and due to the snap shot nature of the images taken, co-localisation and interaction with P-body components may change over the time course of the arsenite induced translational arrest. As previously mentioned, this is illustrated by the difference in distribution between the two P-

body markers themselves in T cells. DEF6 may be interacting with components of P-bodies that are only found in certain subsets, whilst DDX6 and particularly Xrn1 may be found in a more widespread subset of granules^{93,94}. This may also suggest that DEF6 preferentially interacts with DCP1, hence its full co-localisation with this marker. DCP1 and DDX6 do not always co-localise, indicating different subsets of granules^{130,131}.

3.3.3 Translational regulation in activated T cells

Neither PABP nor eIF4E localise as extensively to the central immune synapse as DEF6, but both can be found there in small amounts and can be found more generally in the area peripheral to it. PABP is distributed in a polarised manner towards the immune synapse side of the cell, and is predominantly localised around the immune synapse, with some present in the immune synapse. The distribution falls within the d-SMAC (distal-SMAC) which falls outside the central recognition zone and peripheral adhesion and architectural organization zone of the immune synapse (c-SMAC and p-SMAC). This area is enriched in proteins with long extracellular domains such as the CD43 and CD45 molecules¹³². eIF4E is distributed throughout both the cytoplasm with granules present that are most likely to be stress granules⁹⁵ although eIF4E has also been shown to sometimes be found in P-bodies⁹³. It is present close to the immune synapse localise and is present in small amounts co-localised with DEF6 at the immune synapse. These results suggest that active translation is happening to some extent within the c-SMAC and p-SMAC of the immune synapse, and there appears to be polarisation of translation towards the contact side of the cell or associated with the d-SMAC area.

The polarised localisation of PABP and eIF4E in activated T cells may indicate some polarised control of translation to the immune synapse, but not directly at the c-SMAC or p-SMAC zones of the immune synapse. In neuromuscular junctions PABP and eIF4E form subsynaptic aggregates indicating localised

control of translation at neuromuscular junctions¹³³. The localisation of Xrn1 also adjacent to the immune synapse suggests an ability for the cell to control translation in a polarised manner after T cell activation. DEF6 appears to have a role directly at the immune synapse rather than adjacent to it, suggesting any potential role for DEF6 in translational regulation may be directly linked to the organization of or signalling from the immune synapse, perhaps in organising polarity. However, the location of the granular form of DEF6 is dispersed throughout the cell, not just at the immune synapse. The link between the immune synapse localised form of DEF6 and the granular form is not yet known. Possibilities include the use of granules to deliver mRNA to the immune synapse, or to sequester specific mRNAs from the immune synapse to control local translation. Further work into characterising the DEF6 granules will help define a role for DEF6 in their function by allowing comparison with defined dynamics of other P-body forming proteins.

3.3.4 The use of granules as delivery vehicles

P-bodies have been shown to be capable of delivery to discrete locations in dendrites, usually via transport along microtubules. This delivery has been shown to be modulated by the activity of the neurones, which also controls the release of certain mRNA from the P-bodies¹³⁴. Of particular interest, the P-bodies modulated the local actin cytoskeleton and thus the structure of the synapse by release of mRNAs related to actin cytoskeletal rearrangement, for example the mRNAs for *Nd1* which encodes for a kelch family protein which stabilizes actin filaments, and *Arp2* which forms a subunit of the Arp2/3 complex which nucleates polymerisation of actin filaments¹³⁴.

Myosin Va is a motor protein involved in actin and microtubule dependent transport which is required for the formation and maintenance of P-bodies¹³⁵. It is thought that it transports mRNPs along the cytoskeleton to P-bodies, facilitating the formation of P-bodies. This is consistent with observations of P-

body movement that indicate P-bodies do not scan the cytoplasm for target mRNAs, and suggests mRNAs are instead delivered to the P-bodies⁹². Other P-body proteins have also been shown to link P-bodies to the actin cytoskeleton including the elongation factor eEf1 α and the dynactin subunit p50, leading to suggestions that whilst microtubules are used by mRNP granules for long-distance travel for example along neurones, actin microfilaments are likely more important for translational polarisation for example in developing embryo and somatic cells¹³⁶.

DEF6 could be linked to this network via its role in organization of actin polymerisation at the immune synapse. Actin filament formation at the immune synapse organised by DEF6 activity may be used by myosin Va to deliver mRNPs to polarise regulation of translation both by eIF4E which has been shown to interact with myosin Va during the formation of P-bodies¹³⁵, and the P-bodies themselves as marked by Xrn1. These granules may further modulate the actin cytoskeleton through post transcriptional regulation of specific mRNAs at the immune synapse to aid in synaptic plasticity. The role of DEF6 within the granules themselves will be further assessed later in this thesis.

3.3.5 Mechanisms for DEF6 recruitment to cell junctions

The strong localisation of DEF6 to sites of cell – cell contact in different cell types seen in Figure 22, suggests that rather than F actin binding directing its targeting, DEF6 may interact with specific adhesion molecules or molecules involved in actin organisation at cell-cell junctions. This is supported by a study by Samson *et al.* who showed that ectopically expressed DEF6 localised to focal adhesions where it overlaps with ectopically expressed laminin-binding $\alpha 7\beta 1$ integrin in C2C12 myoblast cells¹³⁷. This study showed that DEF6 can bind to the intracellular domain of the skeletal muscle integrin $\alpha 7A$ ¹³⁷. Interestingly, only full length DEF6 was able to interact with the cytoplasmic tail suggesting that the interaction is dependent on the three dimensional structure of DEF6 and that

changes to this structure may regulate its binding. Additionally, a phosphomimic form of DEF6 with the Y210E mutation was unable to bind suggesting that the phosphorylation state of DEF6 which changes after T cell activation alters its ability to bind integrins. It was suggested in the paper that this is due to the proposed open and closed model of DEF6 proposed by Gupta *et al*³⁵, with the open phosphorylated form abolishing the 3D structure necessary for integrin binding.

Integrins are made up of two non-covalently linked transmembrane chains, the α chain and the β chain. The $\alpha 7 \beta 1$ chain is found predominantly in muscle tissue¹³⁸, and the $\alpha 7$ splice variant is involved in myoblast differentiation¹³⁹. The Samson study suggested that the role of DEF6 in myoblast differentiation is the regulation of Rac1 in a spatially controlled manner via binding the $\alpha 7 A$ chain of the $\alpha 7 \beta 1$ integrin¹³⁷. DEF6 was found to be expressed throughout a variety of muscle types, however due to our focus on DEF6 expressed in T cells, and the exclusive expression of the $\alpha 7 A$ splice variant in skeletal muscle cells, other potential binding partners for DEF6 must be explored. That these cell-cell contacts take place at both T cell - B cell junctions, T cell - T cell junctions, and also at junctions between Cos7 cells suggesting that rather than a T cell-specific adhesion molecule it is likely to be a more general adhesion molecule. Candidate integrins found in T cells include LFA1 which is a broadly distributed integrin found on all leukocytes and is part of the pSMAC on the T cell side of the immune synapse and interacts with ICAM1 on the antigen presenting cell to promote binding¹⁴⁰. LFA1 is also involved in T cell - T cell contacts and contains the integrin chains $\alpha L \beta 2$. Another possibility is the integrin VLA1 ($\alpha 1 \beta 1$) which is involved in cell-cell adhesion and can be found in fibroblasts as well as in T cells¹⁴⁰. A further, non-integrin possibility is ICAM1 itself, an intercellular adhesion molecule which provides the ligand for LFA1, which although it is

generally thought of as only existing on the antigen presenting cell, it is expressed by T cells and B cells, and also by fibroblasts¹⁴⁰.

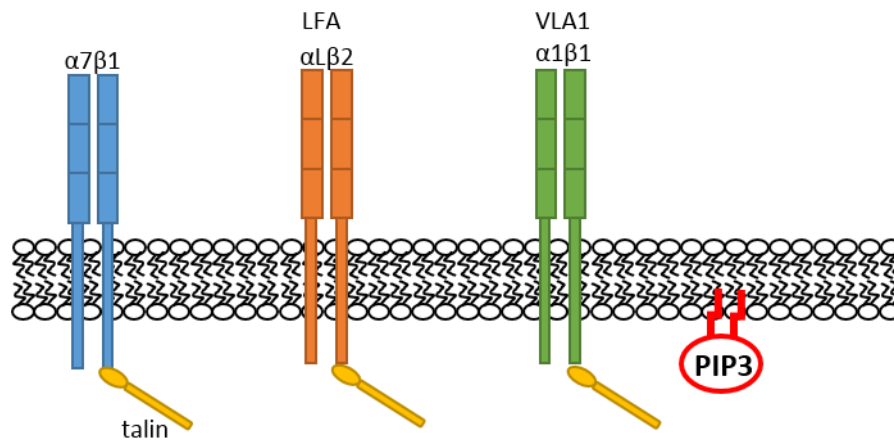


Figure 23 - Potential binding partners for DEF6 at the membrane. DEF6 localises to cell cell junctions, where it has several potential binding partners including the integrins found at T cell junctions including $\alpha 7 \beta 1$, LFA or VLA, or proteins that themselves localise to integrins such as talin. Another possibility is PIP_3 , which has been shown to bind DEF6 and organise its distribution at the immune synapse.

Whilst it is tempting to consider DEF6 interacting directly with the tail region of these integrins, the Samson study showed that DEF6 was unable to bind any other integrin intracellular domains. DEF6 was found to bind specifically to the motif GTVGWDSSSGRST, which is not found in the cytoplasmic tail of integrins other than $\alpha 7$ ¹³⁷. Other possibilities thus include interaction with other proteins that localise with adhesion molecules, in particular those that interact with actin. Cytoskeletal changes are required for the clustering of LFA1 to form the segregated immune synapse. One possibility here is an interaction with the cytoskeletal adaptor protein talin, which localises to integrins. Talin is found at focal adhesions and in lymphocytes at cell - cell contacts and links integrins to the actin cytoskeleton¹⁴¹. Vav1, another T cell Rho-GEF is associated with talin which is in turn bound to inactive VLA1¹⁴². Its dissociation leads to the activation of the VLA1¹⁴³. Unlike the LFA family of integrin like receptors, the VLA family surface expression is not restricted to leukocytes. Vav1 is also linked to the $\beta 2$ integrin subunit, found in LFA1 and MAC1 which induces CDC42, Rac1 and RhoA signalling via Vav1 and Vav3¹⁴⁴. Whilst these molecules provide attractive

potential binding partners for DEF6 that may recruit DEF6 to cell-cell contacts and in particular the immune synapse, there is no data to support the binding of DEF6 to these molecules.

Another potential mechanism for DEF6 localisation to the immune synapse and other cell-cell junctions is via its interaction with $PI(3,4,5)P_3$. DEF6 binds $PI(3,4,5)P_3$ but not $PI(4,5)P_2$ via its PH domain³⁵, an interaction that can be eliminated via mutation of an arginine in the PH domain (R236C). Gupta *et al.* demonstrated DEF6 translocation to the nucleus and via the use of the PI3K inhibitor wortmannin, determined that PI3K activity was necessary for DEF6 translocation to the immune synapse³⁵. However, later work by Becart *et al.* demonstrated that the DEF6 R236C mutant which is unable to bind $PI(3,4,5)P_3$ could translocate to the immune synapse, indicating that $PI(3,4,5)P_3$ binding by the PH domain is not necessary for DEF6 translocation to the immune synapse³⁴. Gupta *et al.* additionally established that phosphorylation of DEF6 at Y210 by Lck was necessary for this translocation. However, Oka *et al.* demonstrated $PI(3,4,5)P_3$ binding without phosphorylation⁶³, though recognised that the phosphoinositide beads used in their study may have been more sensitive than those used in the study by Gupta *et al.*, so it was possible that binding was stronger with phosphorylation. The phosphorylation at Y210 has since been demonstrated to be mediated by Itk rather than Lck by Hey *et al.*¹². Becart *et al.* demonstrated that localisation to the immune synapse required phosphorylation at Y133 and Y144, sites which are phosphorylated by Lck thus indicating that Lck is necessary, but not at the site indicated by Gupta *et al.* Whilst recruitment to the immune synapse may not be mediated by $PI(3,4,5)P_3$ binding, its organization at the immune synapse may be.

A model in which DEF6 is organised at the immune synapse by $PI(3,4,5)P_3$ in order to have a role in organizing actin polymerisation is supported by data showing that actin is organised into a ring formation during immune

synapse formation, a process dependent on Rac1 and Rac2 activity¹⁴⁵ and required for robust engagement with the antigen presenting cell. PI(3,4,5)P₃ demonstrates an annular distribution at the immune synapse accompanying the F actin ring, a distribution which is used to recruit DOCK1 which modulates Rac activity¹⁴⁵. However, the ring of actin persists in the absence of DOCK1 implicating an additional modulator of Rac that can be regulated by PIP3. Le Floc'h *et al.* suggest Tiam1 as a candidate, but their candidate criteria could equally apply to DEF6. This would suggest a model in which DEF6 is recruited to the immune synapse upon T cell activation after phosphorylation by Lck or Itk. Whilst this step may not require PI(3,4,5)P₃ binding, PI(3,4,5)P₃ binding could organise DEF6 into specific patterns after its recruitment, allowing it to organise actin in a PI(3,4,5)P₃ patterned manner. The organisation of DEF6 by PI(3,4,5)P₃ binding by the PH domain could also explain the more widespread distribution of DEF6 seen at the immune synapse than that seen by Singleton *et al.*⁵. In addition to increased Itk levels at the immune synapse in Jurkat cells potentially leading to a wider recruitment of DEF6, the greater amount of PI(3,4,5)P₃ present in the Jurkat cells could account for the widespread rather than highly centralised patterning of DEF6 at the immune synapse seen in this study in these cells. PI(3,4,5)P₃ binding by the PH domain promoting DEF6 localisation patterning although not recruitment could provide polarisation for translational regulation, with further involvement for DEF6 in the regulation via its role in granules. Work has been done on structural changes to the PH domain of SWAP70 upon binding PI(3,4,5)P₃ at the membrane¹⁴⁶. Producing a purified protein construct comprising the PH domain of DEF6 opens the possibility for detailed structural analysis of this part of DEF6s regulatory mechanism. Producing purified protein and furthering the work by Hey *et al.* to study the interactions between the PH domain and Itk feature in the next chapter, whilst more in depth characterisation of the role of DEF6 in granules is described in chapters 5 and 6.

4. Results:

The PH domain of DEF6

4 The PH domain

4.1 Introduction

In addition to its role as a RhoGEF, previous studies have demonstrated that DEF6 can form granules¹². Models for how DEF6 can switch between these two states have been proposed including phosphorylation at the PH domain by Itk leading to the opening of a closed form of DEF6.

The structure of DEF6 is unlikely to be that of a canonical RhoGEF in part due to the domain swap between the PH domain and DHL domain, but also due to the predicted “alpha solenoid” structure of the DHL domain rather than the “chaise longue” structure discussed in section 1.3.2. The predicted solenoid shape of DEF6 where the C-terminus coils back on itself to contact near the PH domain, combined with earlier data showing that DEF6 with phosphomimetic tyrosine mutations at the sites of Itk phosphorylation forms granules, suggests a model in which phosphorylation of DEF6 at the PH domain by Itk allows for a change in conformation that drives the switch in function between GEF activity and granule formation. This work aims to further investigate this potential model, first by investigating the interaction between the PH domain of DEF6 and Itk, and then further investigating the granule forming properties of DEF6.

4.1.1 The PH domain

Pleckstrin Homology (PH) domains were first found as a repeat sequence within Pleckstrin, and has since been found in many proteins¹⁴⁷ providing a wide range of functions with a conserved fold, but little conservation at the sequence level¹⁴⁸. This low sequence identity, and the variety of functions that this one fold can share, has led to the PH domain being described as a superfold¹⁴⁹. Within the PH superfold family are PH domains that bind a variety of ligands including phosphorylated ligands such as inositol phosphates and phosphatidylinositol lipids, polyproline peptides, and Ran, a small GTPase¹⁴⁹. Some PH domains are involved in protein-protein interactions¹⁴⁸. Also within the

PH superfold family are the phosphotyrosine-binding (PTB) domains which bind phosphotyrosine and superimpose onto the PH domain structure¹⁵⁰. These various ligands bind at different sites on the PH domain.

4.1.2 PH domain fold

The PH domain fold consists of a β sandwich comprised of 7 β -strands, and an α -helix that caps the sandwich¹⁵¹. The loops between the β -strands are of variable length, and these loops are where most variations in structure between PH domains occur. Three of these loops are hypervariable and are able to accommodate large inserts, even multiple extra domains as in the insertion of 3 SH domains between the β -3 and β -4 strands in one of the PH domains of PLC γ .

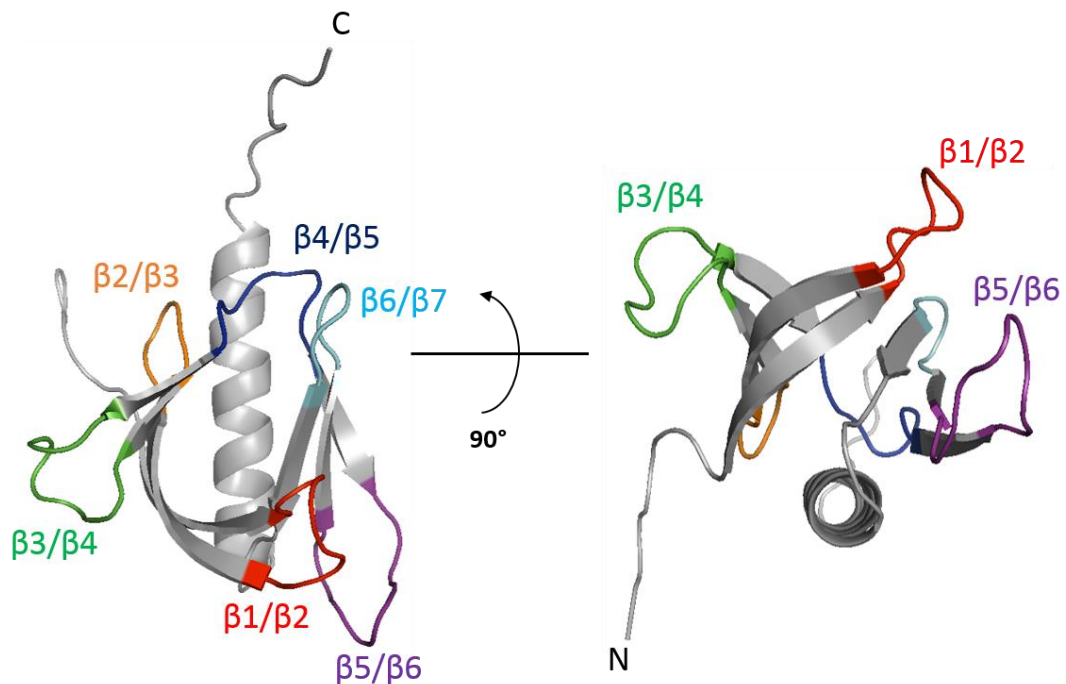


Figure 24 - The structure of the PH domain of SWAP70 (amino acids 211-312) indicating the loop numbering used to describe the PH domain. SWAP70 Protein Data Bank Code: 2DN6⁸¹

The little sequence conservation that exists consists of an almost invariant tryptophan residue in the capping α -helix, positions that usually contain hydrophobic or aromatic groups involved in packing the core of the protein, and a general trend for basic residues on the side of the domain opposite the α -helix,

and acidic residues at the end of the sandwich next to the helix that leads to electrostatic polarisation of the protein¹⁵².

4.1.3 PH domain activity

PH domains have typically been known as phosphatidylinositol binding domains. However, most PH domains only bind phosphoinositides with very low affinity and specificity, if at all¹⁵³. Those that do interact with phosphoinositides or their head groups can use this for recruitment to the membrane, with those specifically binding phosphatidylinositol(3,4,5)-phosphate (PI(3,4,5)P₃) being recruited upon phosphoinositide 3-kinase (PI3K) activation which produces PI(3,4,5)P₃. At the immune synapse PI(3,4,5)P₃ accumulates in an annular formation via the activity of Ras and a class 1A PI3K¹⁴⁵. The PH domain of DEF6 has been shown to bind PI(3,4,5)P₃ via cell pull downs using PI(3,4,5)P₃ beads^{35,63}. Mutational analysis put the binding site at the β 1/ β 2 loop which has several positively charged amino acids.

The previously mentioned electrostatic polarisation allows characterisation of the domains into two groups¹⁴⁸. Those that bind phospholipids have a positive potential around the loops involved in IP₃ binding. Also in this group are others that have not been shown to bind IP₃ but are positively charged in the corresponding loops. The second group contains PH domains linked to DH domains, which have positive potential surrounding the C-terminal helix, and a negative potential around the loops that in IP₃ binding PH domains are positive.

As stated earlier, all Rho family GEFs contain both a PH and DH domain. Like other PH domains, DH associated PH domains in GEFs can have a variety of roles⁷¹. Some appear to be involved in membrane targeting although DH associated PH domains have low phospholipid affinity and specificity, some increase the GEF activity of the DH domain and some are involved in protein-protein interactions. The protein-protein interactions mainly function as docking sites for proteins involved in the signalling cascades regulated by the Rho

GTPase that the GEF is acting on. The PH domain of DEF6 appears to have some regulatory role on the DHL domain, as its removal leads to constitutively active actin polymerisation¹⁵⁴. In Dbs, the PH domain directly contacts the small GTPase CDC42¹⁵⁵, and disruption of this interaction prevents activation of CDC42¹⁵⁶.

4.1.4 Phosphorylation of DEF6 by Itk

Itk is a Tec family kinase that localises to the immune synapse, and has been shown to phosphorylate DEF6 at tyrosine 210 and 222. Tyrosine 222 is within the PH domain, and 210 just N terminal to it¹².

Itk has been shown to require remote docking sites on its target proteins to facilitate phosphorylation^{67,157}. These confer specificity, and increase the affinity of the kinase for the substrate. The specificity is necessary as both the amino acid sequence and the structural context around the target tyrosines differ between protein targets¹⁵⁷. The target tyrosine in phospholipase C γ is found in a linker region between an SH2 and an SH3 domain, the target tyrosine on the autophosphorylated tyrosine on Itk is within its SH3 domain, and the target tyrosines on DEF6 are within its PH domain. The binding site has been mapped on both phospholipase C γ and on Itk for its autophosphorylation. On each of these targets, the binding site comprises of a contiguous patch of basic amino acids remote from the ligand binding pocket⁶⁷. They act by increasing the affinity for the enzyme-substrate complex.

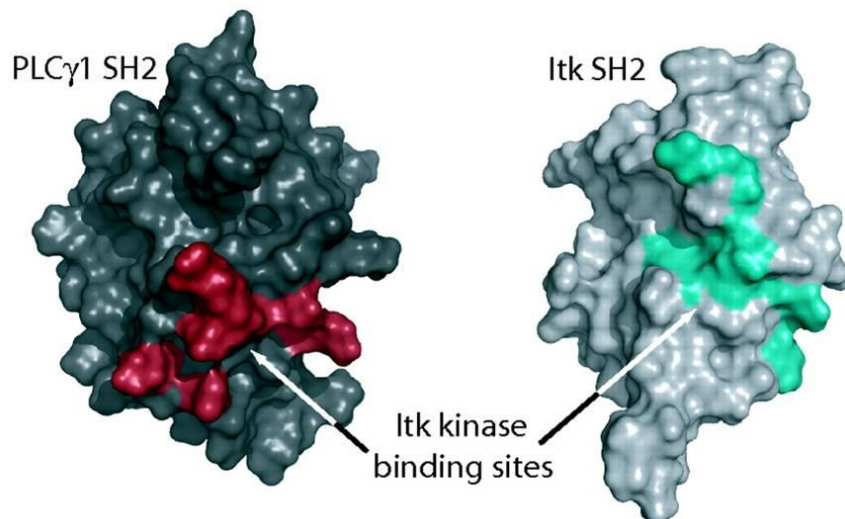


Figure 25 – Itk docks to its substrates at a positively charged binding site. The positively charged binding sites for Itk on the phospholipase Cy SH2C domain (red) and the Itk SH2 domain (cyan) are shown here. Image from Min *et al.* (2009)⁶⁷.

For phospholipaseCy, the corresponding binding site on Itk has been found, and is as expected, a contiguous patch of negatively charged amino acids⁶⁸. Itk is made up of a PH domain, a Tec homology domain, a SH3 domain, a SH2 domain and the kinase domain which is directly responsible for its activity. The binding patch for its interaction with phospholipaseCy is found in the kinase domain, on an alpha helix termed the G helix. The C-terminal lobe, in particular the G helix of the kinase domain is the recognition site on a range of kinases. The G helix is ideal due to its flexibility, its exposure and its proximity to the kinase active site. Itk has other docking sites, for example for the autophosphorylation of the SH3 domain a surface on the back of the kinase domain is used to recognise a basic region on the SH3 domain. Multiple docking surfaces may allow for distinction between substrates.

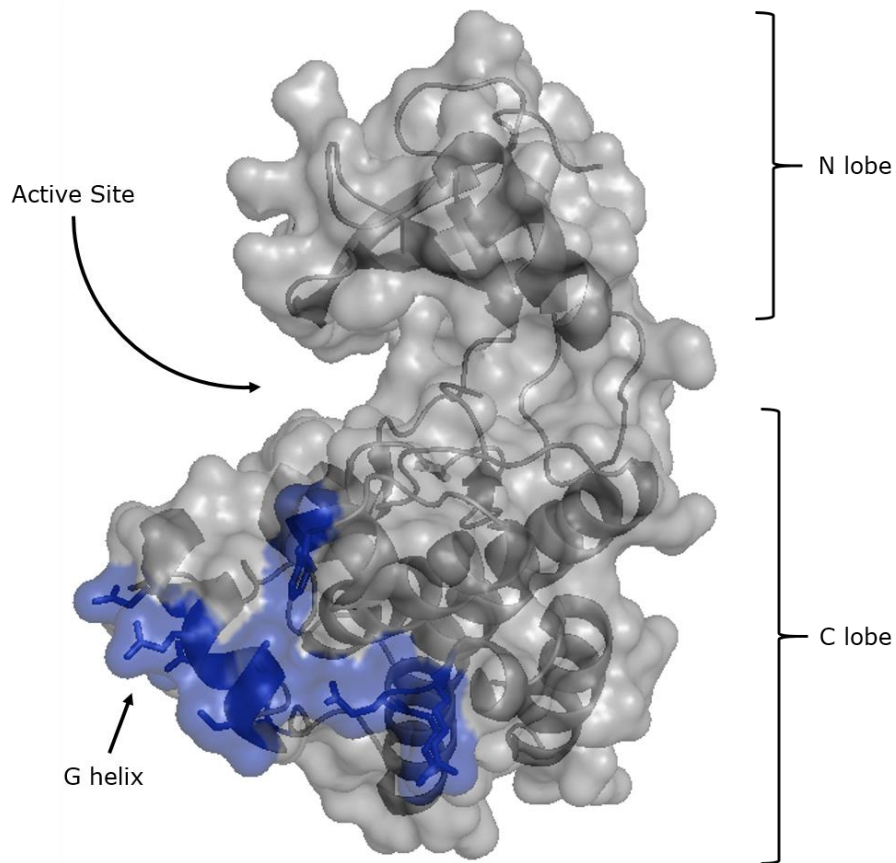


Figure 26 – Itk docks to its substrates using a negatively charged patch on its kinase domain which interacts with a positively charged patch on the substrate. The kinase domain of Itk with the residues involved in docking interactions with PLC γ are shown here coloured in blue. Itk Protein Data Bank Code: 1SNU¹⁵⁸

When considering whether DEF6 similarly has a binding site for Itk separate to the target tyrosines, a helix with the sequence KRREQREQRERRR immediately C terminal of the PH domain was identified (F. Hey, unpublished), which acts as a nuclear localisation signal. This helix is contiguously positively charged along one side of the helix, and so is a good candidate for a binding site for Itk.

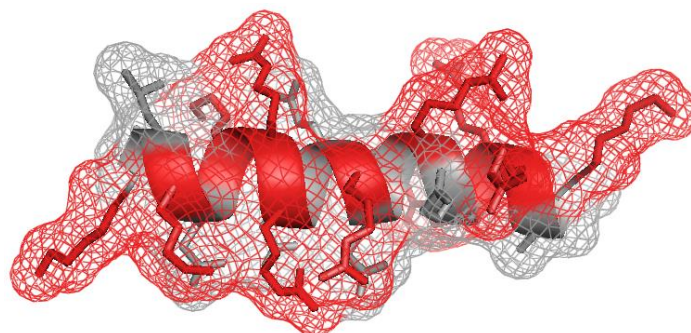


Figure 27 – DEF6 is a substrate for Itk, and has a potential docking site at its PH domain made up of a contiguous patch of positively charged amino acids on the

nuclear localisation signal helix C terminal to the PH domain. These negatively charged amino acids are highlighted here in red. Model produced by submission of the amino acid sequence for 205-342 to I-TASSER

By producing a purified protein construct of the PH domain with and without this helix, we can ascertain whether it is the binding site for Itk, necessary for Itk phosphorylation of the PH domain.

4.2 Results

4.2.1 The PH domain of DEF6 is most similar to that of SWAP70

PH domains, whilst sharing structural similarity do not have strongly conserved sequences (Figure 28). The PH domain of DEF6 is most similar to that of SWAP70, a homolog of DEF6 found in B cells, with 55.67% sequence identity with the next most similar being the pleckstrin PH domain 2, at 32.99%.

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DEF6          DVLKQGYLWKRGHRR-----NWAERWFQLQPSC--LCYFGSEECKEK--RGIIPIDAH- 50
SWAP70        DVLKQGYMMKKGHRRK-----NWTERWFVLKPNI--ISYYVSEDLDK--KGDILLDEN- 50
Pleckstrin_PHdom1 KRIREGYLVKKGSVFN-----TWKPMWVVLLEDG--IEFYKKKSDNSP--KGMIPKGS- 50
Pleckstrin_PHdom2 VTIKQGCLLKQGHRRK-----NWKVRKFILREDPAYLHYYPAGAEDP--LGAIHLRGC- 52
Vav1          RPKIDGELKITSVERR-----SKMDRYAFLLDKA--LLICKRRGDSYD--LKDFVNLHSFQ 52
SKAP_HOM      FVLKAGYLEKRRKDHSLFLG--FEWQKRWCALSKTV--FYYYGSDKDQKQ--KGEFAIDGYS 55
RhoGEF2       KLIHDGCLLWKTATGR-----FKDVLVLLMTDV--LVFLQEKDQKYI--FPTLDKPSV 50
Itk           FILLEEQLIKKSQQKRRTSPSNFKVRFFVLTAKS--LAYFEDRHGKKRTLKGSIELSRI- 57
               :                               *           :           .

DEF6          CCVEVLPDRDGKR----CMFCVKTANR--TYEMSASDTRQRQEWTAAIQMAIR 97
SWAP70        CCVESLPDKDGKK----CLFLVKCFDK--TFEISASDKKKKQEWIQAIHSTIH 97
Pleckstrin_PHdom1 TLTSP-CQDFGKR---MFVFKITTTKQDHEFFQAAFLEERDAWVRDIKKAIK 98
Pleckstrin_PHdom2 VVTSVESNSNGRKSEENLFEIITADEVHYFLQAATPKERTEWIRAIQMASR 104
Vav1          VRDDSSGDRDNKK--WSHMFLIEDQGAQGYELFFKTRELKKKWMQFEMAI 103
SKAP_HOM      VRMNTTLRKDGKK---DCCFEISAPDKRIYQFTAAAPKDAEEWVQQLKFVLQ 104
RhoGEF2       SLQNLIVRDIANQ--EKGMFLLISAAPPEMYEVHTASRDDRSTWIRVIQSVR 100
Itk           KCVEIVKSDISIPCHYKYPFQVVDNYLLVVFAP--DRESQRWVLALKEETR 108
               .                               :           .           *           :

```

Figure 28 – PH domains demonstrate low sequence homology.

Based on this alignment and the known structure for the PH domain of SWAP70, the PH domain of SWAP70 can be used to model amino acid positioning in the PH domain of DEF6.

4.2.1.1 A model of the PH domain of DEF6 can be produced using SWAP70 as a scaffold

A structure has been solved for the PH domain (amino acids 211-312) of SWAP70 by Li *et al.* (PDB number 2DN6, Figure 24)⁸¹. Using this structure to model the structure of the PH domain of DEF6 we can gain a picture of the potential interactions, particularly around the Y222 phosphorylation site. SWAP70 contains a tyrosine at amino acid 216 equivalent with Y222 in DEF6 based on the amino acid alignment but no record of phosphorylation at this site is found on phosphosite.org. The equivalent amino acid in SWAP70 to the tyrosine 210 site in DEF6 is a phenylalanine. As the Y210 site is outside the PH

domain, it did not map onto the SWAP70 PH domain structure which does not cover these amino acids so data was not obtained for its position relative to the rest of the PH domain.

DEF6	204	MAIHEVYQELIQDVLKQGYLWKRGHLRRNWAERWFQLQPSCLCYFGSSECKEKRGIIPLD	263
		MAI+EV+ ELI DVLKQGY+ K+GH R+NW ERWF L+P+ + Y+ SE+ K+K+G I LD	
SWAP70	198	MAINEVFNELILDVLKQGYMMKKGHRKNWTERWFLKPNIIISYYVSEDLKDKKGDILLD	257
		β 1 β 2 β 3 β 4	
DEF6	264	AHCCVEVLPDRDGKRCMFCVKTANRTYEMSASDTRQRQEWTAAIQMAIRLQAEGKTSLHK	323
		+CCVE LPD+DGK+C+F VK ++T+E+SASD +++QEW AI I L G HK	
SWAP70	258	ENCCVESLPDKDGKKCLFLVKCFDKTFEISASDKKKQEWIQAIIHSTIHLKLGSPPHK	317
		β 5 β 6 β 7 helix	
DEF6	324	DLKQKRREQR-----EQRERRRAAKE	344
		+ +Q+R+E R EQ E R KE	
SWAP70	318	EARQRRKELRKKQLAEQEELERQMKE	343

Figure 29 - Amino acid alignment of DEF6 and SWAP70 with the secondary structural features of the known SWAP70 structure indicated.

Using the Li structure as a template, the SWISS-MODEL server was used to predict a structure for the PH domain of DEF6 (Figure 30). SWISS-MODEL is a fully automated server that provides homology modelling of protein structures.

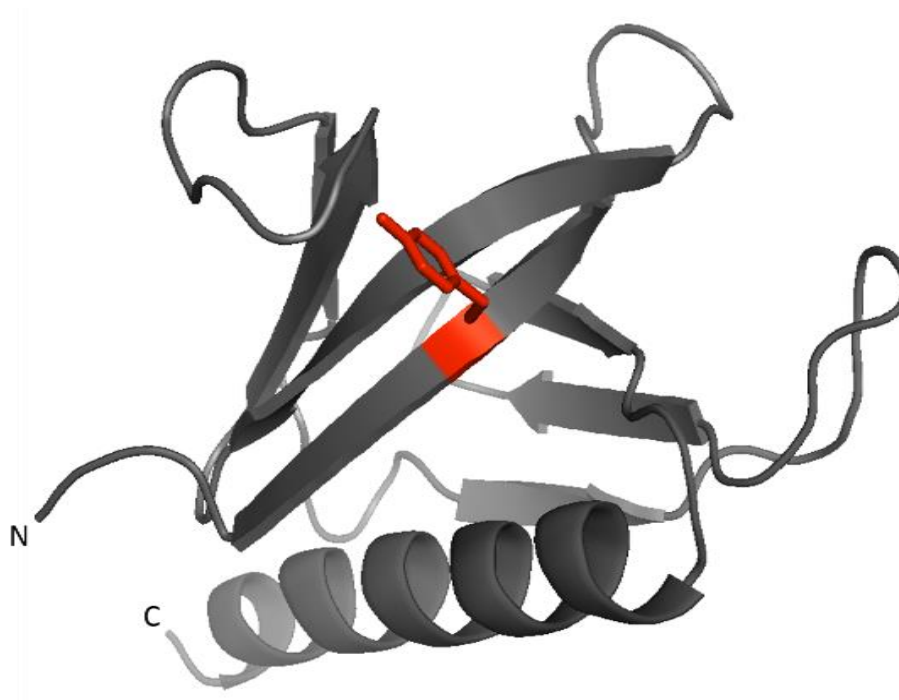


Figure 30 - PH domain of DEF6 (amino acids 215-317) modelled on the PH domain of SWAP70. The model of the PH domain of DEF6 was produced using the SWISS-MODEL server and the PDB file for the PH domain of SWAP70 (2DN6). The tyrosine 222 residue is indicated in red.

This structure is likely to be a good prediction of the PH domain of DEF6 due to the functional and sequence similarities between the two proteins. Both proteins are phosphorylated by Tec family kinases (Itk in the case of DEF6 and Btk in the case of SWAP70), and both proteins bind to membrane bound phosphoinositides via their $\beta 1/\beta 2$ loops^{35,146}. The structure of PH domains are highly conserved and the predicted structure conforms to the expected PH domain fold. Differences are likely to be found within the flexible loop regions, but due to the similarities between the two sequences, the modelling should be fairly accurate.

4.2.2 Production of PH domain protein for interaction studies with Itk

Hey *et al.* showed that Itk phosphorylates DEF6 at tyrosine 210 and 222, that Itk and DEF6 interact *in vitro*, and that *in vivo* Itk interacts with the PH domain of DEF6 when co-transfected in Jurkat T cells and in Cos7 cells¹². To further study this interaction, purified PH domain protein was produced for interaction and structural studies.

Kinases recognise their targets via motifs often outside the target phosphorylation site. Itk often uses SH2 domains as a recognition site, but DEF6 does not contain an SH2 domain. However, it does contain an area of contiguous positive charge, similar to the area on SH2 domains bound by Itk. This is located in the nuclear localisation signal directly C terminal to the PH domain. The only other known protein to contain a nuclear localisation signal in close proximity to a PH domain is BRDG1, a protein phosphorylated by the B cell specific tyrosine kinase, Btk. To study whether this helix is required for Itk binding and phosphorylation of DEF6, protein constructs were made with (205-343) and without (205-315) this helix (Figure 31).

A protein construct was also made with glutamic acids replacing the two tyrosine sites phosphorylated by Itk to mimic the phosphorylated protein. A GFP tagged DNA construct containing the Y210E/Y222E mutations has been made previously by Fiona Hey and used to demonstrate aggregate formation, leading to the

suggestion that the phosphorylation by Itk drives the aggregation of DEF6 into granules. This protein construct is designed to compare with the unphosphorylated PH domain as a mimic of the PH domain phosphorylated by Itk.

Three protein constructs were produced each in the pGEX-3T vector producing N-terminal GST tagged protein with a thrombin cleavage site. The pRSET-A vector was originally used with a His₆ tag, but the GST tag helped produce soluble protein and cleavage was more successful than elution of the His tagged protein for removal from the affinity column.

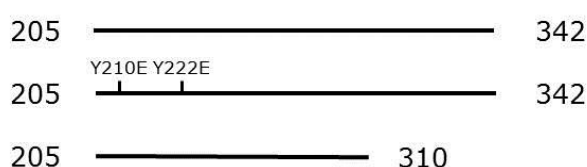


Figure 31 – Schematic representation of the three protein constructs made, with the nuclear localisation signal (top), with the mutations to mimic phosphorylation by Itk (middle) and without the nuclear localisation signal (bottom). Numbers refer to amino acids.

There were ongoing problems with producing soluble protein, similar to those seen by the Andreotti group producing the PH domain of Itk¹⁵⁹. The initial attempts at purification were with the His₆ tagged protein, which had solubility issues with the protein found in the insoluble fraction upon lysis of the cell pellet. Attempts to purify the protein via a refolding method failed due to issues with elution of the protein bound to the beads. Altering the initial growth conditions to use lower temperatures and slow down expression led to an increase in solubly expressed protein, but there were still elution issues once the His₆ tag bound to either a cobalt or nickel column. High salt (0.5M NaCl) conditions helped elution although a large amount of protein was still bound to the column. Due to the issues with elution, cloning into a pGEX vector to have a GST tag with a thrombin cleavage tag was successfully performed, and the protein was both expressed more solubly when expressed at 20°C, and easily cleaved from the

column. However, when switching to M9 media in preparation for production of labelled protein for planned NMR experiments, solubility problems reappeared, but further down the purification process.

In general, growth in the C41 strain of *E.coli* with expression at 20°C produced wet pellets of between 4.6-5.9g. The average purified protein yield for each protein construct was 5mg for 205-342, 3.5mg for 205-342-Y210E Y222E and 3.8mg for 205-315

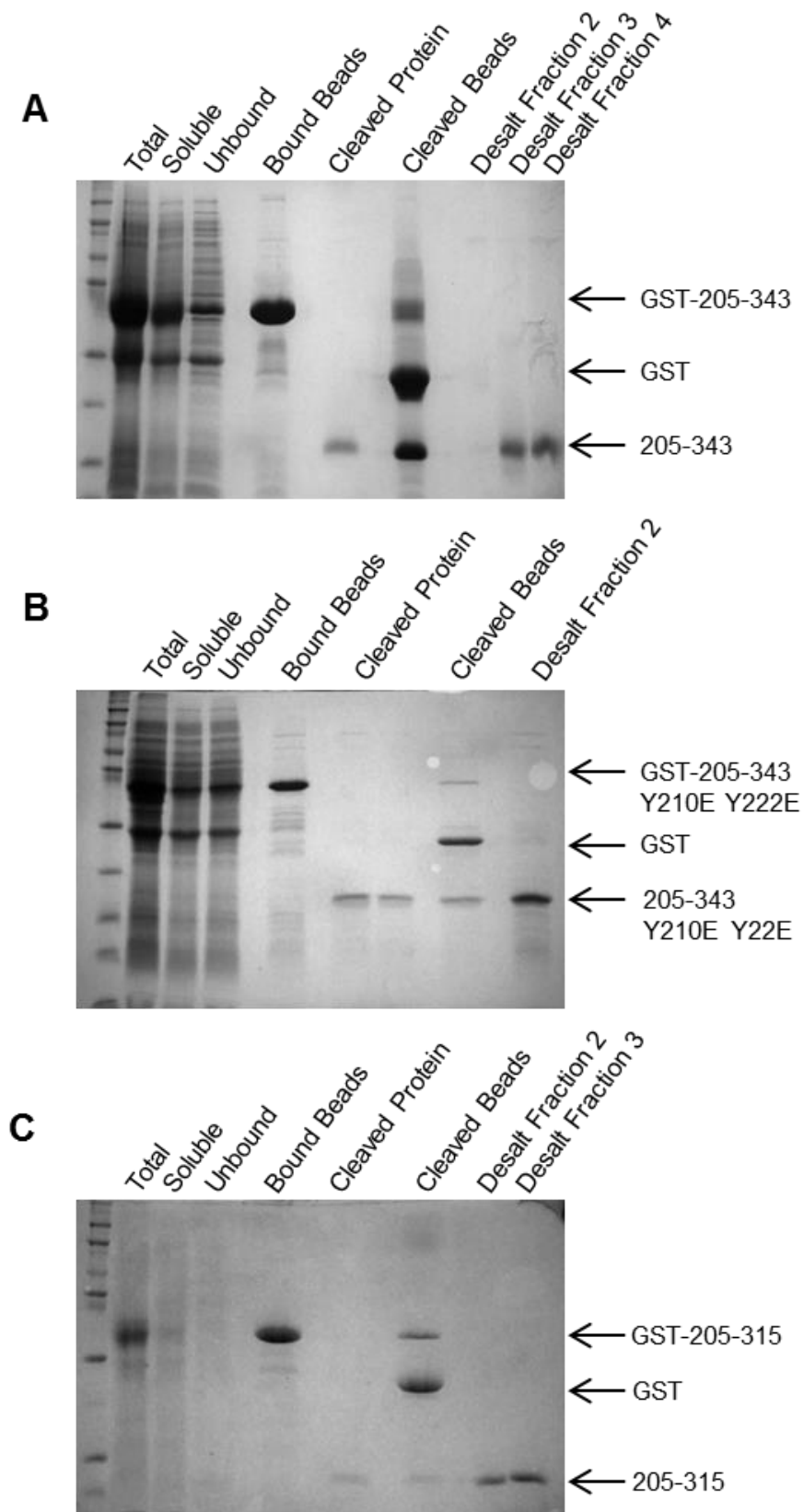


Figure 32 - Purification of GST tagged PH domain constructs. Purification was performed as described in the materials and methods (2.4.3). 3 Protein constructs (A) 205-343 (+NLS), (B) 205-343 Y210E/Y222E and (C) 205-315 (-NLS) were purified and each purification fraction analysed by SDS-PAGE.

The pure protein can be used for direct binding and phosphorylation studies by Itk, direct binding of PH domain to phospholipids, and studies of conformational changes upon phosphorylation.

4.2.3 Validating the purified protein

The purified protein was characterised before studying its interactions, to ensure that the protein constructs used in experiments were the expected size, and were folded correctly.

4.2.3.1 Mass Spectroscopy

The purified proteins were analysed using ESI-TOF mass spectroscopy to confirm their molecular mass. All 3 protein constructs were of the expected mass (Figure 33).

The 205-343 protein construct seen in Figure 33A exhibited higher charge states, which are missing in the spectra of the 205-315 protein construct. This is likely to be due to the extra NLS helix which has a large number of charged residues.

The 205-343 Y210E/Y222E protein construct whilst exhibiting the expected mass in the main charge envelope exhibits a large degree of breakdown. It is also less soluble than the other two protein constructs, suggesting the Y210E/Y222E mutations may destabilize the domain.

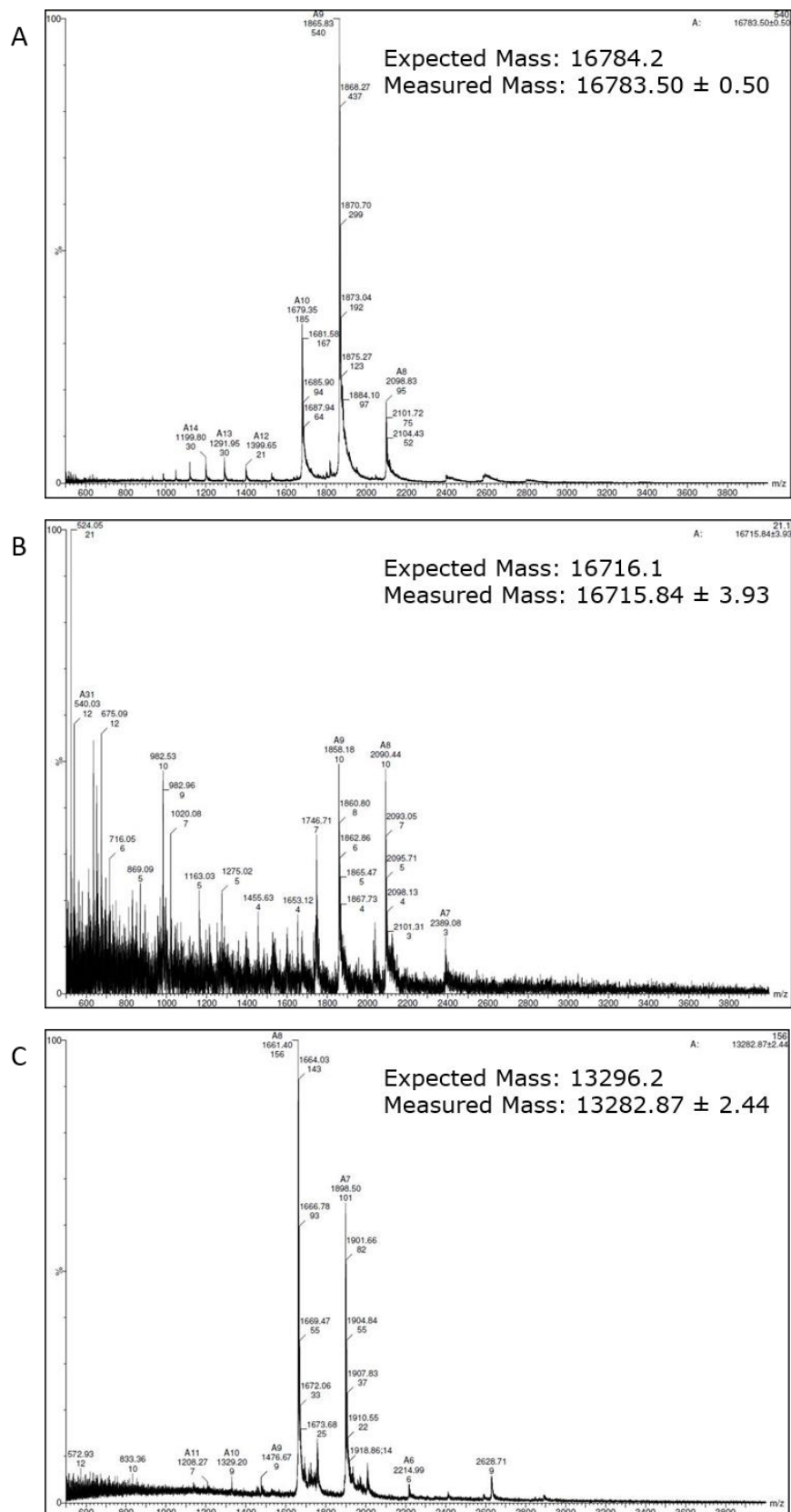


Figure 33 - ESI-TOF mass spectra of (A) 205-343 (B) 205-343 Y210E/Y222E (C) 205-315. The masses measured are in all cases close to the expected mass. The phosphomimic protein construct demonstrates degradation (B), while higher charge states due to the charged NLS are visible in (A)

4.2.3.2 NMR

A ^1H NMR spectrum was obtained for the 205-315 protein construct (without NLS) to confirm the presence of folded protein, and assess the suitability of the protein for further NMR studies.

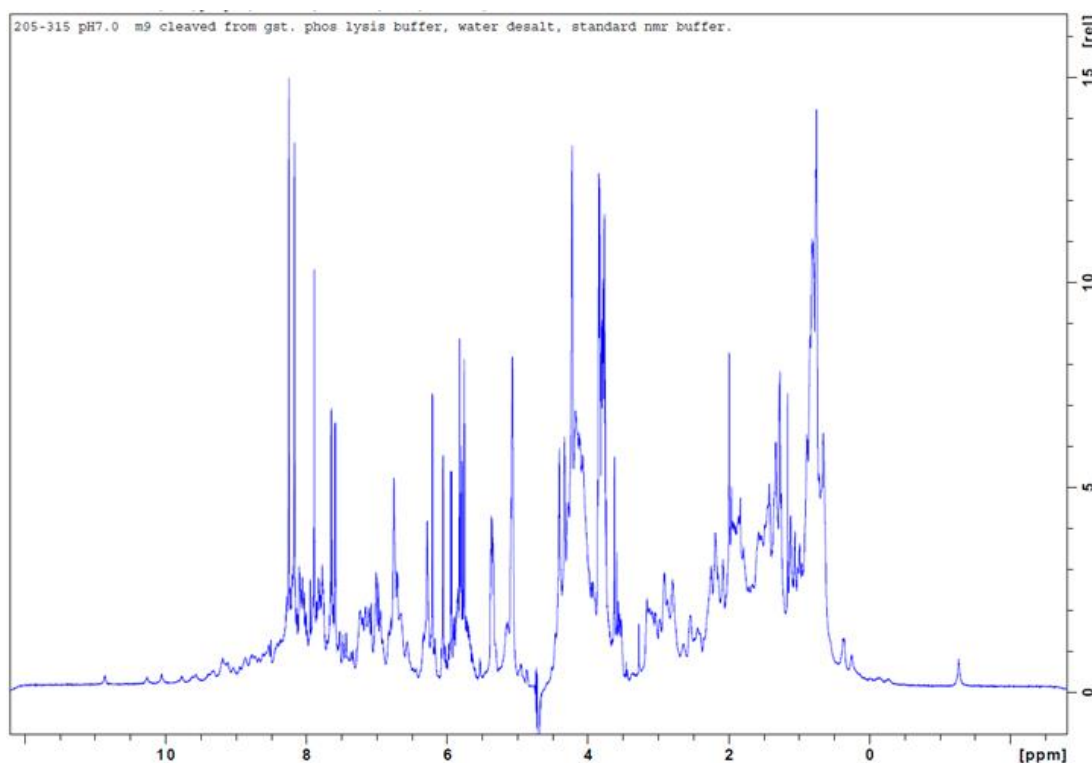


Figure 34 - ^1H NMR spectra of 205-315 PH domain protein construct (without NLS) shown well folded protein as indicated by the distinct, well dispersed peaks.

As can be seen in Figure 34, the protein was folded, exhibiting distinct and well dispersed peaks (NH resonances > 9 ppm, and ring current shifted methyl resonances as low as -2.5 ppm are evidence of hydrogen bonded secondary structure and packing of residue side chains). However, leaving the protein for 5 days, the signal decreased significantly and degradation of the protein sample was evident both in the spectra and in the sample tube with white precipitate forming over time (Figure 35).

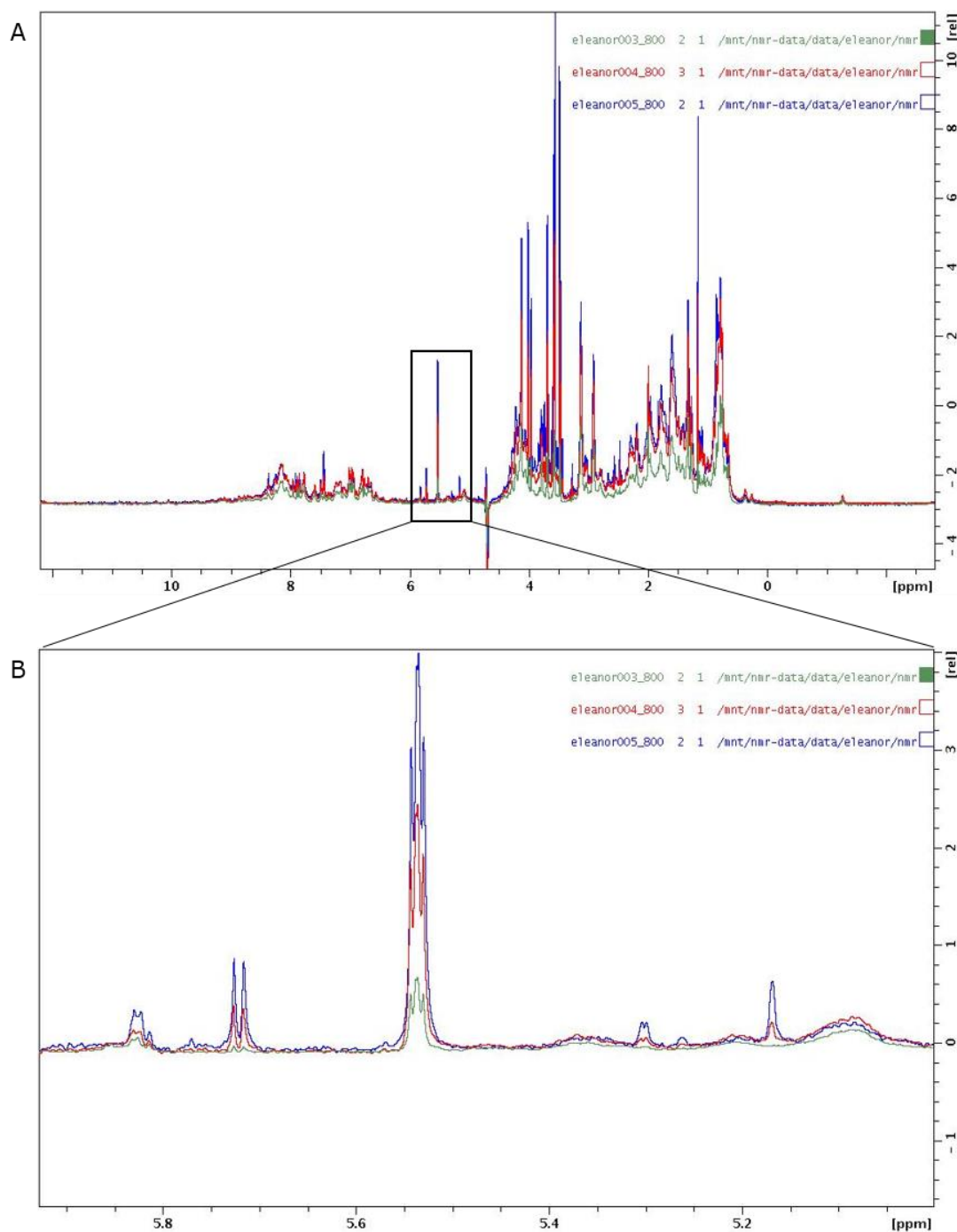


Figure 35 - ¹H NMR spectra of 205-315 protein construct (without NLS) at day 1 (blue) day 4 (red) and day 6 (green) showing a decrease in soluble protein. The section chosen for enlargement was chosen to best show the decrease over time of signal.

Boyken *et al.* found similar problems with precipitation of their PH domain construct of Itk after purification. Their problems were solved by mutating residues in the C-terminal alpha helix of the Itk PH domain to make it more similar to the PH domains of Tec or Btk. Despite the issues this poses with how

representative of the DEF6 PH domain mutations to this region may make the protein construct, for studies involving PI(3,4,5)P₃ binding in particular this route to stability may be worth following in future as the interactions with PI(3,4,5)P₃ are through the loops between β sheets at the opposite side of the protein. For the planned binding and kinase studies with Itk, the degradation of the DEF6 PH domain over several days is not a concern as the experiments happen over a much shorter time scale.

4.2.3.3 Circular Dichroism

To assess the secondary structure of the purified protein, circular dichroism was used and the trace compared to the DichroWeb data base of CD traces for proteins with known secondary structure¹⁶⁰.

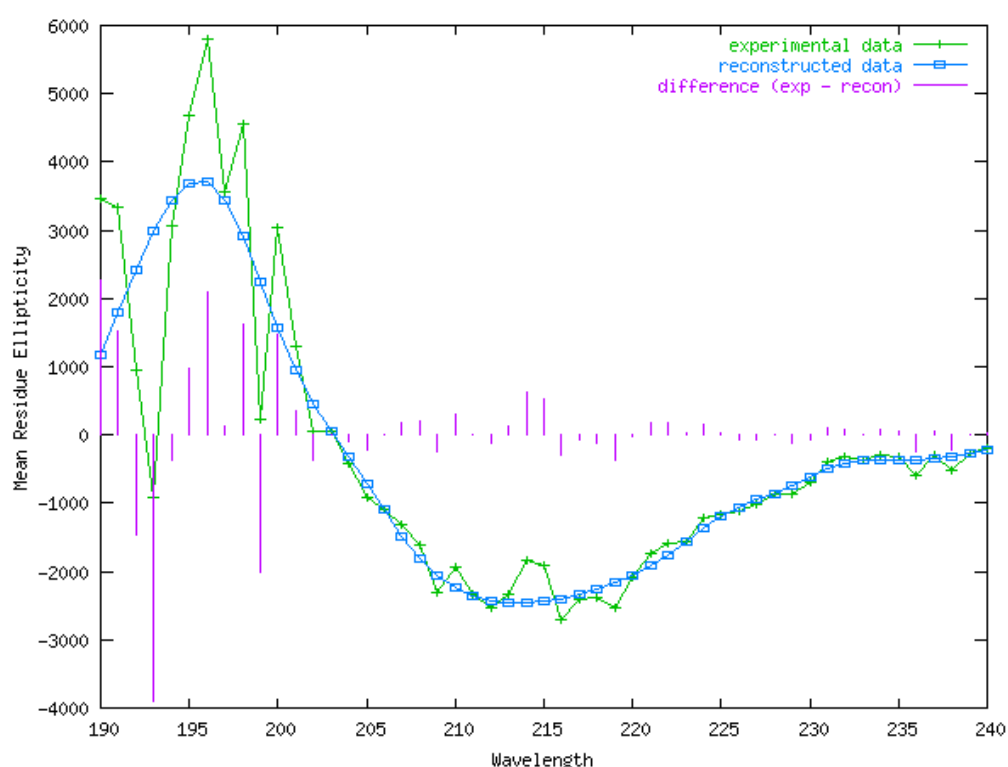


Figure 36 - CD spectrum of 205-315 PH domain protein construct (without NLS) showing the collected data (green) and reconstructed data (blue) for calculating the structural content. The pink lines indicate the differences between the collected and reconstructed data. The data collected was used to calculate the percentage sheet and helical content in the 205-215 PH domain construct.

The experimental data fitted well with the reconstructed data. The calculated secondary structure from this nearest fit compared well with both the

percentage sheet and helix predicted from the secondary structure, and the content in the known structure for SWAP70.

Table 3 - The percentage of sheet and helical structure calculated from the CD results corresponds closely to the values predicted from the sequence for DEF6 and those in the known structure of DEF6

	Sheet	Helix
Calculated from CD	39.5%	8.8%
Predicted from Sequence	42%	15%
Nearest homolog (SWAP70)	34.8%	17.4%

This suggests the purified protein is correctly folded into the PH domain fold, and thus suitable for binding and kinase studies with Itk.

4.2.4 Purified PH domain is sufficient to bind Itk

PH domain protein was produced to probe whether binding of Itk to the PH domain was a direct interaction or mediated by an intermediary. The PH domain protein constructs were covalently bound to cyanogen bromide activated sephradex beads. First the protein constructs were tested for Itk binding in Jurkat cell lysate.

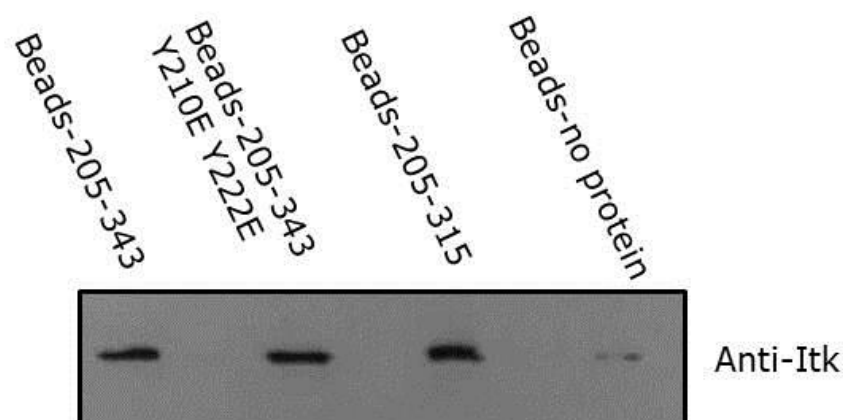


Figure 37 - Itk is able to bind to all three PH domain protein constructs. Jurkat lysate was incubated with purified PH domain protein constructs covalently attached to beads. After incubation and washing, pulldowns were run on SDS-PAGE gel before transferring to membrane via Western-Blotting. Itk was detected with Anti-Itk.

All three protein constructs pulled down endogenous Itk from JURKAT cell lysate (Figure 37). This suggests that the purified protein is correctly folded as Itk is able to recognise and bind to it. Interestingly, both the Y210E/Y222E and 205-315 (without NLS) protein constructs were able to pull down Itk. Measuring the relative intensities of each band, the 205-343 Y210E Y222E protein band was the most intense.

Table 4 - Relative intensity of bands from Figure 37 as measured in ImageJ

	Beads-205-343 (+NLS)	Beads-205-343 Y210E Y222E	Beads-205-315 (-NLS)	Beads-no protein
Relative Intensity	6.8	7.5	6.9	1

That the Y210E/Y222E protein construct is able to bind Itk suggests that the phosphorylation event by Itk does not induce dissociation of Itk. Slow dissociation of kinase after phosphoryl transfer is used by some proteins as a method of inhibiting phosphatase activity¹⁶¹.

The binding of the 205-315 (without NLS) protein construct to Itk suggests that the nuclear localisation signal is not the positively charged patch necessary for Itk binding to DEF6. However, this requires further investigation as it may be the

case that whilst it is not necessary for binding, it may be necessary for correct positioning for kinase activity. To investigate this, the 205-315 protein was then used in an *in vitro* kinase assay to ascertain whether Itk and the PH domain alone are necessary for phosphorylation and whether the nuclear localisation signal is necessary for the phosphorylation of the DEF6 PH domain by Itk. Each purified protein construct was combined with GST tagged Itk active kinase domain and incubated with 6 μ Ci of γ -[³²P]ATP. GST-PLC γ was used as a positive control substrate for Itk phosphorylation³⁰. After incubation with the kinase for 15 minutes the proteins were separated using SDS-PAGE. The polyacrylamide gels were dried and X-ray film exposed to the dried films in the dark. Exposed regions of the film indicate the location of γ -[³²P].

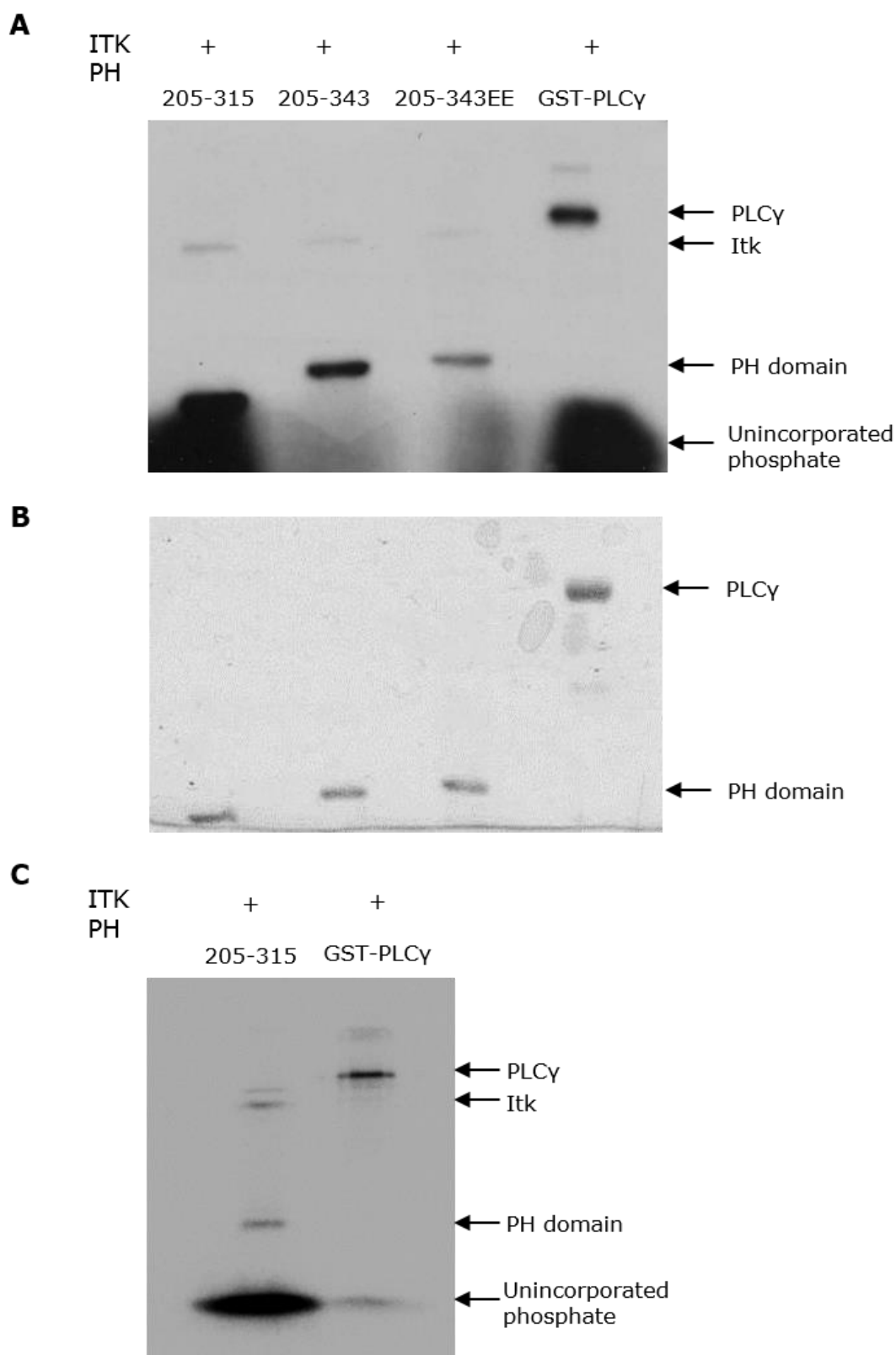


Figure 38 – Itk can phosphorylate all three PH domain protein constructs, although the Y210E Y222E is phosphorylated to a lesser extent. (A) film was exposed to a dried SDS-PAGE gel of separated proteins after the kinase assay (B) proteins were separated by SDS-PAGE gel electrophoresis to show protein loading (same amount of protein as in A), (C) exposure of a repeat of the kinase experiment using 205-315 protein construct due to obscured band in A

Itk is able to phosphorylate all three PH domain protein constructs, indicating that the nuclear localisation signal is not necessary for the kinase activity of Itk on DEF6 (Figure 38A+C). The 205-315 protein construct was partially obscured by the signal from unincorporated phosphate in Figure 38A, so a repeat in which much of the unincorporated phosphate was removed, and the protein run for longer on a higher percentage poly acrylamide gel is shown in Figure 38C to demonstrate that the band in Figure 38A is definitely phosphorylated 205-315. Figure 38B shows an SDS-PAGE gel with the same amount of protein as loaded in Figure 38A, which shows that 205-315 is phosphorylated to the same extent as 205-343. It also shows that whilst 205-343EE can be phosphorylated, it is to a much lesser extent than the wild type protein constructs. That this protein construct can be phosphorylated suggests that it is being phosphorylated by Itk at sites other than 210 and 222. No other phosphorylation sites in the PH domain have been shown for Itk previously, suggesting that this phosphorylation is due to either an excess amount of Itk phosphorylating wherever possible despite a non-favourable interaction, or it could be that the specificity of Itk is partially determined by factors outside the PH domain of DEF6, allowing phosphorylation of non-standard tyrosine residues when these regulatory regions are removed.

4.2.5 Mimicking phosphorylation of DEF6 by Itk leads to aggregate formation

The tyrosine sites phosphorylated by Itk in DEF6 were mutated to glutamic acid residues by Fiona Hey¹². Mutation of tyrosines Y210 and Y222 to glutamic acid to mimic phosphorylation by Itk were found to induce aggregation of DEF6 when expressed in Cos7 cells.

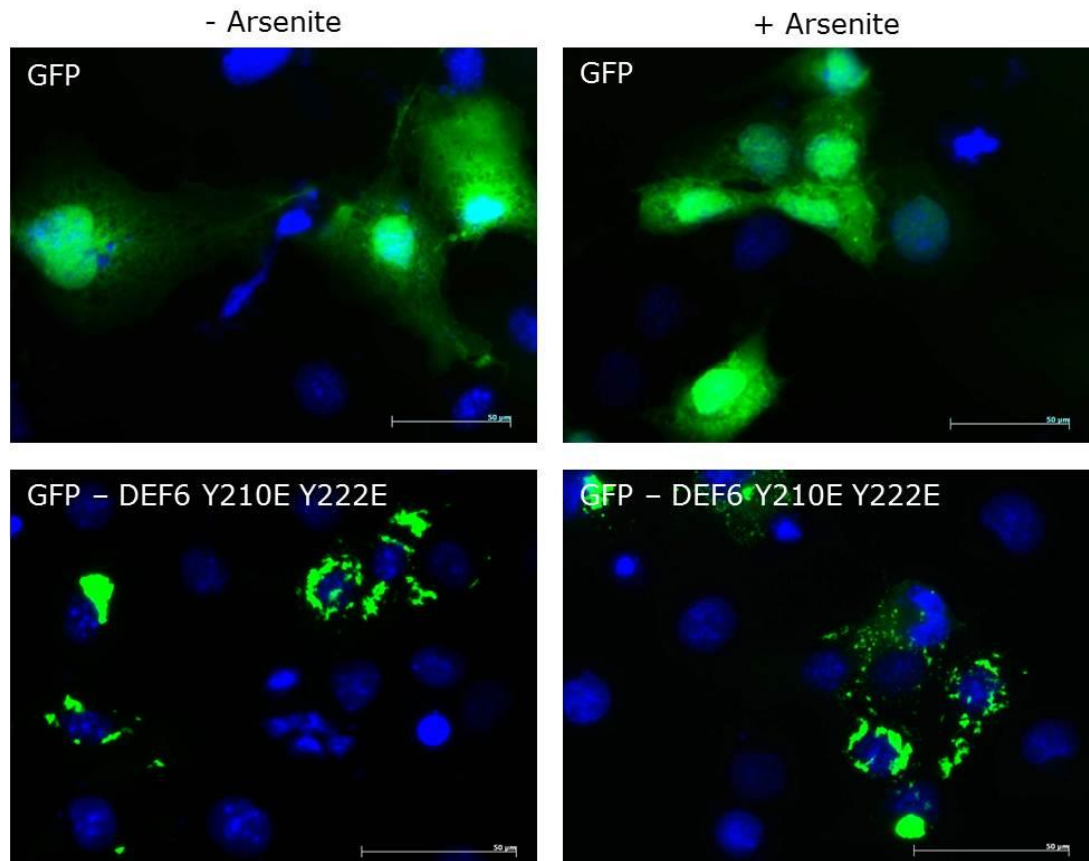


Figure 39 – Ectopically-expressed GFP-DEF6 Y210E/Y222E forms granules with and without arsenite induced translational arrest. GFP-DEF6 Y210E/Y222E and GFP alone were transiently ectopically expressed in Cos7 cells before either no treatment, or treatment with 1μM arsenite for 1hr to induce translational arrest. Cells were fixed with 4% PFA and mounted on coverslips with DAPI containing mountant. Cells were imaged with an Axiovert S100 widefield microscope. The scale bar indicates 50μm.

The aggregation is seen both with and without cellular stress via the addition of arsenite, which is added to promote the global arrest of translation and promote the formation of mRNA regulating granules such as P-bodies and stress granules (Figure 39). Whilst arsenite induced translational arrest is not necessary for the initial formation of granules, further small granules do appear to form with the addition of arsenite. This aggregate formation suggests that phosphorylation of DEF6 by Itk may be a route to granule formation.

To test whether the open/closed model suggested by Gupta *et al*³⁸ for DEF6 was likely, a model lacking the C-terminal 39 amino acids thus breaking the proposed contacts formed by a closed version of DEF6 was produced (GFP-ΔC) (Taina Theodore, 2011). Expressed in Cos7 cells, this also forms granules

without arsenite treatment similar to those seen both with GFP-DEF6 in arsenite treated cells, and with GFP-DEF6 Y210/Y222E (Figure 40A). The granules are smaller than those seen in the Y210E/Y222E construct without arsenite treatment, and co-localise with DCP1, demonstrating interaction with P-bodies in line with the arsenite treated GFP-DEF6 (Figure 40B).

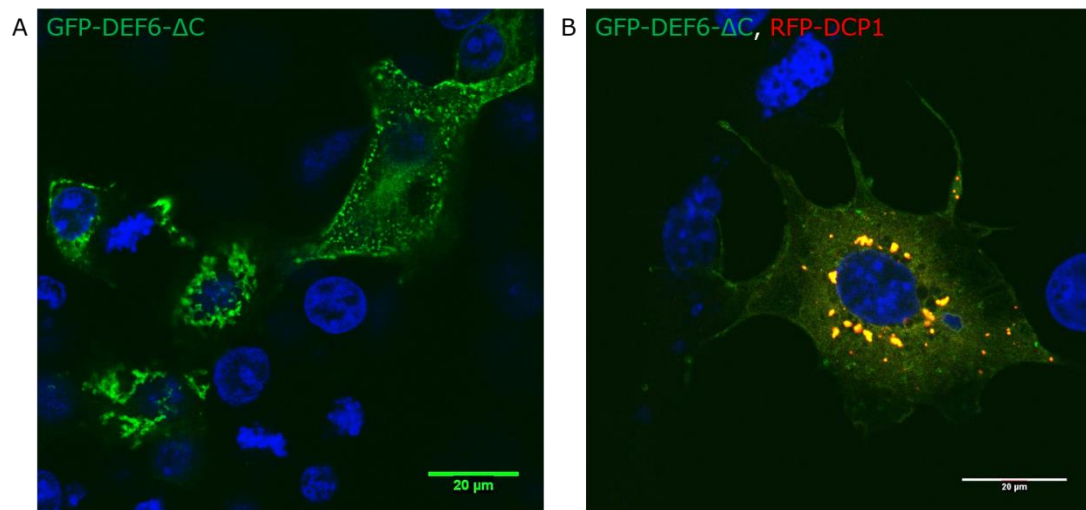


Figure 40 – Ectopically-expressed GFP-DEF6-ΔC forms granules spontaneously in Cos7 cells (A) which are able to co-localise with ectopically expressed DCP1, a marker for P-bodies (B). GFP-DEF6-ΔC was transiently ectopically expressed in Cos7 cells. Cells were fixed with 4% PFA and mounted on coverslips with DAPI containing mountant. Cells were imaged with a LSM710 confocal microscope. A Z plane was chosen to show the widest part of the cell. The scale bar indicates 20μm.

These results suggest that disrupting the C-terminus of DEF6 produces similar results to phosphorylation of DEF6 by Itk. This could be due to both leading to an open form of DEF6 by breaking contacts necessary for holding the closed form closed. However, due to the lack of Itk in Cos7 cells and the ability of GFP-DEF6 to form granules with arsenite treatment despite this lack of Itk, the granule formation of the Y210E Y222E phosphomimic DEF6 was further investigated.

4.2.6 In Cos7 cells phosphorylation of DEF6 by Itk is not the only route to granule formation

Itk is not present in Cos7 cells, so cannot be responsible for the formation of granules of GFP-DEF6 in cells stressed by arsenite. This is supported by analysis of the aggregates formed by the Y210E/Y222E mutant that show that whilst

large aggregates are formed spontaneously, smaller granules appear when the cells are arsenite treated. There are two possibilities for the appearance of smaller foci in phosphomimic transfected cells after the addition of arsenite. It could be due to the breakdown of the larger aggregates present with no stress, or it could be due to cytoplasmic foci forming in addition to the larger aggregates. The latter would support a route to granule formation separate from the phosphorylation of Y210 and Y222. To investigate whether the larger granules are breaking down, or the smaller granules are forming from diffuse protein, Cos7 cells were transfected with the Y210E/Y222E construct, and arsenite added for different amounts of time to assess how the smaller foci appeared.

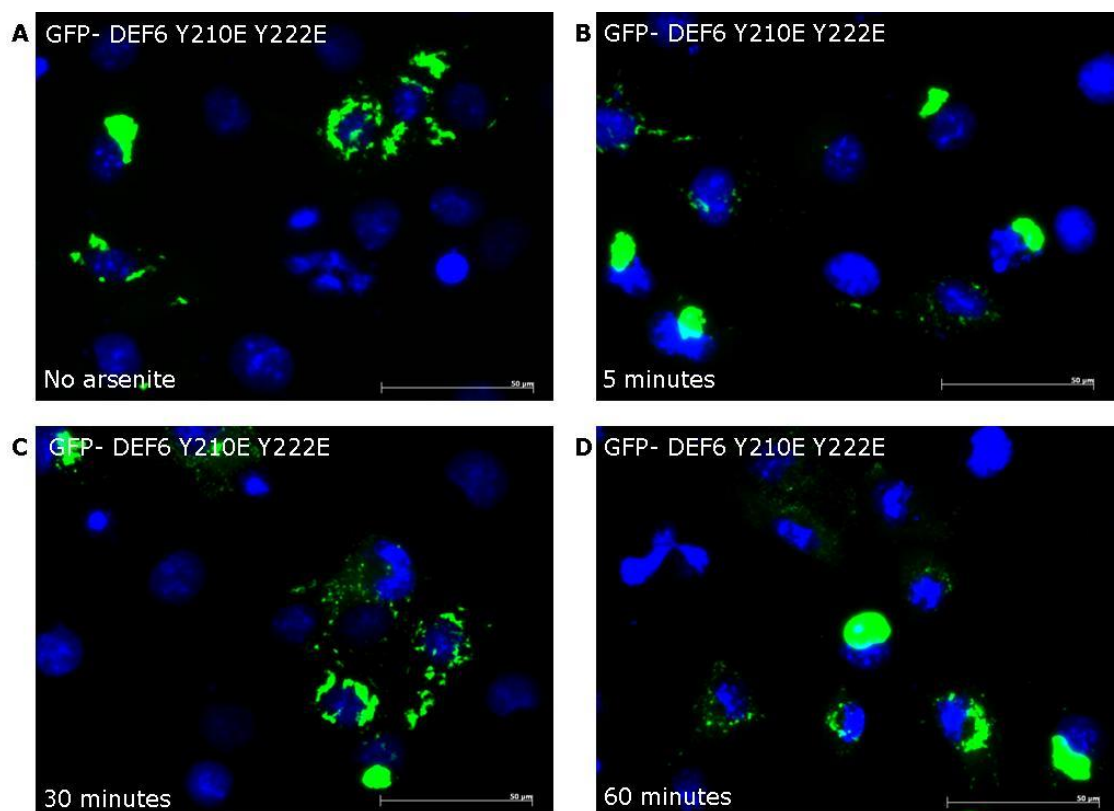


Figure 41 - Effect of arsenite over time on the GFP tagged Y210E/Y222E DEF6 mutant in Cos7 cells GFP-DEF6 Y210E/Y222E was transiently ectopically expressed in Cos7 cells before either no treatment, or treatment with 1µM arsenite to induce translational arrest. Cells were fixed with 4% PFA at the indicated times post arsenite treatment and mounted on coverslips with DAPI containing mountant. Cells were imaged with an Axiovert S100 widefield microscope. The scale bar indicates 50µm.

The number of cells with and without aggregates was counted in randomly selected fields of view, and the cells with aggregates classified as containing either multiple small aggregates or a single large aggregate. The data is preliminary as the experiment was only repeated once. 3 different areas of cells were counted, with the following numbers of cells counted:

Length of arsenite treatment	Total number of cells counted
No Treatment	111
5 Minutes	226
30 Minutes	316
60 Minutes	233

The preliminary data suggests that the observation that the ratio of multiple small to single large aggregates in cells containing aggregates changes with the addition of arsenite is correct, with longer arsenite treatment leading to a higher ratio of smaller aggregates to single large aggregates (Figure 42). To further probe this, the proportion of cells with only diffuse protein to cells with any type of aggregation in was compared.

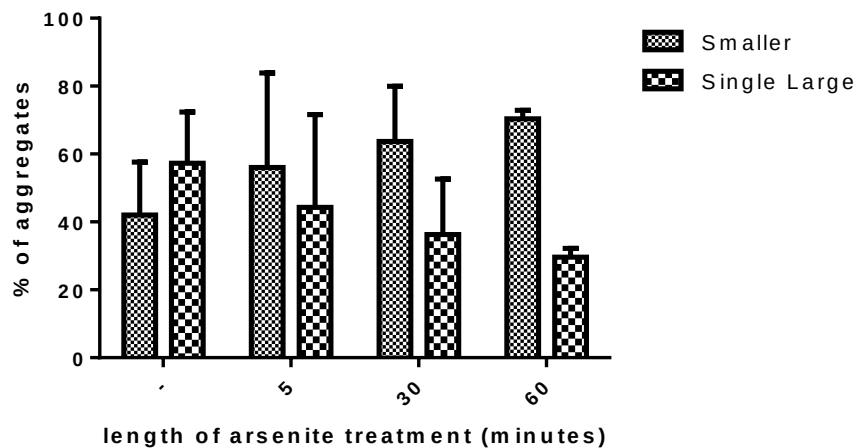


Figure 42 - The ratio of aggregate containing cells with multiple small granules to single large aggregates changes with increased length of exposure to arsenite treatment. Bars show standard deviation

The preliminary data suggests that the proportion of cells containing any type of aggregate or granule of GFP-DEF6 increases over time, suggesting the appearance of new aggregation after the application of arsenite, in addition to that already formed due to the Y210E/Y222E mutation (**Error! Reference source not found.**).

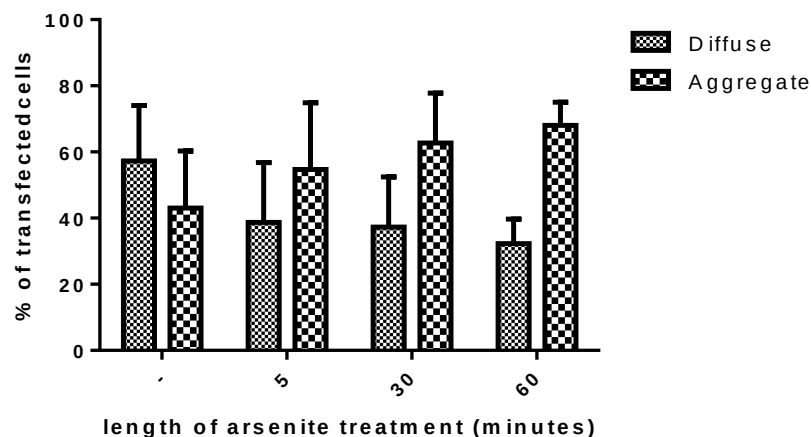


Figure 43 - The ratio of cells with diffuse GFP-DEF6 decreases with increased length of exposure to arsenite treatment, with aggregate formation increasing. Bars show standard deviation

By taking into account the proportion of diffuse cells in addition to small aggregates and large single aggregates, it appears that the proportion of cells

containing single large aggregates remains steady, with smaller foci appearing in previously diffuse cells after the application of arsenite, rather than the appearance of smaller foci being due to the breakup of the large aggregates (Figure 44).

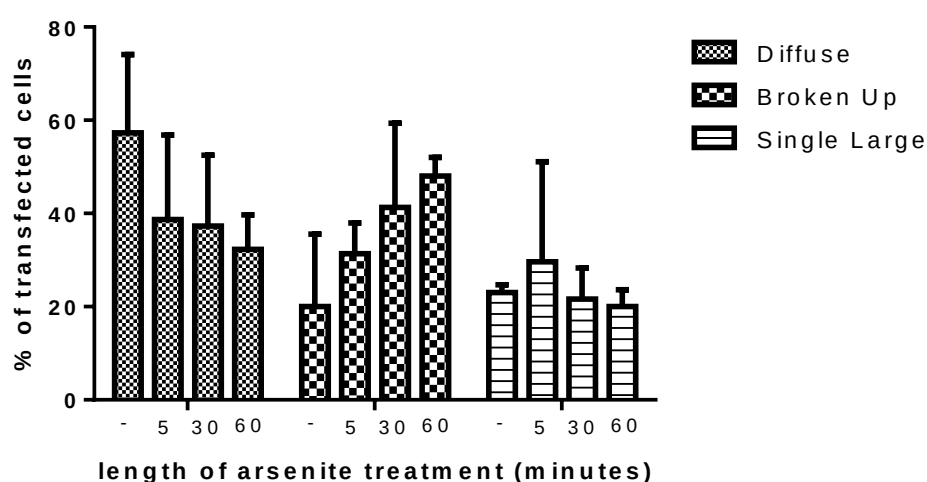


Figure 44 - The proportion of cells with large aggregates stays the same, with the proportion of small aggregates increasing whilst the proportion of cells with diffuse GFP-DEF6 decreasing. Bars show standard deviation.

This preliminary data suggests that the Y210E/Y222E phosphomimic construct can behave similarly to the wild type DEF6, present diffusely throughout the cell until stress causes it to form small ordered granules, but in approximately 1/3 of transfected cells, it aggregates in an uncontrolled manner. Whilst Itk is not responsible for the formation of granules in arsenite stressed cells, there may be other phosphorylation events taking place that phosphorylate DEF6, with the C-terminus a highly likely target.

4.3 Conclusions

4.3.1 The purification of the PH domain of DEF6

Despite some early problems with the initial solubility of the PH domain protein constructs, the PH domain can now be produced in 3-5mg amounts from a 1L LB growth, allowing for its use in binding studies with Itk. However, the PH domain presents solubility problems, crashing out of solution at concentrations necessary for NMR studies in particular ($>100\text{ }\mu\text{M}$), but this may be possible to overcome with further modifications to the protein construct. The PH domain of SWAP70 has been produced and used for biophysical studies, suggesting that with some further alterations to purification the DEF6 PH domain could also be made stable. Unlike the bacterial system used in this study, Li *et al*⁸¹ used a cell free protein synthesis system to produce protein for their structural determination. Tokuda *et al*¹⁴⁶ used a bacterial system for the production of their SWAP70 PH domain protein, using a similarly GST-tagged protein in BL21 derived bacteria. Their conditions for NMR studies used 20mM phosphate buffer (pH7.5)¹⁴⁶, Li *et al*. used 20mM Tris buffer (pH7.0) with 100mM NaCl⁸¹, and studies of the Itk PH domain similarly used 50mM phosphate buffer (pH7.0) with 150mM NaCl¹⁵⁹, all of which are similar to the 20mM phosphate buffer (pH7.4) with 150mM NaCl used in this study suggesting these parameters are acceptable.

The issues with solubility upon lysis and aggregation after purification are not unique to the PH domain of DEF6. Despite the high number of PH domain structures in the literature, some have been shown to be difficult to produce, one example being the PH domain of Itk, which could only be produced in a soluble form by mutating several amino acids to make it more similar to the soluble PH domain of either Btk or Tec¹⁵⁹. It is possible that a similar mutation to DEF6 could improve solubility.

Comparisons between the DEF6, SWAP70, Tec and Btk PH domains in the region altered in Itk to promote its solubility indicate that DEF6 already has the isoleucine in the place of the threonine that causes solubility issues in Itk, but that the other mutation site is variable between all 5 proteins, thus not indicating any particular mutation that may help solubility.

DEF6	D T RQRQEWTA A IQMA I R	312
SWAP70	D K KKKQEWIQAIHST I H	306
Tec	S P QSRDLWVKKLKEE I K	111
Btk	T E ELRKRWIHQLKNV I R	133
Itk	D R ESRQRWVLALKEE T R	111
	. :. * : :	

Figure 45 - Alignment of the alpha helix of the PH domains of DEF6, SWAP70, Tec, Btk and Itk highlighting residues altered in Itk to promote solubility.

The mutation of the Itk PH domain by two amino acids to make it more similar to Btk improved its solubility upon lysis, but upon cleavage the protein still formed aggregates. These however could be separated out by size exclusion chromatography, and the monomeric peak was found to be stable for at least 3 months for NMR studies.

4.3.2 Itk does not require the NLS to bind and phosphorylate DEF6

Contrary to the hypothesis suggested by Hey¹⁶², the contiguous patch of positively charged amino acids made up by the nuclear localisation signal C terminal to the PH domain of DEF6 (amino acids 315-342) is not required for Itk binding for phosphorylation of the PH domain. The other targets for Itk phosphorylation (the Itk SH2 domain and phospholipaseCy) go through a binding step via a positively charged patch on the target before phosphorylation. Other potential binding sites can be located on the PH domain of DEF6.

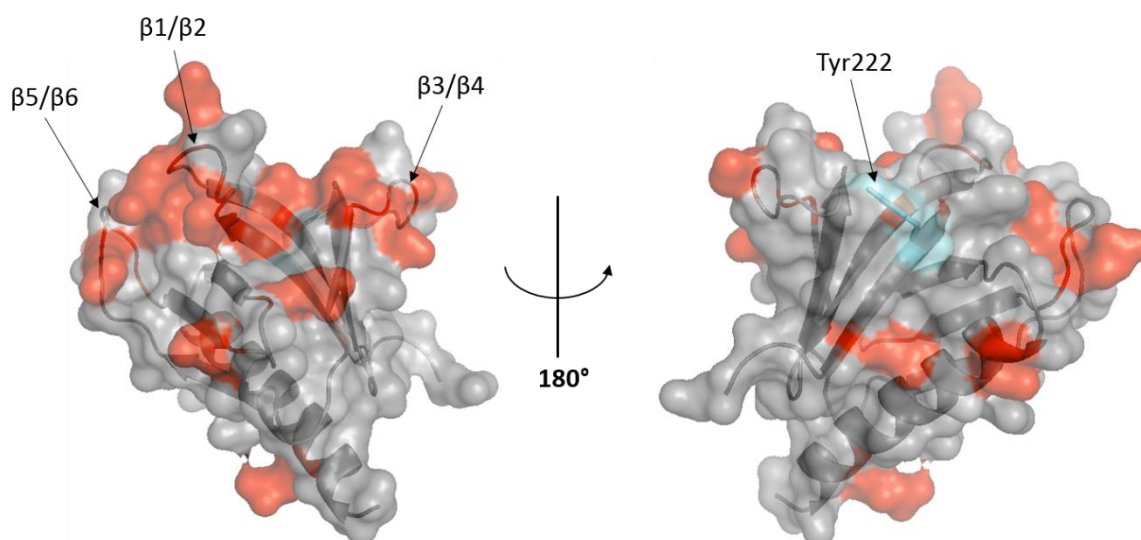


Figure 46 – Itk binds to positively charged patches on its substrate prior to phosphorylation. Potential binding sites on the PH domain of DEF6 are shown here, with positively charged amino acids (red) on the surface of the PH domain of DEF6 indicated along with the target tyrosine 222.

As can be seen in Figure 46 there is a large contiguous patch made up of the $\beta 1/\beta 2$, $\beta 3/\beta 4$, and $\beta 5/\beta 6$ loops. The best studied system for binding between Itk and its target is phospholipaseC γ (PLC γ). This has been shown to contain a positively charged binding patch in its SH2 domain which is remote from the phosphotyrosine binding pocket found in the linker between the SH2 and SH3 domains⁶⁷. The binding site on PLC γ has been shown to have a minimum distance from the target tyrosine of 28Å, a distance which places the positively charged amino acids on the $\beta 1/\beta 2$ and $\beta 5/\beta 6$ loops in range, but not the $\beta 3/\beta 4$ which is too close. By using this minimum distance, the corresponding negatively charged patch on Itk has been identified for PLC γ binding in the G helix, a shared structural feature in several kinases which is emerging as a site for docking interactions⁶⁸. Similar interactions between the kinase G helix and target proteins have been noted between ROCK1 and RhoE, and between PKR and eIF2 α . However, other potential binding sites on the Itk kinase domain have also been identified such as the helix containing E400, D401 and E404 on the N lobe of the kinase domain, which would alter the necessary spacing on the DEF6 PH domain, making selecting a particular set of positively charged amino acids as

the binding site difficult. The charged residues in these other potential docking sites on Itk have been mutated by Xie *et al*⁶⁸ to alanine residues, which could be used to further investigate the localisation of binding patches on both DEF6 and Itk. By determining the localisation of the Itk side of the binding patch using the mutants to find which residues are necessary for interaction, the site on DEF6 could be narrowed down by providing geometric restraints.

This study has also illustrated that Itk binds well to the phosphomimic of the PH domain, indicating that it does not necessarily dissociate immediately after phosphoryl transfer. This is sometimes used as a mechanism to prevent dephosphorylation by phosphatases by providing competition for binding¹⁶¹. Inhibition of phosphatase activity by pervanadate in JURKAT cells was shown by Hey *et al*¹² to redistribute DEF6 protein from the immune synapse to cytoplasmic aggregates. In this paper these results along with data showing the aggregation of GFP-Y210E/Y222E were used to indicate that phosphorylation by Itk leads to the redistribution of DEF6 into aggregates in activated Jurkats. However, pervanadate treatment is not a specific enough treatment to draw the conclusion that Itk drives aggregate formation. It may be the case that whilst Itk is involved in how DEF6 interacts with the immune synapse, it is not the only route to granule formation, with other phosphorylation events providing potential further triggers for aggregate formation. The prevention of dephosphorylation at the Itk target sites may serve to destabilize the interaction between DEF6 and the membrane at the immune synapse leading to its more cytoplasmic distribution when treated with pervanadate, whilst the pervanadate treatment also serves to prevent dephosphorylation of other residues involved in aggregate formation.

During the *in vitro* kinase assay Itk phosphorylated the phosphomimetic protein construct, indicating Itk was able to phosphorylate tyrosine residues other than Y210 and Y222. There are two other tyrosine residues in this construct, Y247

and Y290. In the work by Hey *et al.* the phosphorylation of DEF6 by Itk was shown to be fully abolished when the DEF6 substrate contained both Y210F and Y222F mutations, indicating that these were the only residues phosphorylated¹². Additionally, Y247F and Y290F mutants showed no decrease in phosphorylation. However, the results shown here show the phosphorylation of residues outside Y210 and Y222. The work by Hey *et al.* was performed on full length protein, whilst this work used isolated PH domain protein. Distal docking sites on DEF6 not present in the constructs used in the kinase assay could be involved in providing specificity to the Y210 and Y222 by acting to increase affinity¹⁶³.

Whilst high concentrations of substrate in the kinase assay could allow for phosphorylation of low affinity sites, this is unlikely to be the case here, as the concentration of DEF6 PH domain used as substrate (1.5 μ M) is similar to the measured K_m for PLC γ which is 1.70 μ M $\pm$ 0.43⁶⁸. It was found that at concentrations above 5 μ M for PLC γ in the Itk kinase assay, non-specific phosphorylation could occur, as evidenced by the phosphorylation of PLC γ protein constructs incompatible with docking¹⁶⁴. However, at the DEF6 PH domain concentrations of 1.5 μ M, non-specific or low affinity phosphorylation events are unlikely to occur, unless there is a physical constraint that has been removed.

4.3.3 Phosphorylation by Itk is not the only route to granule formation

Whilst Itk phosphorylation of the PH domain of DEF6 may cause the aggregation of DEF6 *in vitro*, as indicated by the aggregation of the Y210E/Y222E GFP tagged construct and as reported by Hey *et al.*¹², this is not the only route to granule formation. First, this cannot be the only route to granule formation as wild type GFP-DEF6 in Cos7 cells is able to form granules upon arrest of translation by arsenite, despite the lack of Itk in Cos7 cells. Whilst there may be another kinase present also phosphorylating Y210 and Y222 in Cos7 cells allowing granule formation, the behaviour of the Y210E/Y222E construct over time

suggests that whilst this mutation causes widespread aggregation of the protein, there is still a soluble pool that behaves in a similar manner to that of the wild type construct. This suggests that it is not a phosphorylation event at the Y210 and Y222 residues that causes aggregation. Further to this, mutation of these two residues to phenylalanine residues also causes aggregation¹⁶⁵, suggesting that the aggregation seen is merely due to the disruption of the protein and not related to the more ordered formation of the P-body like granules.

A potential mechanism for the effect of phosphorylation on interaction with the immune synapse can be postulated from studies on SWAP70 by Fukui *et al.* relating to the $\beta 3/\beta 4$ loop in the PH domain¹⁶⁶. Using the model of the PH domain of DEF6 based on the known structure of the PH domain of SWAP70, the likely interactions caused by phosphorylation of Y222 can be envisaged.

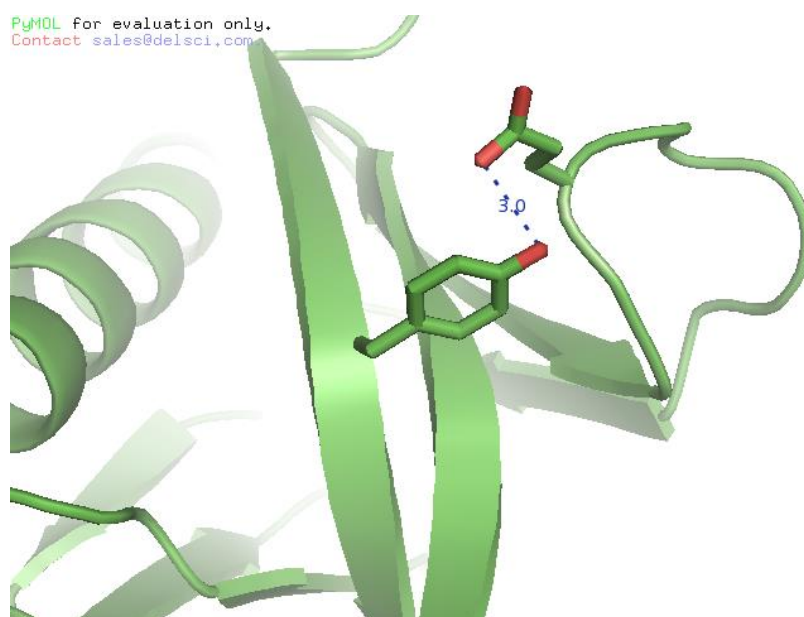


Figure 47 – The $\beta 3/\beta 4$ loop of the PH domain has an effect on the localisation of SWAP70 to membranes. In DEF6, tyrosine 222 is predicted to be located in proximity to the loop, providing a potential for changing interactions upon phosphorylation of tyrosine 222. The figure shows the localisation of tyrosine 222 of DEF6 and its proximity to glutamic acid 251 in the structure predicted from that of SWAP70.

The glutamic acid residue at position 251 on the $\beta 3/\beta 4$ loop is predicted to be in close proximity to Y222. This provides a possibility that phosphorylation of Y222, and thus the addition of a negative charge may cause electrostatic repulsion and

thus change the position of the $\beta 3/\beta 4$ loop, leading to further effects that explain the effect of this phosphorylation on DEF6 location in cells. A search of the literature shows that mutations have been made to the $\beta 3/\beta 4$ loop of SWAP70¹⁶⁶. Of particular interest is a mutation of E245 and D246 to alanines, which prevent localisation of SWAP70 to the membrane despite not affecting phosphoinositide binding. Fukui *et al*¹⁶⁶ suggested that this loop may be involved in stabilizing interactions required for membrane targeting in addition to phosphoinositide binding. This suggests a future experiment of mutating the glutamic acid in DEF6 to an alanine in the Y222E mutant to see if it attenuates the Y222E aggregation effects, and in the wild type in conjunction with experiments overexpressing Itk along with DEF6 in Cos7 cells and observing if the aggregation events occur.

In addition to its phosphorylation by Itk, DEF6 has been shown to be phosphorylated by the tyrosine kinase Lck at residues Y133 and Y144³⁴. Wild type DEF6 is known to localise to the immunological synapse at the membrane¹ upon phosphorylation by Lck in a non PI(3,4,5)P₃ dependent manner³⁴. Therefore a possible model is that DEF6 is phosphorylated by Lck, then localises to the immune synapse, an interaction that may rely on the $\beta 3/\beta 4$ loop in a similar manner to that of the SWAP70 PH domain. Becart *et al*. noted that membrane localisation wasn't sufficient for DEF6 activity, TCR stimulation was also needed. They postulated that Lck phosphorylation of DEF6 and localisation to the membrane could lead to conformational changes and/or the creation of a binding site for further interactions³⁴.

At the membrane, DEF6 is able to bind PI(3,4,5)P₃ via the $\beta 1/\beta 2$ loop of the PH domain^{35,63}. In SWAP70 the binding of membrane bound phosphoinositides by the $\beta 1/\beta 2$ acts as a switch that affects the localisation of SWAP70¹⁴⁶. Binding of the PH domain of SWAP70 to membrane bound phosphatidylinositol lipids but not free phosphoinositides is required for a conformation change that disorders

part of the PH domain, leading to the unfolding of the nuclear localisation signal helix located in the PH domain and allowing nuclear import. Similar conformational changes to DEF6, or merely its localisation at the IS with Itk could allow for the phosphorylation of Y210 and Y222 by Itk. This phosphorylation is likely to disrupt the $\beta 3/\beta 4$ loop, which could lead to dissociation from the membrane due to lack of stabilization, as in SWAP70 PH domains with mutations in this loop region. Downstream from this, phosphorylation signals related to arrest of translation could act to open the DEF6 conformation to promote aggregation and interact with P-bodies to mediate T cell mRNA expression. The role of Itk phosphorylation of DEF6 may thus be in disrupting the interaction between DEF6 and the membrane.

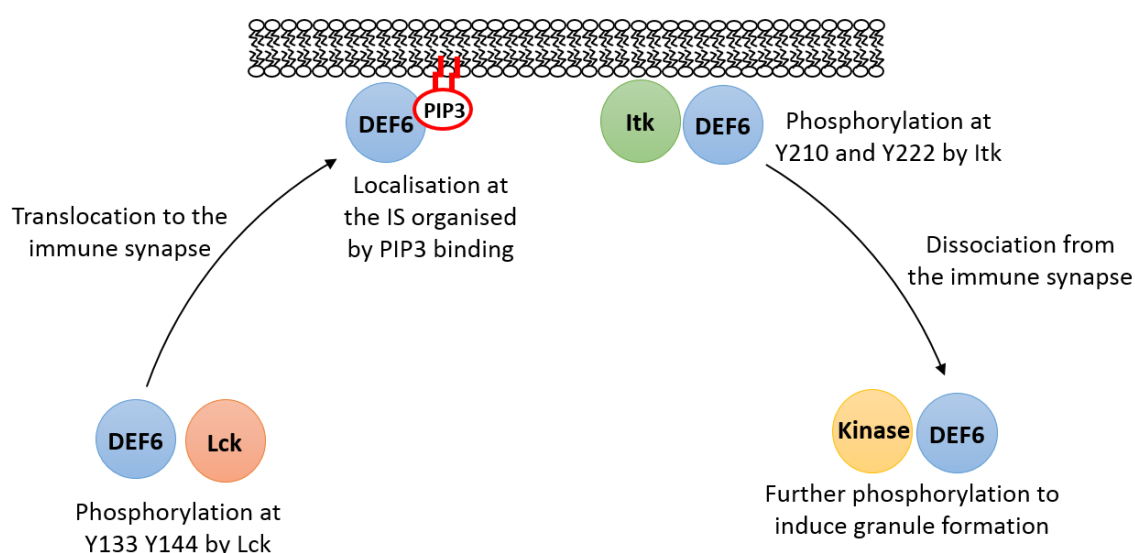


Figure 48 - Potential order and location of phosphorylation events on DEF6

4.3.4 Other routes to granule formation

Returning to the closed model for DEF6 discussed in section 1.3 of the introduction, if phosphorylation at the PH domain isn't the route to opening up DEF6, the C-terminal end that provides the contact to keep DEF6 closed could provide a potential target. The ΔC GFP tagged mutant illustrates that opening the conformation of DEF6 can lead to aggregate formation by removing interactions between the C-terminus and the rest of the protein that keep DEF6

in a closed, non-aggregating formation and that it is within the final 39 amino acids that the contact point resides for keeping DEF6 in a closed conformation in the model prediction. Whilst the previous model was that phosphorylation of the PH domain would disrupt this interaction, it is possible that phosphorylation at the C-terminus is the route to granule formation. Several phosphorylation sites at the C-terminus of DEF6 within the region cut off by the ΔC mutant have been identified by proteomic mass spectrometry including T595, S597, S600, S606, S618, T619¹⁶⁷. DEF6 may have several methods of opening the inhibitory closed conformation. Different methods may allow for different functions, as opening the closed inhibited formation has also been proposed to be the method for allowing both Rho-GEF activity, and phosphoinositide binding³⁵. It would also allow for an activating response to several different signals.

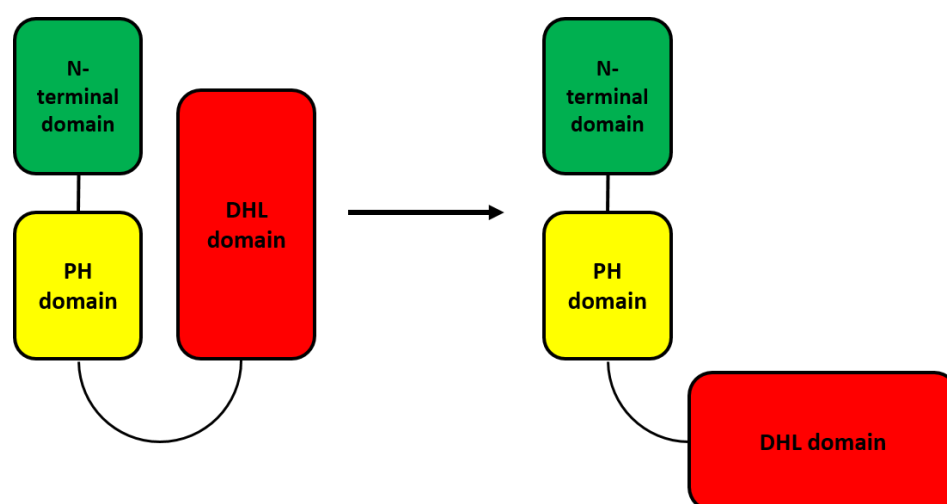


Figure 49 - A model of the proposed closed and open forms of DEF6. Interactions between the DHL domain and either the PH or N-terminal domain may keep DEF6 in a closed, non-aggregating form. A phosphorylation event could open the protein, allowing aggregation into granules.

The GFP- ΔC aggregates can co-localise with DCP1 indicating an ability to interact with P-bodies. Opening the closed DEF6 formation may expose a region of DEF6 which provides interactions with other P-body components, driving its aggregation into granules. The next chapter of this study investigates the role of different domains of DEF6 in its interaction with P-bodies, and the dynamics of DEF6 once it has formed granules.

5. Results:

The dynamics of DEF6 granules

5 Granule dynamics

5.1 Introduction

Several proteins that are found in P-bodies have been shown to contain distinct domains that contribute to foci formation including glutamine/asparagine rich domains^{101,102}, arginine/glycine rich domains⁸⁹, the YjeF domain¹⁰³, an NTF₂ domain⁸⁹, proline rich domains⁸⁹, the Sm fold¹⁰³, a fold similar to ARM and HEAT repeats¹⁶⁸ and the ROQ domain¹⁶⁹. Identification of the role of each of the domains of DEF6 in its interaction with P-bodies will help to provide an understanding of how the role of DEF6 in P-bodies combines with its previously identified roles. In particular, differences between the localisation and the movement of GFP tagged constructs of individual domains of DEF6 and both the full length protein and other P-body forming proteins will help elucidate how DEF6 both interacts with P-bodies, and performs its role as a Rho-GEF. Studying the dynamics of the DEF6 granules will provide insight into the type of role DEF6 has in the P-bodies by comparing its rates of diffusion in and out of the granules, and the movement of DEF6 granules in the cytoplasm with that of other P-body forming proteins.

5.1.1 Domain features of P-bodies and stress granules

As previously described in section 1.3.2, many proteins involved in P-body formation contain glutamine and asparagine rich regions which are thought to drive the aggregation of the P-body components. Glutamine/asparagine rich regions have been demonstrated in Ccr4p and Pop2p, components of a deadenylase complex in *S.cerevisiae*; the helicase Dhh1p, the RNA binding protein Lsm4p and GW182, a scaffold protein involved in RNA mediated gene silencing; all of which have a role in P-body formation^{101,102,170}. DEF6 has been shown to form cytoplasmic foci and contains a glutamine/asparagine rich region at its C-terminus¹⁷¹. DEF6 has been shown to interact with P-bodies in a dynamic manner, demonstrated by its partial co-localisation with DCP1, a marker for P-

bodies¹⁷¹. The presence of a glutamine/asparagine rich region provides further support for the classification of DEF6 as a protein involved in P-bodies due to similarities in amino acid sequence¹⁰², and suggests a dual role for the C-terminal domain in addition to its role as a DHL domain in contributing to the GEF activity of DEF6.

Support for the function of glutamine/asparagine-rich domains in heterologous protein/protein interactions is demonstrated by the interaction of Pan1p and S1a1p (proteins involved in the assembly and organisation of actin cytoskeletal structures in yeast) via their glutamine/asparagine rich domains¹⁷², and by the binding of the transcription factor Sp1 via its glutamine rich region to the transcription factor dTAFII₁₁₀¹⁷³. It has been suggested that extensive hydrogen bond networks in glutamine/asparagine-rich regions contribute to the formation of a “polar zipper” that facilitates protein-protein interactions¹⁷⁴. Glutamine/asparagine-rich domains have further been shown to promote the aggregation of heterologous proteins with similar domains, for example the promotion of conversion to the prion form of the glutamine/asparagine rich protein Sup35 by the glutamine/asparagine rich protein Lsm4p^{175,176}. These interactions have also been shown to be interchangeable, with the glutamine/asparagine rich domain of Lsm4p replaced by the glutamine/asparagine rich domain of Rnq1 and the protein remaining functional¹⁰¹.

Due to their role in the regulation of translation via regulation of the availability of mRNA for translation, in addition to domains involved in protein – protein interactions P-body proteins often contain domains or regions that allow interaction with RNA. In line with this, proteins containing glutamine/asparagine rich regions have been shown to be biased towards certain functions, including the regulation of transcription and translation, RNA processing and RNA binding^{69,177}.

5.1.2 **Proteins in P-bodies exchange with the cytoplasmic pool**

The rate of diffusion of proteins in and out of P-bodies and stress granules has been measured using fluorescence recovery after photo bleaching (FRAP). Different components have different rates of movement in and out of the granules, thought to be related to their role within the granule. Those that have very long exchange times are core fixed components, likely to be involved in the organization of the P-body structure. In addition, as some proteins have fairly fast recovery times, but don't fully recover, it has been suggested that this indicates that the P-body proteins have two populations, a stationary population providing a core structure that does not exchange with the cytoplasm, and a dynamic population forming an outer layer in the P-bodies from which proteins can exchange with the cytoplasmic pool⁹².

This core and outer structure model is supported by electron microscopy data which shows that whilst DCP1a and Xrn1 are found throughout P-bodies, DDX6 is found predominantly at the periphery¹⁷⁸. In this case however, the peripheral DDX6 provides a stabilizing role, suggesting the core is not necessarily the organizing centre. Similarly, whilst DCP1a is found throughout P-bodies and has a slow FRAP recovery rate suggesting a core role in P-bodies^{92,93}, its disruption did not have as strong an effect on the stability of P-bodies as the disruption of the peripheral DDX6 did¹⁷⁸. FAST does not recover quickly, and is found in both stress granules and P-bodies suggesting a scaffolding role⁹³. It functions as a promoter of cell survival in cells exposed to adverse environmental conditions, interacting with the translational repressor Tia-1 to increase the translation of inhibitors of apoptosis¹⁷⁹. The presence of a stable protein found in both P-bodies and stress granules leads to the possibility that there may be similarities in the composition of the core components of stress granules and P-bodies⁹³.

Components that exchange quickly with the cytoplasm such as the RNA binding protein TTP which promotes mRNA degradation and exchanges quickly in both P-

bodies and stress granules have been suggested to act as transient tethers between mRNA and the decay machinery present in the P-bodies⁹³. Components with intermediate recovery such as DCP1 and the initiation factor PABP1 are suggested to be unattached to mRNA but not structural components, shuttling in and out of P-bodies and stress granules independently of mRNA.

By measuring the dynamics of DEF6 exchange between the granule and cytoplasmic pools via recovery from photo bleaching, its properties can be compared with those known for other P-body component proteins, and a greater picture of the role of DEF6 in its interactions with P-bodies may emerge.

5.1.3 The movement of P-bodies within cells

P-bodies and stress granules are dynamic structures that are able to move to an extent in the cytoplasm, assemble and disassemble based on the presence of an intact microtubule network, and show differential diffusion of component proteins in and out of the granule structure. Whilst P-bodies appear to move faster than stress granules, they both exhibit a range of types of motion from being constrained to an area, to occasional rapid directed movement¹⁸⁰. Stress granules and P-bodies have been shown to associate with and move along microtubules^{90,180}, and the disassembly of stress granules relies on this association¹⁸⁰. Whilst actin appears to have no role in the movement of stress granules or P-bodies, the stationary population is thought to be associated with actin⁹⁰. Using mean squared displacement analysis, it has been demonstrated that the granules mainly exhibit diffusion like movement, but also show corralled diffusion⁹². From this analysis, it was calculated that the diffusion rate for P-bodies were mostly between $10^{-3} - 10^{-2} \mu\text{m}^2/\text{s}$ and they can move with a velocity of up to $0.5-1.1 \mu\text{m}/\text{s}$ in the case of granules with directed movement.

It is currently unknown whether DEF6 granules exhibit movement in the cytoplasm as live imaging studies have not been performed. Due to their similarities with other foci, and their interactions with P-bodies, it is likely that

movement of DEF6 granules is possible. In this study live imaging will be used to determine if DEF6 granules move within the cytoplasm, and if movement is seen, single particle tracking and analysis of their speed of movement and how much of the cytoplasm they visit can be compared with the values measured for the granules formed by other granule forming proteins, such as those measured by Aizer *et al*⁹².

5.2 Results

5.2.1 The DHL domain of DEF6 contains sequences characteristic of P-body proteins

The presence of a glutamine/asparagine rich region within the DHL domain (amino acids 342-631) of DEF6 as indicated in Figure 50 is consistent with its ability to interact with P-bodies¹⁸¹.

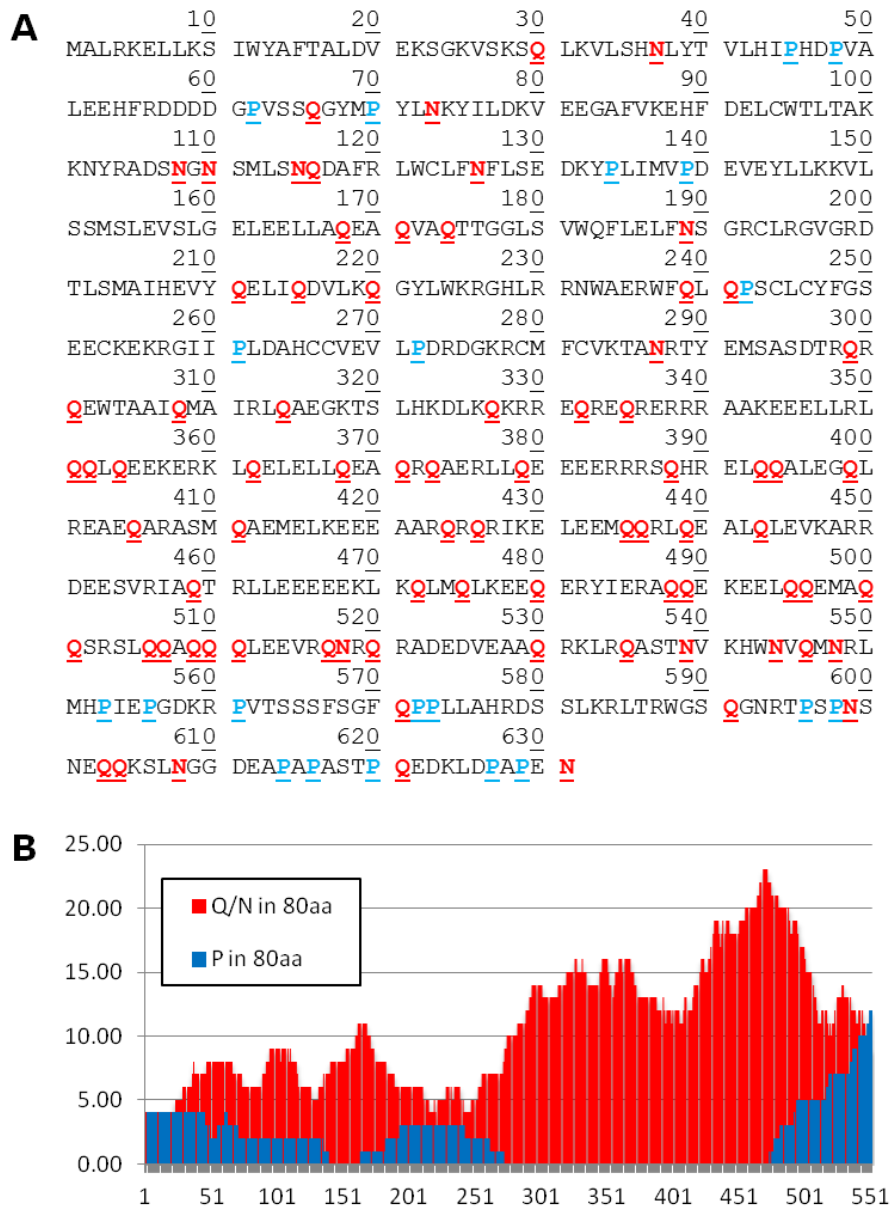


Figure 50 - DEF6 contains a high number of glutamine and asparagine residues (red). The region with the highest concentration of glutamine and asparagine residues lacks proline residues (blue). The number of each type of residue per 80mer in the sequence of DEF6 was calculated and plotted against residue number to show regions rich or lacking in either glutamine and asparagine or proline residues. A graph analysing the Q/N and P content of GFP can be found in the appendix, section 8.9.

A further characteristic of these regions in P-body forming proteins is that they generally lack prolines within the glutamine/asparagine rich region, and are often flanked by a proline rich region at their C-terminus.

To compare these regions between proteins, the number of glutamines and asparagines in the most rich 80mer can be compared. This method can also be used to reveal the lack of prolines found within the glutamine/asparagine rich regions, and the proline content of the proline rich region often found C-terminal to the glutamine/asparagine rich region. A plot of the number of glutamine and asparagine residues, and of proline residues per 80 amino acids is shown in Figure 50B, indicating the relative glutamine/asparagine richness in the DHL domain region, and both the lack of proline residues within this region, and the regions rich in proline residues flanking the glutamine/asparagine rich region.

Table 5 - Frequency of glutamine and asparagine residues in the most glutamine/asparagine rich 80mer, prolines in the most glutamine/asparagine rich 80mer, and proline in the most proline rich 80mer in P-body forming proteins

Protein	Q/N	P in Q/N rich	P
Lsm4	38	1	2
Dcp2	24	8	20
Def6	23	0	12
DCP1	22	2	4
DDX6	18	7	7
Xrn1	15	1	24
Lsm1	14	1	2

The average number of glutamine and asparagine residues in a protein per 80mer is 7.7. Reijns *et al.* found that core components of P-bodies scored higher than the average¹⁰². DEF6 as a whole contains 10.77 per 80mer, placing it

higher than the average. The C-terminal region of DEF6 from 300 – 631 contained 15.09 per 80mer, whilst the N-terminal region of DEF6 from 1 – 300 contained 7.13 per 80, near the average and thereby suggesting a specialised role for the C-terminal region in the accumulation of DEF6 into cytoplasmic granules.

5.2.2 The DHL domain can form spontaneous granules

The identification of the DHL domain of DEF6 as containing a glutamine/asparagine rich region similar to other P-body forming proteins suggests that this domain is highly likely to be the DEF6 domain that promotes the interaction of DEF6 with P-bodies. A GFP tagged construct containing amino acids 357-631 of DEF6 (GFP-DHL) had previously been made¹. This plasmid construct was used to demonstrate that the DHL domain is able to form granules similar to those formed by DEF6. GFP-DHL was expressed in Cos7 cells which were treated with arsenite to produce cellular stress and the global arrest of translation in the cells^{120,121}. This has been previously demonstrated to be a method of inducing P-bodies^{95,125}. Granule formation by the DHL domain alone will suggest that the domain is sufficient for aggregation. Spontaneous formation of foci without global arrest of translation induced by arsenite treatment would suggest the ability to form separate to a response to stress. GFP-DEF6 only form foci in response to arsenite stress, and not in untreated cells suggesting that it responds to arrest of translation (Figure 17).

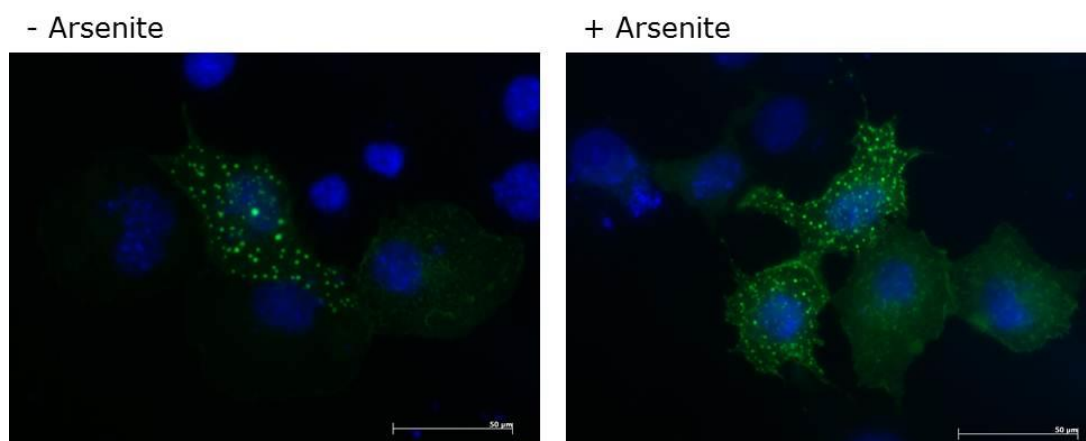


Figure 51 – The DHL domain (amino acids 357-631) of DEF6 can form granules without arsenite treatment. Cos7 cells were transfected with GFP-DHL and either left untreated, or treated with 1µM arsenite for 1 hour to induce translational arrest. Cells were fixed with 4% PFA and mounted on coverslips with DAPI containing mountant. Cells were imaged with an Axiovert S100 widefield microscope. The scale bar indicates 50µm.

GFP-DHL forms cytoplasmic foci both with and without arsenite treatment (Figure 51), indicating that the domain is sufficient for aggregation and that it does not require the signals for foci formation that the full length protein does. Transfected cells expressing GFP-DHL were classified as containing diffuse protein (no foci present in the cell), containing protein in granular form (foci present in the cell), or as being rounded up and dying (Figure 52). This is preliminary data as it was performed on cells from one experiment. The following numbers of cells counted:

Experiment	Total number of transfected cells counted
GFP – arsenite	174
GFP + arsenite	224
GFP- WT – arsenite	97
GFP-WT + arsenite	188
GFP-DHL - arsenite	118
GFP-DHL + arsenite	119

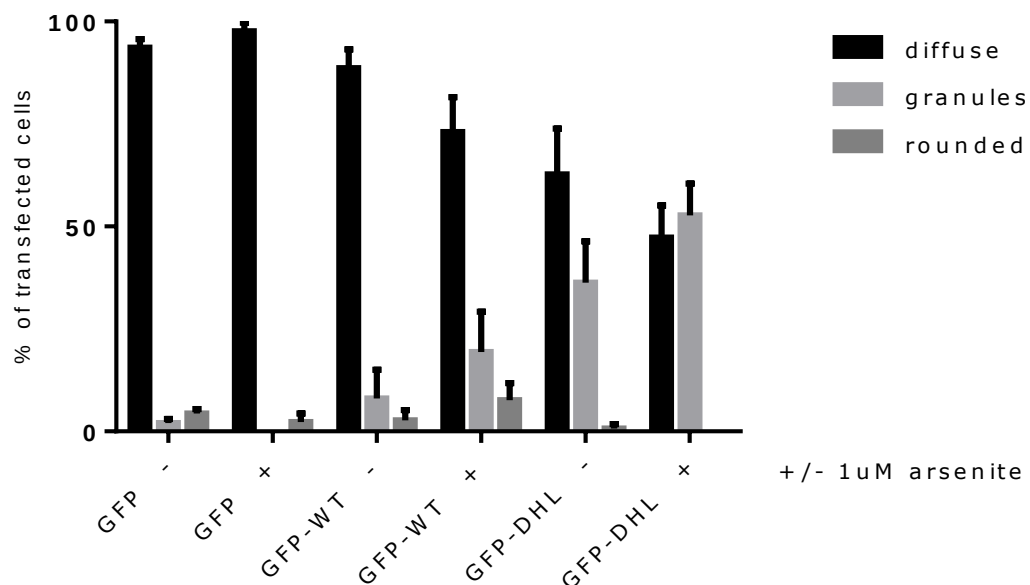


Figure 52 - Cells transfected with the GFP-DHL (amino acids 357-631) construct spontaneously form granules when transfected into Cos7 cells. Images were taken of 3 randomly selected areas of the cover slip at 10x magnification and the number of cells expressing GFP counted. These were classified as being diffuse, containing granules, or being rounded up. Bars show standard deviation

This preliminary data suggests that the granules form spontaneously, without arsenite stress, and are seen in a large proportion of cells; 36% of cells expressing the construct when untreated, and 53% when treated with arsenite. The granules are present in a higher number of cells than for GFP-DEF6, indicating that the full length protein even when responding to stress has some kind of inhibition to forming granules that is relieved in the DHL domain alone.

5.2.2.1 DHL domain granules do not extensively co-localise with DCP1

Given the ability of the DHL domain to form granules that are morphologically similar to the granules that full length GFP-DEF6 forms which interact with P-bodies as marked by DCP1, co-localisation of the plasmid construct with DCP1, a marker for P-bodies was examined. Whilst the DHL domain is able to form granules indicating an ability to aggregate, it is unknown where in the structure of DEF6 its ability to interact with P-bodies originates. Interaction with P-bodies similar to that of the full length DEF6 would suggest that both of these functions related to granule formation are contained within the C-terminus, whilst less or

no interaction would suggest that this role is carried out elsewhere in DEF6, and the aggregation of granules and their interaction with P-bodies are separated.

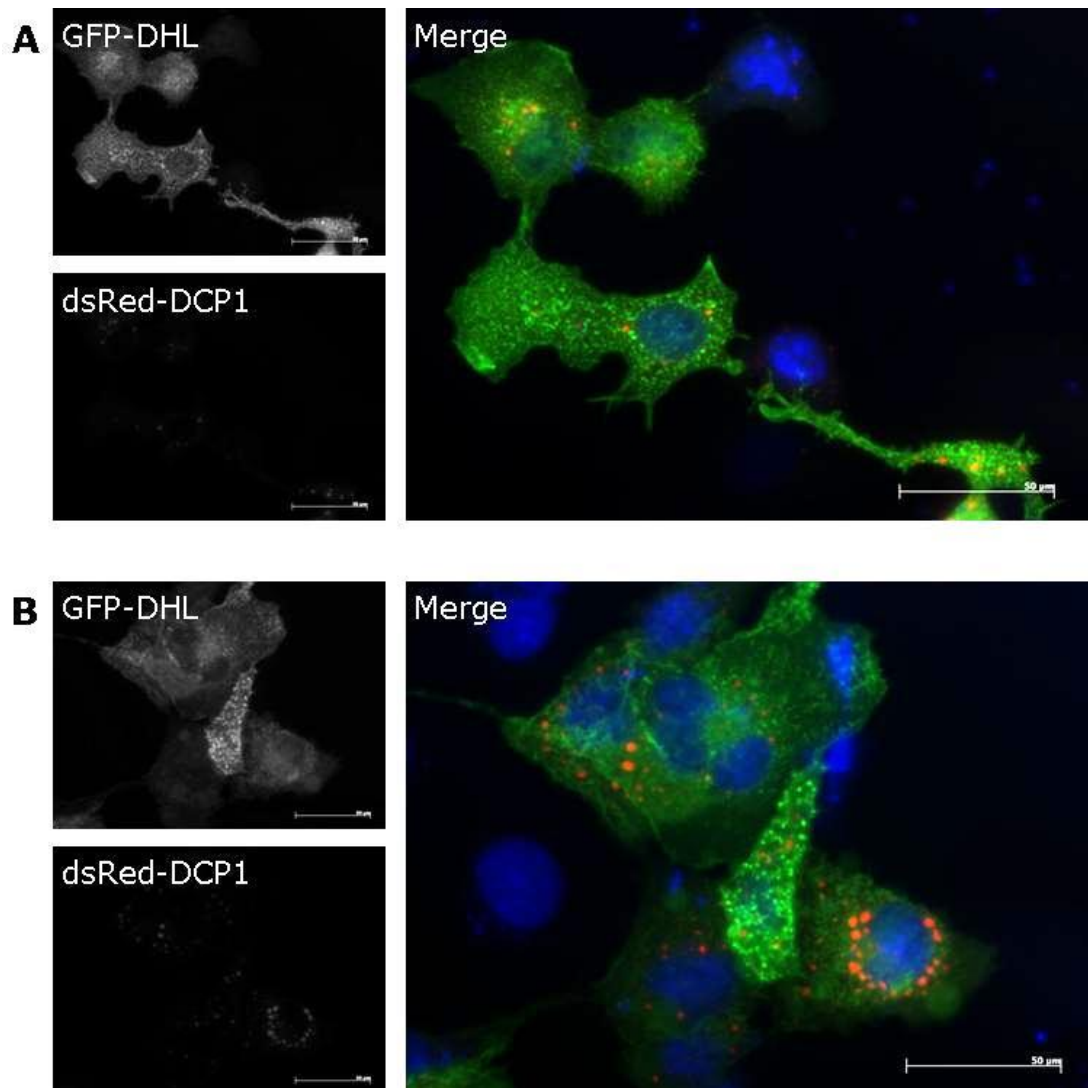


Figure 53 - GFP-DHL (amino acids 357-631)(green) does not co-localise with DCP1 (red). Cos7 cells were transfected with GFP-DHL and dsRed-DCP1 plasmid constructs and either left untreated (A) or treated with 1μM arsenite to induce formation of P-bodies for 30 minutes (B). Cells were fixed and stained with DAPI containing mountant (blue). An AxioVertS100 microscope was used to take widefield images of the fixed cells. The scale bar indicates 50μm.

In this experiment DHL domain granules do not appear to co-localise extensively with DCP1, suggesting that the DHL domain whilst able to drive aggregation requires other sequences in DEF6 for effective targeting to P-bodies. This is similar to glutamine/asparagine rich aggregating regions in other P-body proteins such as ROQUIN¹⁶⁹, Lsm4p¹⁰¹ and GW182¹⁸² that require a separate domain for P-body localisation. However, whilst most cells demonstrated a lack

of co-localisation between DCP1 and the DHL domain of DEF6, analysis by confocal microscopy revealed that in some cells the DHL domain sometimes clustered around the DCP1 granules.

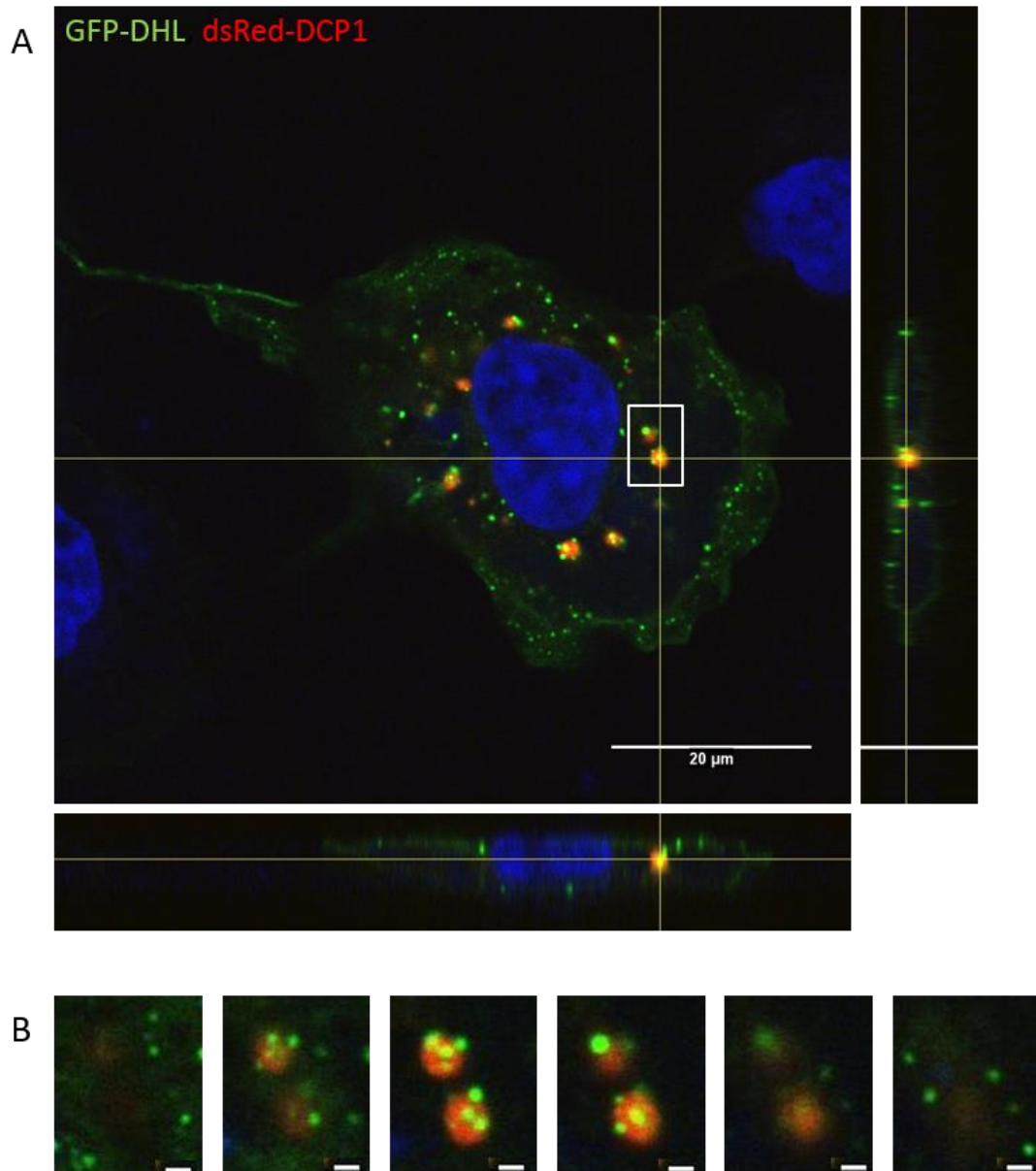


Figure 54 – In some cells the DHL domain (amino acids 357-631) (green) docks with and is contained within DCP1 (red). Cos7 cells were transfected with GFP-DHL and dsRed-DCP1 plasmid constructs. Z stacks were obtained using a Zeiss LSM710 confocal microscope. Presented is a representative Z stack with a xz and a yz projection (A), the area indicated in (A) is presented enlarged and with all the Z slices containing the indicated granule(B). Scale bar is 20 μm in (A) and 1 μm in (B). Each slices is 0.65 μm

It is also apparent that the DHL domain granules can be present within the DCP1 granules as seen in panel B of Figure 54 which shows granules present within the DCP1 granule in Z slices taken from the centre of the granule, but in a form

able to retain their own structure and shape as a granule, suggesting they are immiscible with the DCP1 marked P-bodies. This suggests a partial ability to interact with P-body components, but a failure to become mixed with other P-body components and integrated into the P-body throughout its structure. To fully interact with P-body components and form heterogeneous granules, other regions of DEF6 appear to be necessary. Because of its high degree of sequence conservation across evolution and because of its unknown function one potential region for this was the N-terminus of DEF6.

5.2.3 The N-terminus of DEF6 forms spontaneous granules

The lack of full interaction between the DHL domain and DCP1 suggests that another domain of DEF6 is responsible for interactions with P-body components and the co-localisation of the full length protein with DCP1. The sequence of the N-terminal domain is highly conserved across evolution as seen in Figure 55. This figure shows the sequence conservation between human DEF6 and orthologs of both DEF6 and its paralog SWAP70. It was produced using the ConSurf server which estimates the conservation of amino acids via analysis of residue location between homologs¹⁸³. The figure displayed indicates the conservation scores for each residue of human DEF6. The full colour coded multiple sequence alignment can be found in the appendix (Section 8.6). The sequences were chosen to provide an evolutionarily diverse set of protein sequences related to DEF6 and the DEF6 paralog SWAP70 (Table 5)⁸³. SWAP70 in humans is found in B cells, and is related to DEF6 through a gene duplication event⁸³.

Table 6 - Proteins used for multiple sequence alignment and amino acid conservation calculation indicating % sequence identity with H.Sapiens DEF6

Species	Protein Name	NCBI Reference	%Sequence Identity
<i>H.sapiens</i>	DEF6	NP_071330.3	100.0
<i>M.musculus</i>	DEF6	NP_081461.2	92.6
<i>G.gallus</i>	DEF6	XP_418019.3	75.8
<i>X.laevis</i>	DEF6	NP_001083435.1	68.0
<i>D.rerio</i>	DEF6	NP_957334.1	63.0
<i>H.sapiens</i>	SWAP70	NP_055870.2	45.6
<i>A.gambiae</i>	AGAP012332-PA	XP_001238272.2	29.0
<i>S.purpuratus</i>	SWAP70-like	XP_003728414	31.1
<i>T.adherens</i>	Putative uncharacterised protein	XP_002117356.1	18.4

High conservation of the N-terminal domain is seen even in the putative SWAP70 like protein found in *Trichoplax adherens*, the simplest non-parasitic multicellular animal which overall only has 18.4% sequence identity with human DEF6⁸³. This suggests a conserved role for the domain prior to its role in the adaptive immune system, which both *T.adherens* and *S.purpuratus* lack. The N-terminal domain does not currently have a known function, although it has been proposed to be an EF hand.

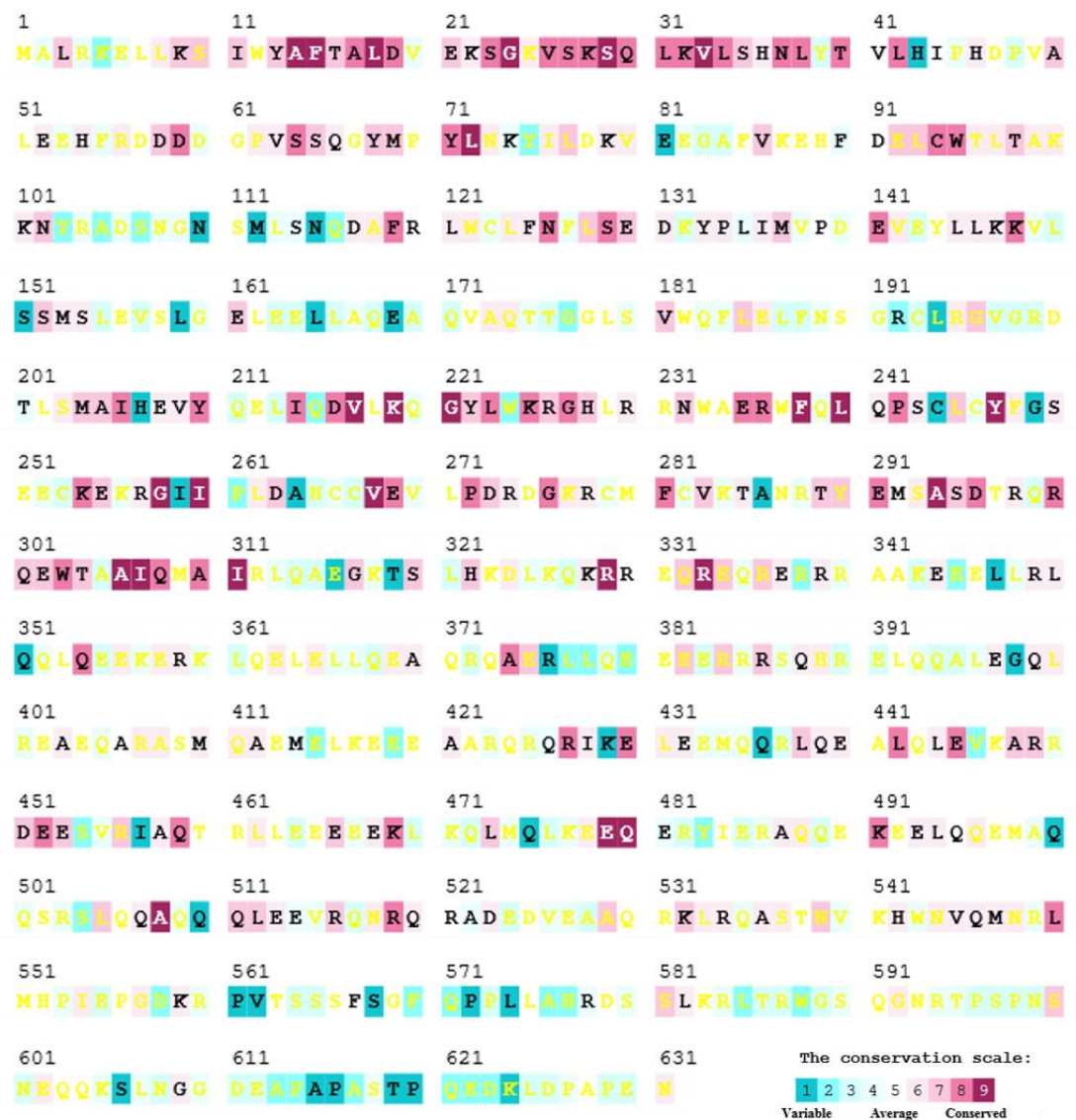


Figure 55 – The DEF6 amino acid sequence colour coded by ConSurf¹⁸³ to show sequence conservation between DEF6 and a selection of its paralogs indicating sequence conservation at the N-terminus.

Preliminary unpublished data has suggested that the N-terminal domain of DEF6 is able to bind RNA binding protein. This suggests it may be the domain of DEF6 that drives localisation of DEF6 into P-bodies. To test whether the N-terminus of DEF6 is responsible for localisation of the full length protein to P-bodies, a GFP tagged plasmid construct containing amino acids 1-109 was made. First, the ability of the GFP-N-terminus plasmid construct to produce protein that accumulates into foci was assessed. Cos7 cells were transfected with the GFP-N-terminus construct and were either untreated or treated with arsenite to induce global arrest of translation to promote granule formation.

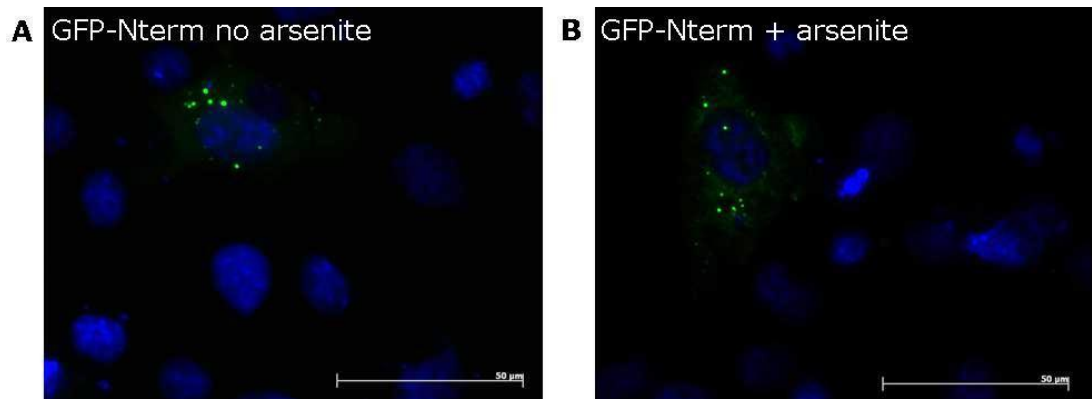


Figure 56 – The N-terminus (amino acids 1-109) of DEF6 forms granules. Cos7 cells were transfected with GFP-Nterminus and either left untreated (A) or treated with 1 μ M arsenite for 1 hour (B). Cells were fixed and stained with DAPI containing mountant (blue). An AxioVertS100 microscope was used to take widefield images of the fixed cells. Scale bar is 50 μ M.

The N-terminus is able to form granules, both under conditions of arrested translation via arsenite treatment, and spontaneously without arrest of translation. Whilst it does not contain the aggregating sequences present in the DHL domain that allow the DHL domain plasmid construct to form granules, if it is able to interact with P-body components, it may localise to endogenous granules and thus appear in a granular form. To test its ability to interact with P-bodies, its co-localisation with the P-body marker DCP1 was studied.

5.2.3.1 The N-terminus of DEF6 localises to DCP1 bodies

To assess whether the N-terminal protein was localising to P-bodies, the GFP-tagged N-terminal domain of DEF6 was transiently co-expressed with dsRed-DCP1 in Cos 7 cells (Figure 57).

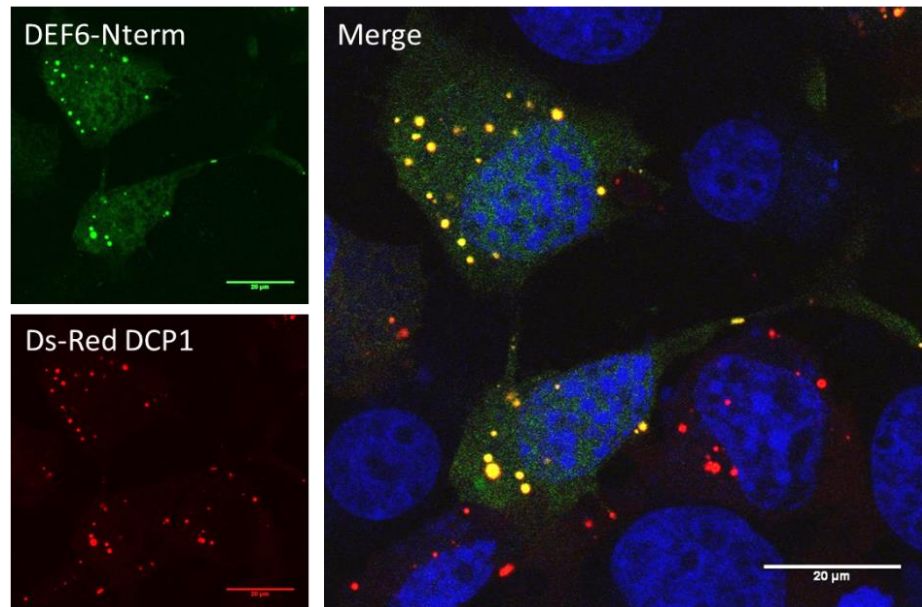


Figure 57 – The N-terminus of DEF6 co-localises with DCP1. Cos7 cells were transfected with GFP-N-terminus and dsRed-DCP1. A single Z plane image was obtained using a Zeiss LSM 710 confocal microscope. Scale bar is 20μM.

The N-terminus of DEF6 co-localises with DCP1 in P-bodies, and is homogenously incorporated throughout their structure suggesting that it is the domain of DEF6 responsible for localisation to P-bodies.

5.2.4 Movement in and out of granules

The exchange of proteins found in P-bodies and stress granules with the cytoplasm has been measured previously using fluorescence recovery after photobleaching (FRAP). This has shown that different proteins exchange with the cytoplasm at different rates, suggesting different roles for the proteins in the granules. It has been suggested that those that exchange quickly are tethered to mRNA, those that exchange slowly are core structural components and those with intermediate dynamics shuttle between the granule and cytoplasm but are not bound to mRNA⁹³. FRAP on granules bleaches individual granules within cells, by following the change in fluorescent intensity of the granule over time, the exchange of bleached molecules within the granule with unbleached molecules outside the granule can be measured. Using FRAP on granules formed by the GFP-DEF6 plasmid construct, its rate of exchange with the cytoplasm can be measured and compared to the rates measured for other proteins. The GFP-

DEF6 fusion protein was transiently transfected into Cos7 cells and treated with arsenite to induce translational arrest and the formation of DEF6 granules. Granules were selected that were of average size and targeted with the laser to photo bleach the granule. Measurements were taken from single granules in 8 different cells, grown in 3 different dishes and the results averaged. The strength of the laser was such that the granules were bleached to approximately 50% their original intensity so the granule was still visible to confirm it did not move out of the selected area over time. The recovery of fluorescence within the selected area was followed by measuring the intensity of fluorescence. Identically sized areas within the cytoplasm and outside the cytoplasm were also measured for fluorescence over the time course of the experiment. The area outside the cell was used to subtract any background signal not due to GFP fluorescence in the cell from both the granule measurement and the cytoplasm measurement. The cytoplasmic measurement was used to follow decreases in fluorescence not due to the directed bleaching but due to time related photo bleaching due to exposure. These measurements were used to correct the measured fluorescence of the granule over time. The kinetics of diffusion could then be compared with the published rates for other P-body proteins. Data is shown below as both the relative intensity of the selected granule with 1.0 being the starting intensity, and the granule bleached to approximately 0.5 relative intensity, and the % recovery, with the initial intensity set as 100%, and the signal immediately after bleaching set as 0%.

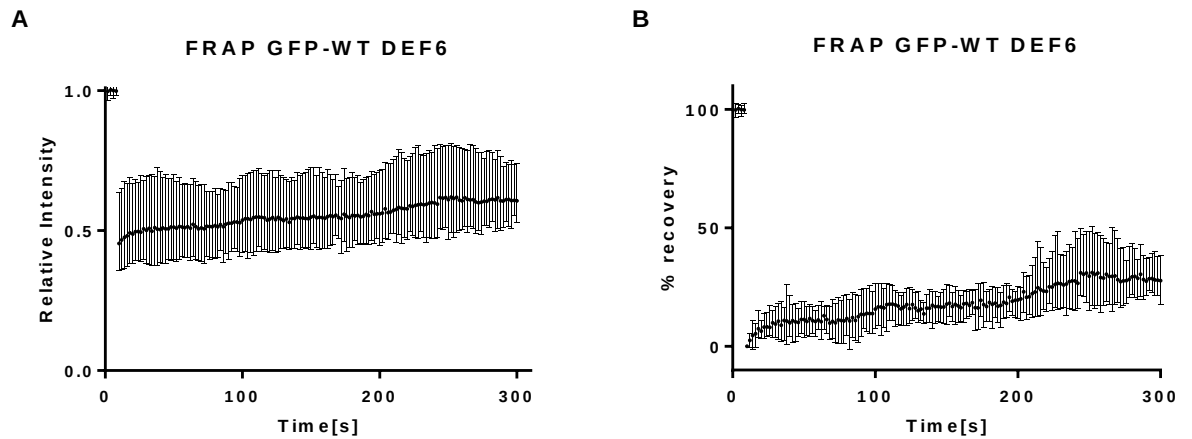


Figure 58 - Data from FRAP experiment on GFP-WT DEF6 granules formed in Cos7 cells after 60minutes arsenite treatment (A) Relative intensity (B) % recovery. Error bars show range of measurements from 8 granules from 3 transfections.

The majority of GFP-DEF6 did not recover fluorescence, with only 34.7% recovery of fluorescence in 5 minutes. This suggests a core fixed role for DEF6 in P-bodies, rather than a role shuttling mRNA or other components in and out of granules.

Table 7 - FRAP rates for P-body and stress granule forming proteins

Protein	% recovery in 30s	% recovery total
Ago2 ¹⁸⁴	~50	~50
DCP1a ^{92,93}	77 ⁹² , 71 ⁹³	86
DCP1b ⁹²	51	67
DCP2 ⁹²	No recovery	No recovery
DDX6 ¹⁸⁴	~40	~45
DEF6	9.4	34.7
Dhh1 ¹⁸⁵	~35	~42
eiF4e ¹⁸⁶	~20	45
eiF4e-T ¹⁸⁶	~40	89

FAST ⁹³	~12	Not reported
G3BP1 ⁹³	~97	100%
GW182 ⁹³	~21	Not reported
Lsm4 ¹⁸⁴	~60	~70
Lsm6 ¹⁸⁶	~30	73
PABP1 ⁹³	~42	Not reported
Staufen ¹⁸⁷	~5	40
Tia1 ⁹³	~100	~100
TTP ⁹³	~83	Not reported

The recovery of DEF6 is similar to that of Ago2, DDX6, eIF4E, FAST, GW182, and Staufen, which have been reported as exhibiting slow exchange with the cytoplasm and thus classified as core or scaffold proteins in P-bodies. Speed of recovery has been linked to the role of the protein in P-bodies, with slow recovery indicating a structural role and fast recovery suggesting a role as transient tethers for mRNA. Recovery has been noted in some cases to depend on granule size, for example the recovery rate of DCP1a has been reported to be rapid in small P-bodies but slow in larger ones⁹³. The slow recovery of DEF6 suggests that it provides a structural rather than tethering role within granules. This could be due to the glutamine/asparagine rich C-terminal region of DEF6, which in the essential core protein Lsm4 is necessary for aggregation. It has been noted that these glutamine/asparagine rich regions are common in P-body and stress granule proteins and may provide a means for proteins outside the 3 essential core proteins (EDC3, Lsm4 and Pat1, which are required for P-body formation) to participate in the aggregation process⁹⁰.

Considering a link between glutamine/asparagine rich regions; of the proteins with similar rates of recovery as DEF6, GW182 exhibits similar slow recovery and contains a glutamine/asparagine rich region. DDX6 is slow but not particularly glutamine/asparagine rich, although it has a small glutamine/asparagine rich section near its N-terminus, however its yeast homolog Dhh1 is classified as glutamine/asparagine rich and exhibits very similar dynamics to those of DDX6 and DEF6. eIF4E, FAST, Ago2 and Staufen however do not contain glutamine/asparagine rich regions (supplementary material section 8.7) indicating that a glutamine/asparagine rich region is not necessary for slow exchange. These proteins are likely to have other domains that allow them to interact to remain in P-bodies in a relatively stable manner. Tia1 and Lsm4 on the other hand also contains a glutamine/asparagine rich region (supplementary material section 8.7), but recover quickly and in the case of Tia1, fully. The fast recovery of Tia1 is consistent with its role in mRNA binding, but the relatively quick recovery of Lsm4 is interesting due to both its glutamine/asparagine rich region, and its role as a core protein necessary for P-body aggregation. This suggests that glutamine/asparagine rich regions, whilst able to interact and promote aggregation must have a certain degree of reversibility to their aggregation propensity. Whilst targeting granules for FRAP, it was observed that the granules exhibited movement within the cytoplasm. Whilst this was in a relatively localised manner for the full length DEF6, the GFP-N-terminus (amino acids 1-109) plasmid construct was observed to move rapidly. This meant that determination of whether the smaller N-terminal construct could move in and out of granules faster than the full length due to the loss of the aggregating domain via FRAP on GFP-Nterminus was unsuccessful, as due to rapid movement it was not possible to target granules accurately for FRAP, and any that were bleached could not have their recovery reliably measured due to movement. These observations are the first evidence that

DEF6 exhibits movement within the cytoplasm similar to the observations of movement of other P-body components including DCP1 and DCP2⁹².

5.2.5 Movement of granules

To follow the dynamics of DEF6 granules in living cells, GFP-tagged DEF6 and N-terminus (amino acids 1-109) were overexpressed in Cos7 cells which were treated with arsenite for 1 hour to induce translational arrest before imaging. In general, the GFP-DEF6 exhibited small localised movements, whilst in contrast the GFP-Nterminus exhibited faster localised movements with occasional fast directed movement. To quantitatively describe the dynamics, imaging was followed by single particle tracking using the point tracking software Mosaic Particle Tracker 2D/3D which provides coordinates at each time point for numbered particles, allowing analysis of the area the granules visited, their speed, and their displacement for MSD (Mean Square Displacement) analysis.

5.2.5.1 Example traces

Tracking software provided X and Y coordinates for each detected particle with time data for each coordinate. These were used to produce example traces of the particles.

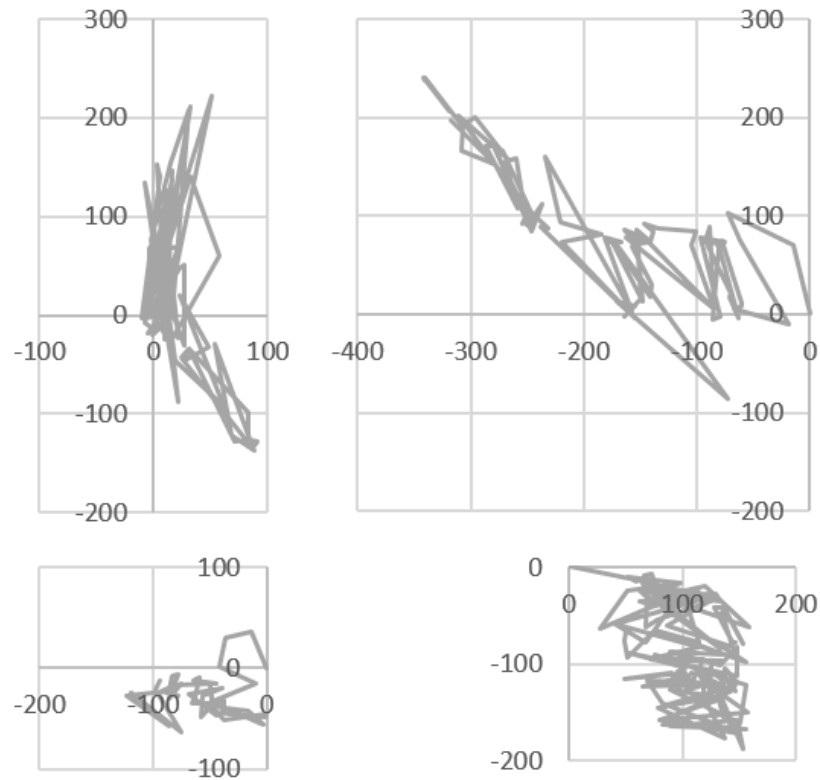


Figure 59 Granules formed by GFP-DEF6 exhibit movement within cells. Granules were live imaged and tracked over time and example traces are shown here for individual granules from one randomly selected cell to demonstrate that movement is in multiple directions and thus not due to cell movement. Cos7 Cells were transfected with GFP-DEF6, and treated with 1 μ M sodium arsenite for one hour, 24 hours after transfection. Cells were live imaged on a Zeiss LSM 710 confocal microscope with 2 seconds between images. Units are in nm. Cell and traces were selected randomly.

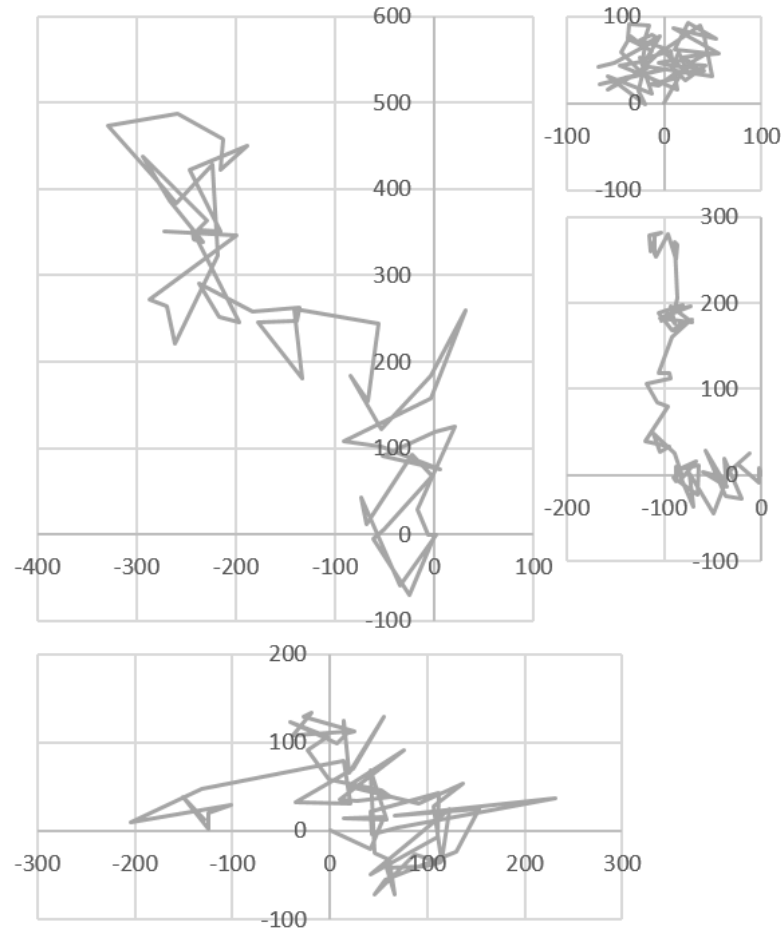


Figure 60 - Granules formed by GFP-N-terminus exhibit movement within cells. Granules were live imaged and tracked over time and example traces are shown here for individual granules from one randomly selected cell to demonstrate that movement is in multiple directions and thus not due to cell movement. Cos7 Cells were transfected with GFP-N-terminus, and live imaged on a Zeiss LSM 710 confocal microscope with 2 seconds between images 24 hours after transfection. Units are in nm. Cell and traces were selected randomly.

As can be seen in Figure 59 and Figure 60 the granules generally exhibited constrained movements, with a few more directed movements over longer distances. The figure also demonstrates that the movement measured is due to granule movement and not due to cell drift, which was corrected for using the imageJ plugin "stackreg"¹⁸⁸.

5.2.5.2 Area visited

Using the data described above, the area visited in the cytoplasm was calculated for each particle detected by calculating the largest possible circle that

constrains the X and Y coordinates for each track. To do this, the largest and smallest of each X and Y coordinate were found, and the distance between these calculated. The largest distance was used as the diameter of a circle, which would be the circular area that constrained all of the X and Y coordinates measured for the granules movement. These calculations were performed in excel. In total the area visited by 719 GFP-DEF granules and 773 GFP-N-terminus granules from 5 separate cells for each experiment was measured.

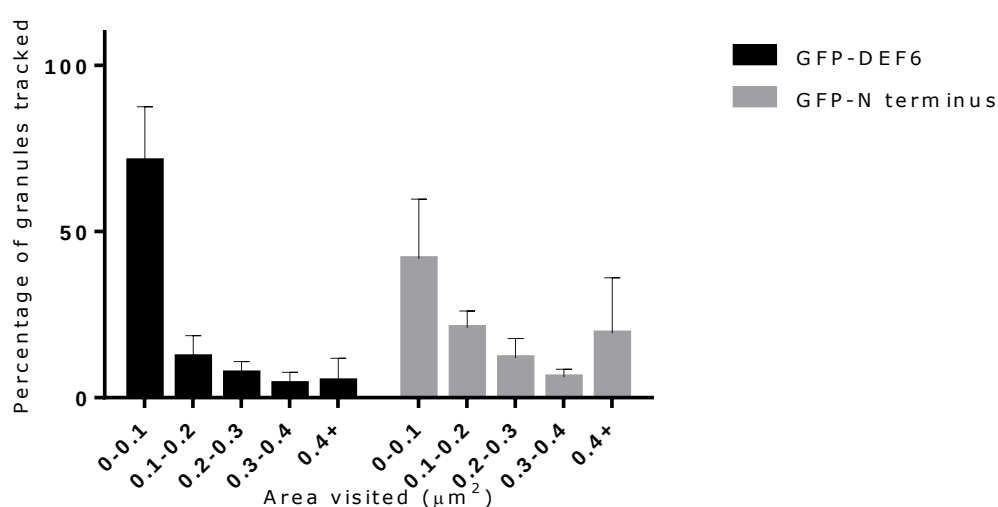


Figure 61 - GFP-DEF6 visits a very small area, whilst GFP-Nterminus is able to visit larger areas. The largest circle to contain the traces of each granule was calculated for the granules from 5 images. Bars show standard deviation

The average area visited by GFP-DEF6 was $0.10\mu\text{m}^2$, whilst the average area visited by GFP-Nterminus was $0.33\mu\text{m}^2$. This compares with an average granule area of $0.36\mu\text{m}^2$ for GFP-DEF6 and $0.09\mu\text{m}^2$ for GFP-Nterminus. This shows that the GFP-DEF6 granules are remaining relatively stationary, moving in an area smaller than their own area, whilst the GFP-Nterminus granules are visiting a larger area than their own area. This could imply a tethering role for the C-terminus of DEF6 in keeping the granules more closely tied to one spot that is lost in the GFP-N-terminus which only contains the amino acids 1-109. Both the GFP-DEF6 and GFP-N-terminus granules moved in a much more constrained area than those measured by Aizer *et al.* 100% of the GFP-DEF6 granules and

97.7% of GFP-N-terminus granules moved within an area of less than $2\mu\text{m}^2$, exhibiting more constrained movement than DCP2, a marker of P-body granules, which Aizer *et al.* measured as 48% of granules moving within an area of greater than $2\mu\text{m}^2$. DCP1a and DCP1b act similarly to DCP2, with 46% and 40% respectively moving within an area of greater than $2\mu\text{m}^2$.

5.2.5.3 Speed of movement

The average speed of each granule along its trajectory was calculated by dividing the total length travelled by the time it took to travel that distance. To calculate the total length travelled, the length travelled between each time point was calculated by working out the change in Y and the change in X, then using Pythagoras' theorem to calculate the length travelled. These were summed for all the measured time points to get the total distance travelled. These calculations were performed in excel. In total the average speed of 719 GFP-DEF granules and 773 GFP-N-terminus granules was measured.

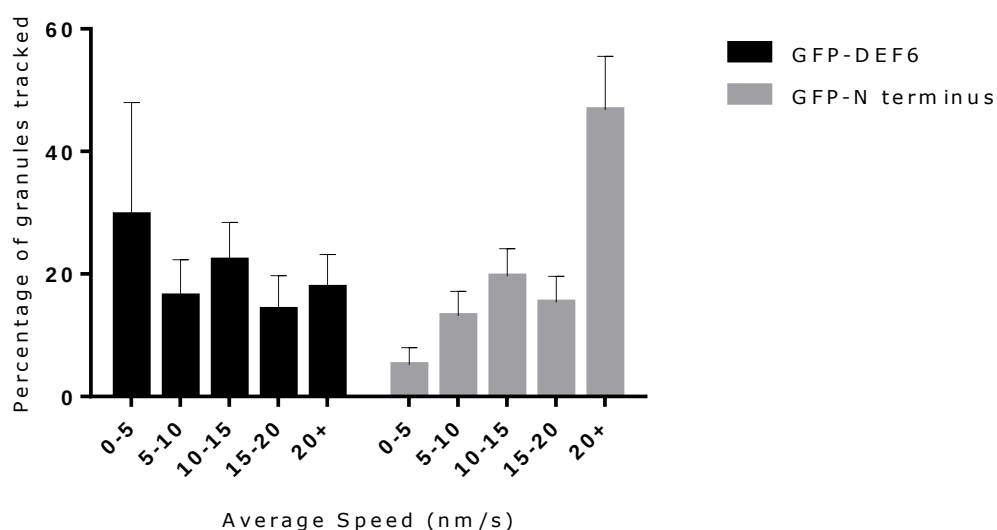


Figure 62 - The N-terminus travels with a faster speed than full length DEF6. Distance/time was calculated for each granule using the total distance travelled over the length of the trajectory. Bars show standard deviation

GFP-N-terminus granules exhibited a higher number of faster moving granules than full length DEF6 granules in line with what can be seen in the videos of the

granule movement (attached CD). The GFP-N-terminus granules moved at an average speed of 24.7nm/s, twice the mean average speed of the GFP-DEF6 granules at 11.4nm/s. Thus not only is GFP-DEF6 moving in a smaller area than GFP-N-terminus, but it is doing so more slowly. The average speed however does not provide a full picture of whether the movements are rapid back and forth in one spot, or in a directed manner. It is possible that due to their lower speed, the GFP-DEF6 granules visit a smaller area than the GFP-N-terminus granules because of their slower speed, this would demonstrate slow but directed movement. To compare the directed movement of the granules, average velocity was also measured for each granule. If the average speed is similar to the average velocity, the movement is more directional, whilst if the average speed is much faster than the average velocity, the granule is likely to be moving back and forth in a confined manner.

The average velocity of each granule was calculated by calculating the change in X and Y between the starting and finishing time point, and calculating the distance travelled using Pythagoras' theorem. This gives the displacement of the granule from its starting point, which is divided by the time taken to give the average velocity the granule moved with, which is a directional measurement (how fast the granule moved in a particular direction).

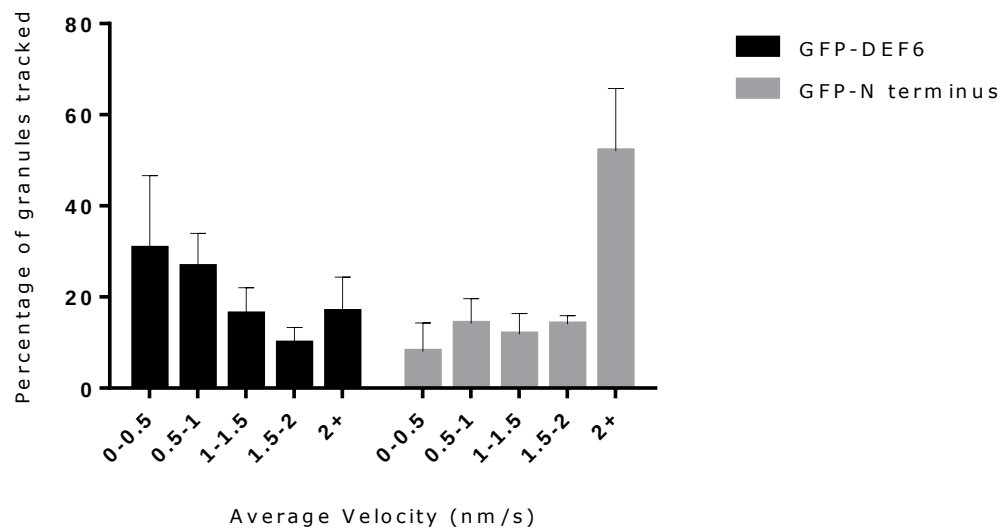


Figure 63 - GFP-N-terminus moved with faster velocity than GFP-DEF6. Displacement/time was calculated for each granule by taking the total X and total Y displacements, summing them to get a displacement from the origin and dividing by the length of the trajectory in time. Bars show standard deviation

Again, the N-terminus of DEF6 exhibited higher velocities than the full length DEF6 construct, indicating faster displacement of the N-terminal granules from their point of origin. This supports the data showing that GFP-N-terminus visits a larger area than GFP-DEF6 as the velocity of the GFP-DEF6 will be diminished as its movement is back and forth in a confined area.

The mean of the average velocities for the granules were 1.22nm/s for the GFP-DEF6 construct, whilst the GFP-N-terminal granules had a mean average velocity 3 times faster at 3.87nm/s. The difference between velocities is higher than the difference between speeds, suggesting that the N-terminal granules have more directional movement than the DEF6 granules. Additionally, whilst the average speed of the GFP-N-terminal granules is 6x that of their average velocity, the average speed of the GFP-DEF6 granules is 9x that of their average velocity, again pointing to back and forth movement in a confined area. Further supporting this, in the GFP-N-terminus transfected cells, granules exhibiting fast directed motion were also present, as illustrated in Figure 64.

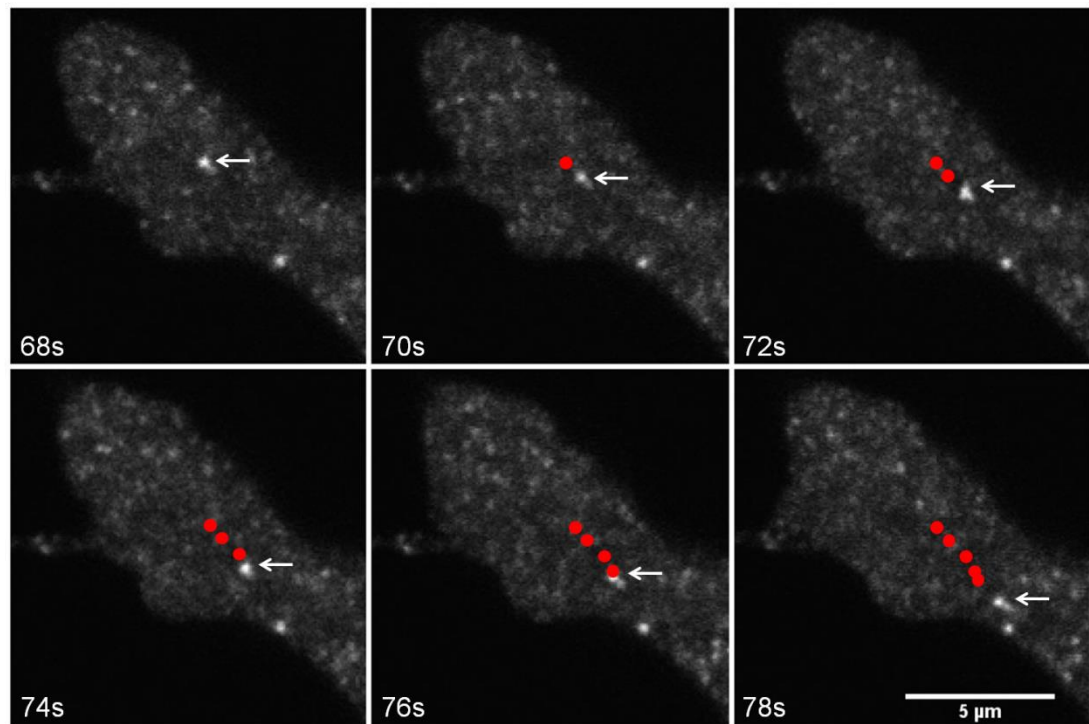


Figure 64 – An example of quick directed movement of a GFP-N-terminus granule. Cells were transfected with GFP-Nterminus and treated with arsenite. This is a series of images taken 2 seconds apart which show the movement of an N-terminal DEF6 granule indicated by the white arrow moving rapidly in a linear manner. The red dots show where the granule was previous to the current image. The total distance covered in the images show in 4μm.

The granule in Figure 64 exhibited a velocity of 0.4μm/s, similar to the 0.5-1.1μm/s measured by Aizer *et al.* in their granules with fast directional movement. These fast directional movements by GFP-N-terminus are the first indication that DEF6 is likely to be associated with the tubulin cytoskeleton. This is significant as it provides a mechanism for DEF6 to rapidly move throughout the cells, and polarise in activated T cells. T cells polarisation involves the repositioning of the microtubule organising centre to a position between the nucleus and the immune synapse. This contributes to the formation of the synapse, and the polarised release of cytokines¹⁸⁹ and cytolytic granules¹⁹⁰. Association with and movement along the microtubule network provides a mechanism for the polarisation of DEF6 seen in activated T cells.

5.2.5.4 Mean Squared Displacement Analysis

To describe the extent of movement of the granules, mean squared displacement was calculated. Mean squared displacement measures the movement of a “random walker” and the area that it visits, this gives an idea of the properties of the environment it is moving in. For normal diffusion, the MSD grows proportionally to Δt (the change in time in seconds) as illustrated by the blue trace in Figure 65. If the environment constrains the movement of the particle, the increase in MSD increases slowly with respect Δt and is referred to as subdiffusion. Finally, if the increase in MSD is faster as Δt increases, the particle is exhibiting superdiffusion and in a cellular environment is likely to be moving via active transport. For granules in cells, this analysis can give an idea of whether the granules are able to move quickly and directionally, suggesting active transport potentially along microtubules, exhibit free diffusion within the cytoplasm, or if they are corralled to an area, for example by tethering to the actin cytoskeleton.

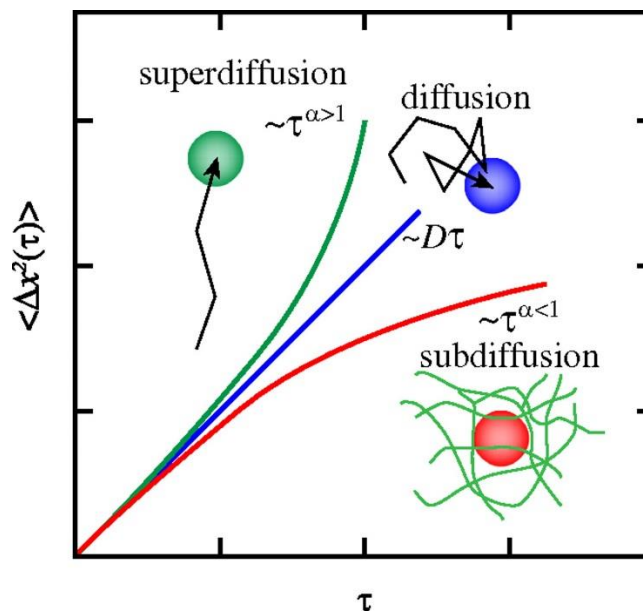


Figure 65 - Examples of MSDs of particles indicating normal diffusion (blue), subdiffusion (red) and superdiffusion (green). The axes shown the mean squared displacement (MSD, $\Delta x^2(\tau)$) against the change in time τ . Diagram from F. MacKintosh¹⁹¹

The data obtained from the particle tracking gives positions X_i and Y_i at different time points. The displacement between positions is calculated for different time

lags, Δt starting with $\Delta t = 2s$ up to $\Delta t = 50s$. Both X and Y displacements are squared, and summed to give the square displacement r^2 at time lag Δt (Equation 1). The displacements for each time lag are averaged to give the mean square displacement (Equation 2), which are plotted against Δt .

$$\langle r^2 \rangle = (\Delta X_1(\Delta t))^2 + (\Delta Y_1(\Delta t))^2 \quad (\text{Equation 1})$$

$$\langle r^2 \rangle = \frac{1}{n} \sum_{i=1}^n r_i^2(\Delta t) \quad (\text{Equation 2})$$

MSD was calculated for 5 randomly chosen traces for each cell from each plasmid construct, between $\Delta t=2s$ and $\Delta t=50s$ where the traces were longer than 60s. The curves obtained suggested the presence of 4 types of movement, active transport, diffusion, confined diffusion and little to no movement (Figure 66 and Figure 67). A combination of the types of movement can also be seen, typically with a trace that starts diffusing, plateaus indicating confined movement but in later time lags demonstrates an increase in displacement again. Active transport suggests the movement of granules along microtubules, diffusion suggests the granules are free within the cytoplasm, whilst confined diffusion suggest the granules are connected to the actin cytoskeleton and thus confined to one area. Classification was done by fitting a linear equation (Equation 3) and a quadratic equation (Equation 4, where v is the mean velocity) to the plots and selecting the curve with the best fit for diffusion and active transport, whilst the traces demonstrating corralled diffusion were classified on the basis of the trace plateauing indicating a lack of increase in displacement after a certain amount of time. Some traces demonstrating confined diffusion could be fitted to the equation for confined diffusion (Equation

5, where r_c^2 is an approximation of the size of the confinement, and the constants A_1 and A_2 are defined by the geometry of confinement), but many could not due to the mixed nature of the movement (starting out with normal diffusion but plateauing relatively late). Several traces showed very little displacement throughout the time course, indicating little to no movement.

$$\langle r^2 \rangle = 4D\Delta t \quad (\text{Equation 3})$$

$$\langle r^2 \rangle = v^2\Delta t^2 + 4D\Delta t \quad (\text{Equation 4})$$

$$\langle r^2 \rangle = \langle r_c^2 \rangle \left[1 - A_1 \left(-\frac{4A_2 D \Delta t}{\langle r_c^2 \rangle} \right) \right] \quad (\text{Equation 5})$$

The MSD curves for all 25 GFP-DEF6 trajectories can be seen in the appendix. Examples of the different traces of the 4 different types of movement are shown in Figure 66.

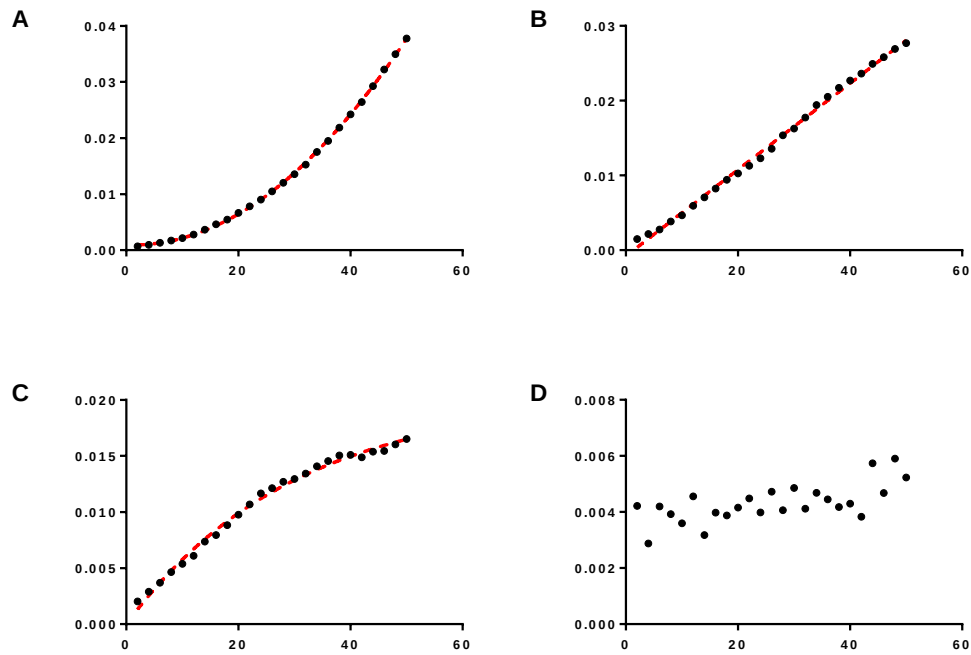


Figure 66 - MSD traces for 4 GFP-DEF6 trajectories indicating (A) active transport (B) normal diffusion (C) confined diffusion and (D) little to no movement. The fitted curves for active transport, normal diffusion and confined diffusion are shown in red.

GFP-DEF6 exhibited mainly normal diffusion, with 40% of MSD traces fitting best to a linear regression, closely followed by active transport with 32% of the MSD traces fitting best to a non-linear second order polynomial regression. Confined diffusion and no movement were demonstrated by the same number of MSD curves with 12% each. One trace could not be classified according to my system, which showed a decrease in the rate of increase of displacement at higher time lags similar to confined diffusion, but not a full plateauing. This distribution of types of movement would seem to suggest that GFP-DEF6 is relatively free to move within the cytoplasm, as indicated by the number following normal diffusion, and the lack of a significant number demonstrating constrained movement. Despite this freedom, the granules do not move widely or quickly as they have no means of active transport.

Using (Equation 4) values for the velocity of transport and the diffusion rate could be calculated. The values obtained in the case of active transport gave

diffusion rates of between $5.5 \times 10^{-6} \mu\text{m}^2/\text{s}$ and $2.1 \times 10^{-5} \mu\text{m}^2/\text{s}$, which is in the range for small molecules to proteins diffusing in water. A faster diffusion coefficient was measured from the trajectories demonstrating normal diffusion between $1.3 \times 10^{-5} \mu\text{m}^2/\text{s}$ and $1.4 \times 10^{-4} \mu\text{m}^2/\text{s}$. The velocities obtained were between 0.9nm/s and 3.9nm/s, which fits with the average velocities measured directly from the granules the average of which was 1.22nm/s in Figure 63.

The MSD curves for all 25 GFP-N-terminus trajectories can be seen in the supplemental data in the appendix section 8.10. Examples of shown active diffusion, normal diffusion and confined movement are shown in Figure 67, along with a trajectory showing a combination of types of movement.

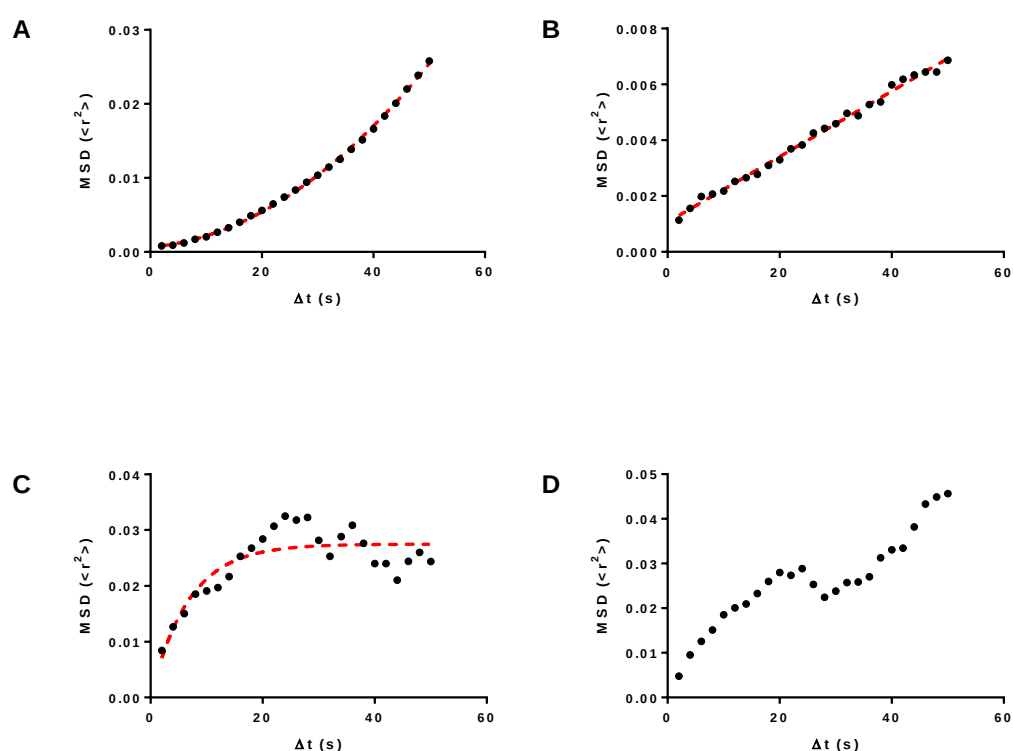


Figure 67 - MSD traces for 4 GFP-N-terminus trajectories indicating (A) active transport (B) normal diffusion (C) confined diffusion and (D) mixed modes of movement. The fitted curves for active transport, normal diffusion and confined diffusion are shown in red.

GFP-N-terminus exhibited mainly active diffusion, with 40% of MSD traces fitting best to a non-linear second order polynomial regression, closely followed by confined movement with 28% of the MSD traces exhibiting a plateauing in

increase in MSD. Mixed movement was seen in 20% of the trajectories, and was indicated by a trace that formed a plateau after a short increase in displacement, followed by a renewed increase in displacement after the plateau. Normal diffusion was demonstrated by only 12% of the trajectories. This distribution of types of movement would seem to suggest that the GFP-N-terminus granules are not free to diffuse within the cytoplasm, as indicated by the low number of granules demonstrating this type of movement. Instead, they are constrained potentially by association with parts of the cytoskeleton, along which they can then be actively moved accounting for the high number of granules indicating either active or constrained movement. The existence of mixed movement suggests a stop start movement along the microtubules, which constrain diffusion away when they are not actively moving.

Using (Equation 4) values for the velocity of transport and the diffusion rate could be calculated. The values obtained in the case of active transport gave diffusion rates of between $1.7 \times 10^{-5} \mu\text{m}^2/\text{s}$ and $1.9 \times 10^{-3} \mu\text{m}^2/\text{s}$, which is faster than the range for small molecules to proteins diffusing in water indicating very fast diffusion. A slower diffusion coefficient was measured from the trajectories demonstrating normal diffusion, with a range of $2.9 \times 10^{-5} \mu\text{m}^2/\text{s}$ and $2 \times 10^{-4} \mu\text{m}^2/\text{s}$. The velocities obtained were between 1.1nm/s and 6.1nm/s, which fits with the average velocities measured directly from the granules the average of which was 3.87nm/s in Figure 63.

5.3 Discussion

5.3.1 Domain differentiation

Furthering the experiments by Hey *et al.*¹⁶², the DHL domain was confirmed to be able to form foci independent of arsenite induced translational arrest. Initial experiments demonstrated that the DHL domain foci rarely co-localised with the P-body marker DCP1, indicating that whilst the DHL domain can drive aggregation, it is not sufficient for efficient co-localisation with other P-body components. However, more detailed examination of the foci using confocal microscopy suggested that whilst the DHL foci and the DCP1 foci did not fully co-localise, the DHL domain foci could be found clustered around the DCP1 foci, with some mixing of the two proteins at the edges of their respective foci. The DHL foci could also be seen to exist within the DCP1 foci, but manage to remain distinct. This could suggest that the DHL aggregation interactions are strong, and that other regions of DEF6 is required to promote mixing with other P-body components. P-bodies and stress granules have been shown to compartmentalise from the cytoplasm despite their lack of a membrane due to liquid-liquid unmixing like behaviours of the constituent proteins, in particular driven by interaction between low complexity regions^{88,192,193}. The coiled coil interactions may allow for similar unmixing of the protein from the surrounding cytoplasm, and only allow for incorporation into other P-bodies when another domain interacts with components within these other granules. Complex phase behaviour has been observed in synthetic polymer systems in which phase separation of charged polyelectrolytes is influenced by ionic strength, a system which is thought to allow segregation of cellular components in biological systems^{194,195}. Structural changes are able to alter the phase behaviour of proteins, which normally exhibit phase behaviour at high concentrations. The critical concentration is higher for globular proteins than for random coil making them more likely to coalesce into the liquid like phase that describes granules such as P-bodies. More than two phases can be obtained with extra types of

polymer are present^{194,196}, and the coiled coil propensity of the DHL domain may provide it with different enough properties from the globular proteins that for granules that it can separate into another separate phase, thus remaining separate from other foci.

Here it has been shown that the N-terminal domain of DEF6 allows integration of DEF6 into P-bodies. Surprisingly, the GFP-tagged, N-terminal construct comprising amino acids 1-109 of DEF6 formed granules independently of arsenite-induced translation arrest. The larger granules co-localised with DCP1 indicating that the N-terminus can localise to P-bodies. This suggests that the N-terminus is able to target, and interact with P-body components in a way that promotes inclusion and mixing of DEF6 into P-bodies. Its formation of granules independently of the C-terminus suggests that it may localise to endogenous granules which present independent of arsenite induced translational arrest as it does not contain aggregating sequences. It is possible that the smaller granules that do not contain DCP1 contain other P-body forming proteins and that the N-terminus is able to associate with several P-body component proteins.

A division between protein sequences involved aggregation and localisation has been demonstrated by Reijns *et al.*¹⁰² who showed that glutamine/asparagine-rich regions contribute to the accumulation of P-body components into P-bodies, but that they are not essential for P-body formation. Similar to the results seen with DEF6, they showed that whilst the glutamine/asparagine-rich region of Ccr4p forms granules without inducing global arrest of translation, it is unable to co-localise with Dcp2. In Pop2p and Dhh1p, they showed that whilst the glutamine/asparagine-rich region was unable to form granules alone, it was necessary for the formation of granules of the full length protein and their co-localisation with P-bodies. Similarly Behm-Ansmant *et al.* showed that the Q-rich domain of GW182 contributes to, but is not sufficient for its localisation into P-bodies¹⁷⁰. Additionally the glutamine/asparagine-rich region of Lsm4p has been

shown to be required for P-body assembly in the absence of the P-body scaffold protein Edc3p, supporting a role for glutamine/asparagine-rich regions in the aggregation of P-body components into granules¹⁰¹. In Lsm4p the glutamine/asparagine-rich region can be replaced by other prion forming domains and retain its aggregating ability.

5.3.2 DEF6 is a stable component of granules

The FRAP data for GFP-DEF6 shows that the majority of DEF6 within granules does not exchange with the cytoplasmic pool of protein, and the small pool of protein that does, does so slowly. Slow movement in and out of granules suggests that the role of DEF6 in granules requires it to be static, and may be part of the stabilization of the granules. This is supported by the glutamine/asparagine rich region present at the C-terminus of DEF6, which is a characteristic of both several P-body proteins, and of prion proteins. In prion proteins this stretch is involved in aggregation, and this role is also present for the glutamine/asparagine rich region of Lsm4, where it is necessary for the aggregation of P-bodies.

Fast exchanging proteins in P-bodies such as the RNA binding protein TTP have been suggested to play a role in tethering mRNA and bringing it to the P-bodies for degradation⁹³. The kinetics of DEF6 recovery rule this out as a role for the full length protein, however there is a possibility that the N-terminus may be involved in mRNA binding either within the P-bodies whilst securely integrated via the C-terminus of DEF6, or potentially cleaved from DEF6. There is a predicted caspase 1 cleavage site at amino acid 106 within the N-terminus that may allow the N-terminus to act independently of the rest of DEF6.

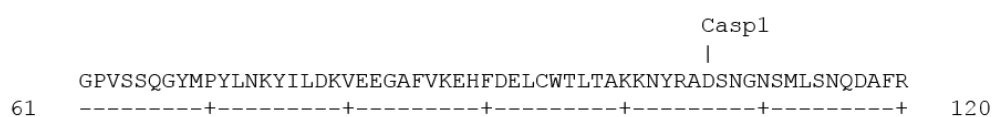


Figure 68 - A caspase 1 cleavage site is predicted at amino acid position 106 in DEF6 by the ExPASy PeptideCutter tool

If this happens prior to the incorporation of DEF6 into granules, it could provide two roles for DEF6 in the granules, a stabilizing and a tethering role. The kinetics of recovery of the N-terminus of DEF6 in granules was not achieved in this study due to difficulty following fast moving bleached protein, but use of a photoactivatable form of the N-terminal construct may provide a better way of following its exchange. This fast movement is significant as it is the first time that DEF6 has been observed moving in cells, and provides some evidence that it may be trafficked along microtubules. Active transport of DEF6 would allow for rapid polarisation in activated T cells.

5.3.3 DEF6 granules exhibit confined movements in cells

Live imaging data provided measurements for the area DEF6 visited, along with velocity, diffusion rates and types of movement. These all suggested that DEF6 granules remain relatively static in the cytoplasm, visiting a very small area of on average $0.10\mu\text{m}^2$ in all cases, an area smaller than the size of the granules which had an average cross sectional area of $0.36\mu\text{m}^2$. The velocity and speed of movement were also relatively slow, all pointing to DEF6 granules being immobile, and potentially not free to interact with the microtubule network in the way other P-bodies are able. Once again, this stability within the cytoplasm may be due to the glutamine/asparagine rich C-terminal region, as the results for the N-terminal portion of DEF6 only suggested much faster dynamic granules more in line with those formed by other P-body proteins.

Despite the seemingly low speeds achieved by DEF6 granules and small areas visited, the type of movement determined by mean squared displacement analysis was predominantly normal diffusion (40%), closely followed by active transport (32%). A total of 24% of the granules exhibited no movement or confined movement, indicating confinement by the actin cytoskeleton similar to what is seen with P-bodies. This mixture of different types of movement

supports an ability to be moved quickly to set up polarisation, and normal diffusion within the cytoplasm when this is not happening.

Diffusion rates of GFP-DEF6 and GFP-Nterminus within the cytoplasm were in the range of 5.5×10^{-6} – 1.4×10^{-4} $\mu\text{m}^2/\text{s}$ and 1.7×10^{-6} – 2×10^{-4} $\mu\text{m}^2/\text{s}$ respectively, slower than those for the Dcp proteins⁹² which were in the range of 10^{-3} – 10^{-2} $\mu\text{m}^2/\text{s}$, but closer to the range for other cytoplasmic organelles such as mitochondria¹⁹⁷ at 5×10^{-4} $\mu\text{m}^2/\text{s}$ and secretory vesicles¹⁹⁸ at 3.9×10^{-4} – 7.4×10^{-3} $\mu\text{m}^2/\text{s}$.

5.3.4 The N-terminus of DEF6 has kinetic parameters that are similar to other P-body components

The N-terminal domain of DEF6 moved with greater speed and velocity than the full-length DEF6 protein. In particular the differences in velocities were marked, showing that the N-terminal domain not only moved faster, but its movement was more directional than that of full-length DEF6 rather than rapid back and forth movements in one place. Consistent with this directional movement, the N-terminal domain was able to visit a larger area of the cell with the average area visited by GFP-Nterminus granules $0.33 \mu\text{m}^2$ compared to $0.10 \mu\text{m}^2$ for GFP-DEF6 granules. In both cases this is a much smaller area visited than by the DCP proteins measured by Aizer *et al*⁹², which visited an area of greater than $2 \mu\text{m}^2$ in over 40% of cases. Additionally, whilst the overall average velocity was still relatively slow at 3.9 nm/s for the GFP-N-terminus granules and 1.2 nm/s for the GFP-DEF granules, some of the faster moving GFP-N-terminus granules exhibiting directed movement could be observed to move with a velocity of $0.4 \mu\text{m/s}$ which is comparable with velocities measured by Aizer *et al* for the fast moving directed DCP1b granules which demonstrated velocities in the range of 0.5 – $1.1 \mu\text{m}^2/\text{s}$. This may again support a separate role for the N in P-bodies from the rest of the protein.

In line with its visiting of greater cytoplasmic areas, mean squared displacement analysis suggested mainly active transport for the N-terminal granules (40%). This however was closely followed by confined movement (28%), with very few (12%) of granules exhibiting normal diffusion. This suggests a model in which the N-terminus is tethered to the microtubule network, allowing active transport but also preventing normal diffusion within the cytoplasm thus demonstrating confined movement when active transport is not happening. The N-terminus has been previously shown to interact with tubulin indirectly (PhD thesis Hey¹⁶²), and may provide the link to the microtubule network that allows the location of the full length protein to polarise after T cell activation.

5.3.5 Potential structure of the C-terminus

With two roles identified for the C-terminal DHL domain of DEF6 as the domain providing Rho-GEF activity, and as an aggregation-promoting region, the C-terminus of DEF6 has several and potentially multiple interesting structural possibilities. Using the I-TASSER modelling server¹¹³, a platform that produces 3D models based on multiple threading alignments and iterative fragment assemble, a 3D model for full length DEF6 can be produced. In this model the C-terminus of DEF6 is predicted to form an extended alpha helical structure in which the helices are organised in helix-turn-helix hairpins stacked to give a superhelical architecture. This has been described as an alpha solenoid shape.

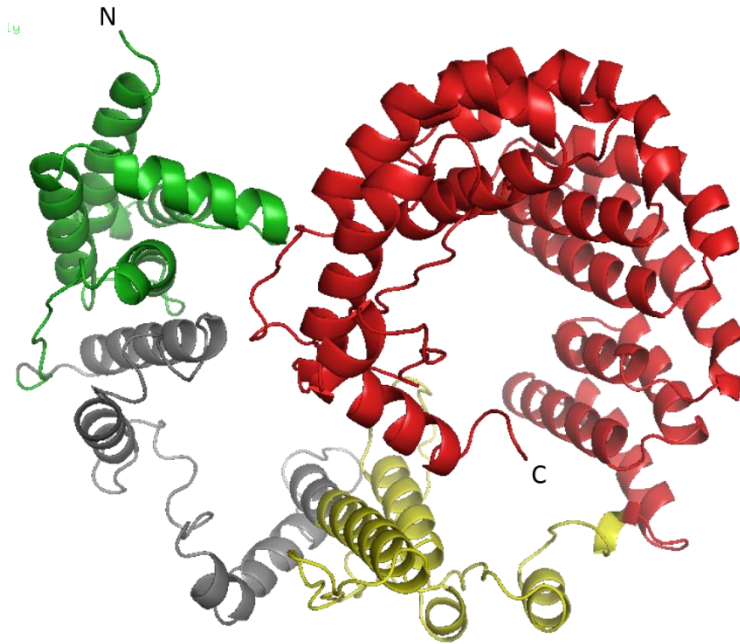


Figure 69 - I-TASSER model of DEF6 indicating the predicted alpha solenoid shape of the C-terminal domain. N-terminal domain (green), PH domain (yellow), C-terminal domain (red)⁸³.

Whilst there are issues with this model, most obviously that the PH domain is not modelled as a PH domain structure, the alpha solenoid shape has precedence in both granule-forming proteins such as Ge-1¹⁶⁸ and GTPase binding proteins such as β -importin²⁰⁰. Using the DALI server²⁰¹ to compare the coordinates of the structure obtained via I-TASSER for the C-terminus against structures contained in the Protein Data Bank for similar structures reveals multiple proteins containing similar alpha helical repeats including Huntington-elongation-A-subunit-TOR (HEAT) repeats and armadillo (ARM) repeats. HEAT repeats are sequence repeats made up of two α -helices and two turns which in tandem form alpha solenoid domains and are involved in protein-protein interactions^{202,203}, including in proteins involved in translation²⁰⁴. The closest structural homologue returned by the DALI server is importin-beta, a Ran-GTPase binding nuclear importin which superimposes with an RMSD of 2.5Å over 290 amino acids (Figure 70). This provides important support for the ability of the alpha solenoid shape to bind GTPases, allowing the possibility of GEF activity

in this conformation despite the structural differences to the normal “chaise longue” shape of Rho-GEF DH domains.

Another structural homologue returned by the DALI server is importin-alpha, which superimposes with an RMSD of 11.4Å over 302 amino acids. Importin-alpha has previously been noted to be structurally similar to the C-terminal domain of Ge-1¹⁶⁸, a decapping activator that interacts with DCP1, DCP2 and DDX6²⁰⁵ with which it co-localises in P-bodies^{66,206}. The C-terminus of Ge-1 is necessary and sufficient for its localisation to P-bodies²⁰⁷ and mediates the interaction with DCP1 and DCP2 as well as self-interaction²⁰⁸. Although DALI does not return the Ge-1 structure when the predicted structure of DEF6 is submitted, the helical repeats are highly similar between the C-terminus of DEF6 and the C-terminus of Ge-1.

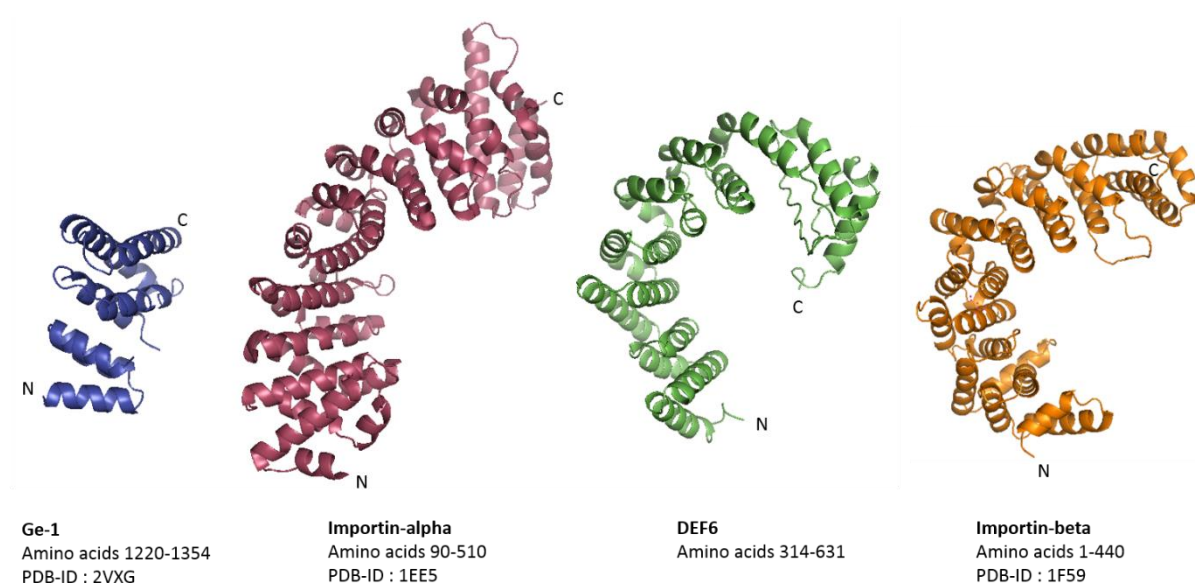


Figure 70 - An illustration of the repeated helix-turn-helix architecture in the Ge-1 Cterminus (blue), importin-alpha (pink), the DEF6 Cterminus (green) and the importin-beta N-terminus (orange). Ge-1 superimposed onto importin alpha with an RMSD of 3.3Å and DEF6 superimposed onto importin alpha with an RMSD of 11.4Å and onto importin alpha with an RMSD of 2.5Å.

As both the Rho-GTPase binding protein beta importin and the aggregating-protein Ge-1 contain alpha solenoid structures involved in these functions, the alpha solenoid in DEF6 has the potential to function as a GEF and assemble into protein aggregates. Whether these roles in DEF6 are mutually exclusive remains

to be determined. Data indicating that DEF6 has open and closed conformations supports the potential for at least two different structures³⁵. These open and closed forms could provide a mechanism for the switch between a soluble form and an aggregating form that DEF6 demonstrates. Aggregation could be triggered by the release of constraints holding the closed form of DEF6, potentially via a phosphorylation event such as those discussed in chapter 4, which could expose aggregating sequences, or could trigger the refolding of the closed protein into a form that promotes protein-protein interactions. Based on the structural prediction by I-TASSER, the alpha solenoid form is a good candidate for the closed form of DEF6. A potential different structure for the C-terminus of DEF6 which could provide an aggregating function is a coiled coil structure. The glutamine/asparagine rich regions like that are found in the C-terminus of DEF6 have been linked to prion formation^{69,175}, but also the formation of coiled coils⁷⁰. Both these structures can be used to promote protein-protein interactions and could provide a potentially reversible mechanism for the condensation of DEF6 into granules. The following chapter explores further the potential for a coiled coil structure of the C-terminal DHL domain of DEF6, and possibilities for its mechanism of aggregation into cytoplasmic foci.

6. Results:

The C-terminus of DEF6

6 The C-terminus

6.1 Introduction

6.1.1 The association between glutamine/asparagine rich regions in proteins and aggregation

The DHL domain of DEF6 contains a glutamine/asparagine rich region that is found in many P-body forming proteins¹⁰². Glutamine/asparagine rich regions are well known as prion-like⁶⁹ and prone to forming β -amyloid structures^{209,210}, and for most P-body proteins it is thought that it is through prion-like aggregation that the glutamine/asparagine rich aggregating domains coalesce¹⁰².

In studies searching both the yeast and human proteomes for prion-like domains, many proteins identified also contained RNA-binding domains^{177,211,212}. These RNA-binding domain containing proteins could form visible aggregates with amyloid-like properties, but unlike conventional amyloid aggregates, such as those seen in protein disorder, macromolecular collectives these were more dynamic and able to depolymerise²¹³. Other domains that have been shown to be enriched in prion-like domain containing proteins include pumilio repeats²¹¹, part of the ARM repeat family discussed in chapter 5 which show similarities with the predicted structure of the closed, non-coiled coil form of DEF6. Searches for functions associated with prion domain containing proteins show enrichment for RNA processing, binding, transport and transcription, plus formation of RNP complexes and RNA granules, and interactions with the cytoskeleton and cortical actin network²¹¹.

Although prion-like domain containing proteins show high occurrence in RNA granules, it is unlikely that amyloid-like oligomerisation is present in these structures due to their dynamic nature and reversibility. These prion-like regions are more likely to be involved in weaker interactions that run the risk of forming irreversible insoluble aggregates as evidenced by the inclusion of granule

proteins in pathogenic aggregates^{212,214}. On the other hand, a core portion may use amyloid aggregation and be less dynamic and reversible, but normally be controlled by granulophagy (a form of autophagy specialised to the removal of stress granules and P-bodies) to remove aggregates at a sustainable rate, unless defects arise in the clearance mechanisms, such as the mutations in the ubiquitin segregase VCP that lead again to pathogenic inclusions²¹⁵. Coiled coils present an alternative method of oligomerisation that has the potential to be more reversible than formation of amyloid fibrils, and as demonstrated by Fiumara *et al*, can form from domains labelled as prion-like due to their glutamine/asparagine rich regions⁷⁰.

Unlike most proteins containing glutamine/asparagine rich regions which are able to form amyloid fibril structures, DEF6 is not predicted to form β -sheet in the DHL region, rather it is predicted to be highly helical. Whilst β -amyloid formation can derive from helical regions, recent evidence has shown that glutamine/asparagine-rich regions can also be associated with coiled coil-forming regions⁷⁰. This leads to the possibility that the glutamine/asparagine rich region in DEF6 may form a coiled coil, and its aggregation into P-bodies could be driven by coiled coil interactions.

6.1.2 Structure and features of coiled coils

Coiled coils are protein motifs where two or more alpha helices coil around each other to form a supercoil (Figure 71B)^{216,217}. The residues from one helix pack into the space between residues on the other, forming the characteristic “knobs in holes” packing. Because of the requirement for certain amino acids at the interface to form a hydrophobic core, and the periodicity of 3.5 amino acids per turn, a heptad pattern is evident in the sequences of coiled coil proteins which can be labelled a-g, where positions a and d are hydrophobic and form the packing interaction at the core of the supercoil (Figure 71A). Coiled coils are most commonly found as dimers or trimers, and can run parallel or anti-parallel

to each other. The supercoil is usually oligomeric with hetero-oligomers and homo-oligomers possible, but can also less commonly form from helices in the same chain²¹⁷. In this case, they are almost always found to be anti-parallel.

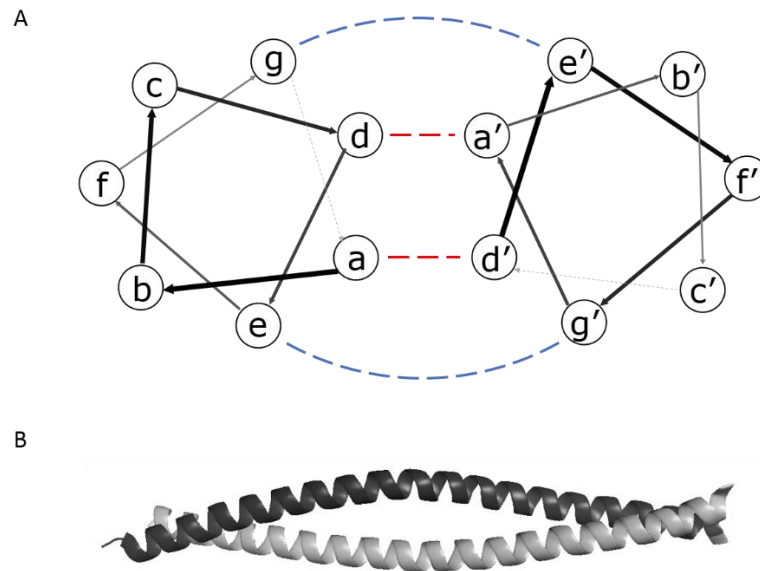


Figure 71 – Coiled coil structures demonstrate interactions between specific residues in a heptad pattern labelled a-g. A) Helical wheel diagram looking down the helix from the N to the C-terminus showing interacting residues in a parallel dimeric coiled coil. d-a' and a-d' interactions between hydrophobic residues and g-e' and e-g' interactions between hydrophilic residues. B) Structure of an example coiled coil (The Rho-kinase Rho-A binding domain, structure from PDB 1UIX))

Coiled coil domains have been found in a wide range of proteins with diverse functions²¹⁶, including transcription factors, motor proteins and cytoskeletal proteins. Coiled coils can also function as switches, for example via the pH dependent conformational change of in macrophage scavenger receptors²¹⁸.

6.1.3 Coiled coils in P-body and stress granule proteins

Whilst a large number of stress granule and P-body forming proteins have been shown to contain glutamine/asparagine rich regions¹⁰², and these regions have been shown to be capable of forming coiled coil structures to aid in aggregation⁷⁰, few P-body or stress granules proteins have been shown to contain coiled coil regions. One example is Pdc1, a yeast homologue of Edc4, part of the decapping complex with Dcp1 and Dcp2, that self-interacts via its coiled coil domain which is required for foci accumulation in cells²¹⁹. PABPN1, a

protein necessary for efficient polymerisation of poly(A) tails on mRNA also forms inclusions that contain RNA reliant on its coiled coil domain²²⁰, but neither PABPN1 or Pdc1 contain a glutamine/asparagine rich region. One example of a protein that contains both a coiled coil and a glutamine/asparagine rich region, and is involved in mRNA containing granules is heatshock transcription factor 2 (HSF2), which is found in nuclear stress bodies, another subset of ribonuclear protein that are initiated by heatshock transcription factor 1 (HSF1)^{221,222}. HSF2 is relatively rich in glutamine and asparagine residues, with 17 in its richest 80mer, and the coiled coil of HSF2 is essential for aggregation²²³.

The presence of a coiled coil in DEF6 would thus both provide a mechanism for its aggregation into cytoplasmic foci, linking the properties of glutamine/asparagine enrichment in P-body proteins, with the ability for these regions to form coiled coils to provide a reversible mechanism for granule aggregation.

6.2 Results

6.2.1 The DHL domain of DEF6 is predicted to form a coiled coil

Due to the association of glutamine/asparagine rich regions with coiled coil forming regions, the amino acid sequence for DEF6 was submitted to two coiled coil predicting servers, PairCoil2 and Coils. Coils²²⁴ is a sequence based program that compares the sequence of the protein of interest to a database of sequences for known two- stranded, coiled-coil forming proteins and from there computing the probability of the sequence of interest forming a coiled coil. PairCoil2¹¹⁶ predicts the location of coiled-coil regions within a sequence using pairwise residue probabilities and the PairCoil algorithm²²⁵ against a database of coiled-coils. For each a score is produced based on similarity to coiled coil forming proteins, which is used to classify regions of the protein as likely or unlikely to form a coiled coil.

The sequences for Vav1, a T cell Rho-GEF with no known coiled coil region, and tropomyosin, a well-known coiled coil protein were also analysed as comparisons. Additionally importin-beta was analysed to determine if coiled coil is predicted for helical repeat sequences such as the HEAT repeats found in importin-beta, and seen in the alpha-solenoid model of DEF6¹².

To predict the type of coiled coil likely to form, MultiCoil²²⁶ was used to predict the likelihood of either dimeric or trimeric coiled coil formation in DEF6. This program is also based on the PairCoil algorithm, but extends it and uses a database with trimeric known coiled-coils in addition to the dimeric ones used in PairCoil2. By comparing the sequence with both sets of coiled coil types, it produces a score for the query sequence based on similarity to either set.

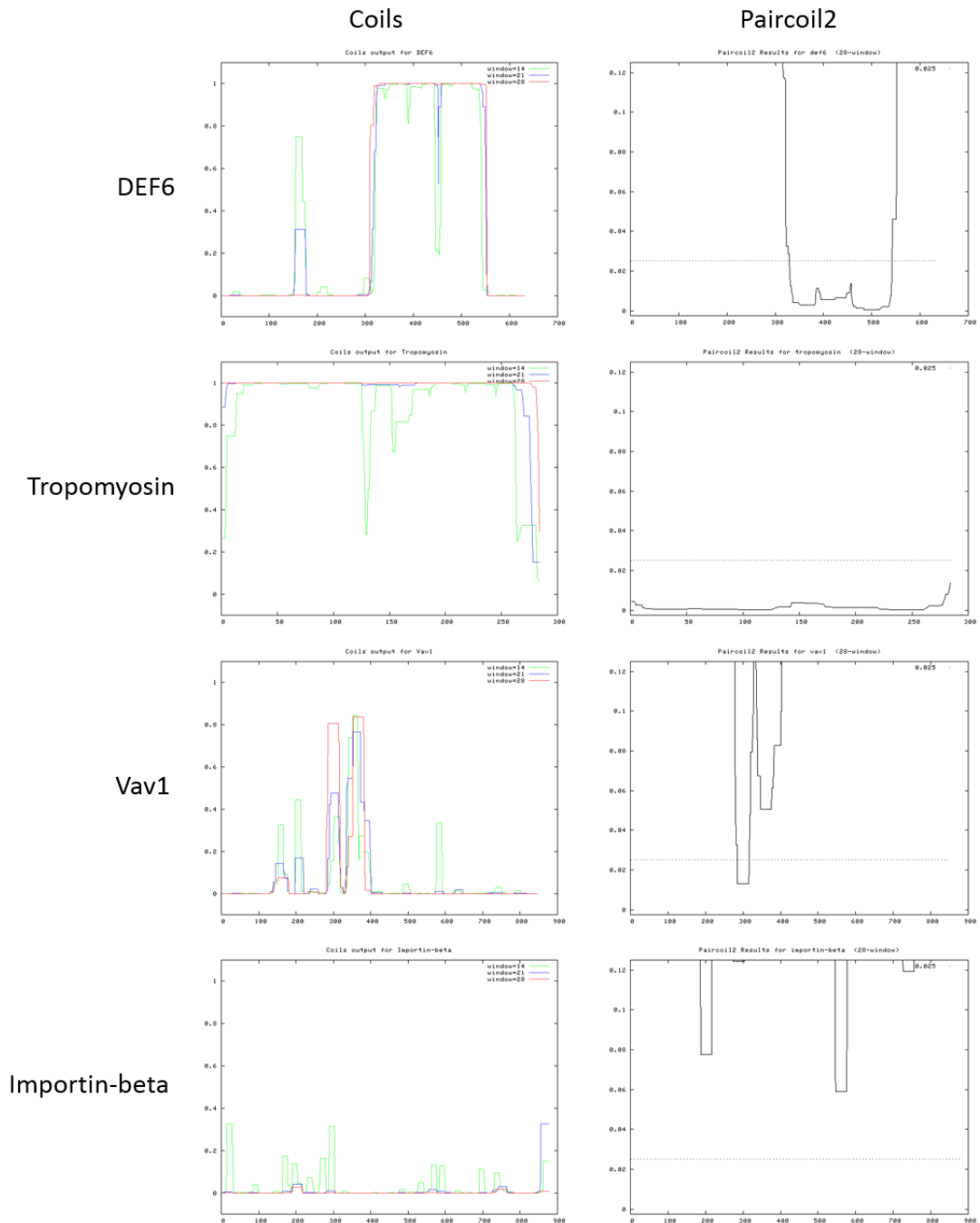


Figure 72 - DEF6 is predicted to form a coiled coil in its C-terminal region. DEF6 is predicted by both Paircoil2 and Coils to contain a coiled coil region between amino acids 330 and 550. For comparison the propensity for Tropomyosin (highly coiled coil), Vav1 (A Rho GEF with no coiled coil) and Importin-beta (forms a highly helical but not coiled coil structure) to form coiled coils was also calculated. Adapted from Hey *et al*¹².

DEF6 has a region predicted to form a coiled coil between amino acids 330 and 550 (Figure 72). For predictions by the program coils, scores above 0.8 indicate likely coiled coil formation, whilst for Paircoil2 scores below 0.035 indicate likely coiled coil formation. Paircoil2 additionally assigns the heptad register to the

amino acid sequence, as shown in Figure 73 for the region predicted to form a coiled coil.

	abcdefg
321	LHKDL
	KQKRREQ
	REQRERR
	RAAKEEE
	LL R
	LQQLQEE
	KERKLQE
	LELLQEA
	QRQAERL
	LQEEEEER
	RRSQH
	R
	EL
	QQALEGQ
	LREAEQA
	RASMQAE
	MELKEEE
	AARQRQR
	IKELEEM
	QQRLEEA
	LQLEVKA
	RRDEESV
	RIAQTRL
	LEEEEEK
	LKQLMQL
	KEEQERY
	IERAQQE
	KEELQQE
	MAQQSRS
	LQQAQQQ
	LEEVRQN
	RQRADED
	VEAAQRK
	LRQASTN
	VKHWNVQ
	MNRLMHP 553

Figure 73 - The assignment of the DEF6 amino acid sequence between residues 321 and 553 to the heptad register by Paircoil2. Adapted from Hey *et al*¹².

Tropomyosin is correctly predicted to form an extended coiled coil demonstrating the ability of both servers to correctly predict coiled coils. Vav1, as expected, is not predicted to contain a coiled coil region, demonstrating that the presence of an extended coiled-coil forming region in Rho-GEFs is atypical. Importin-beta is likewise not predicted to contain any coiled coil forming regions, indicating that the server, correctly, does not assign the highly-helical HEAT repeats present in

importin-beta as coiled coil. Consequently, the coiled coil prediction for DEF6 is unlikely to be a mistaken assignment of the highly helical HEAT- like repeats seen in the model produced by I-TASSER as coiled coil.

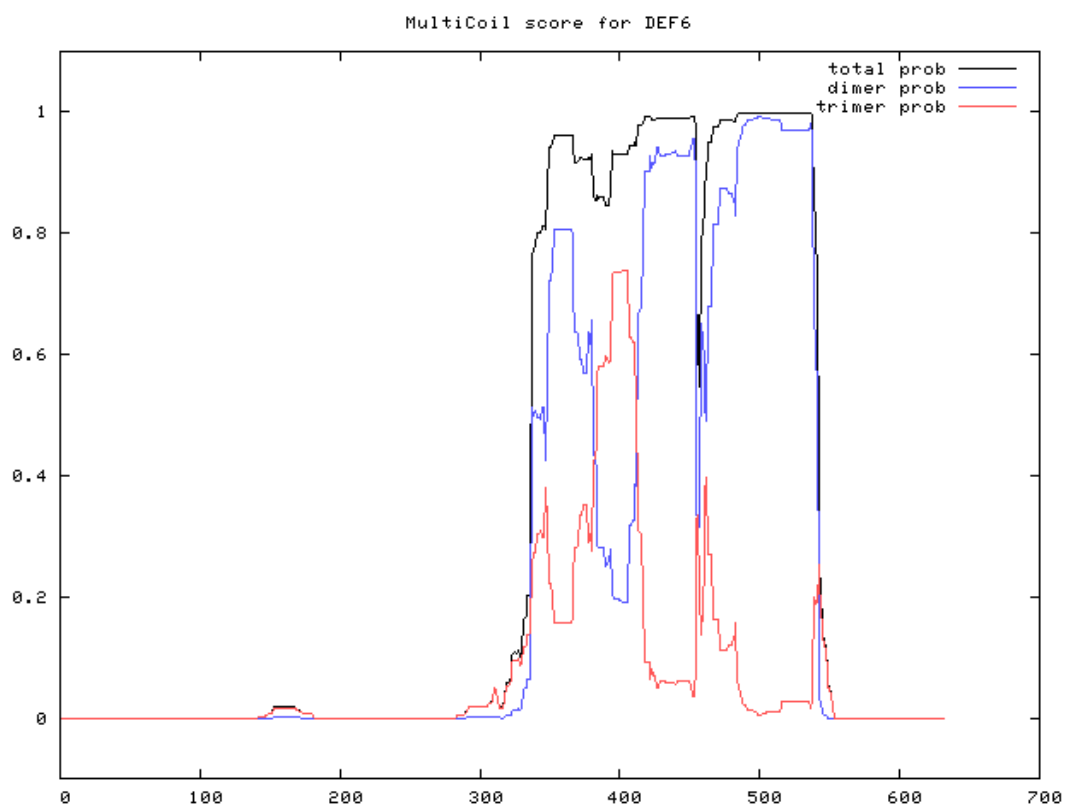


Figure 74 – DEF6 is predicted to have a high probability of forming a dimeric coiled coil. MultiCoil was used to predict the likeliness of dimeric or trimeric coiled coil formation from the sequence of DEF6.

Since DEF6 is predicted to contain an extensive coiled coil region, the question as to whether DEF6 exists as a monomer or multimer within cells also arises. The analyses of the probability of DEF6 to form either a dimeric or trimeric coiled coil via the MultiCoil program suggests that DEF6 is most likely to form a dimeric coiled-coil (Figure 74). Similar to the program coils, scores above 0.8 indicate high probability. The total probability for coiled coil formation is high, and the score for the coiled coil region is higher for trimer probability than dimer probability. Generally, dimeric coiled coils are found in intracellular proteins while trimeric coiled coils are more common in extracellular proteins²¹⁷. This is consistent with the intracellular location of DEF6.

6.2.2 Production and purification of recombinant protein containing the predicted coiled coil section of DEF6

To determine whether the predicted coiled coil region of DEF6 actually forms a coiled coil, amino acids 313-550 were expressed in *E.coli* as a GST fusion protein C-terminal of the affinity tag. The expected size of the protein is 55kDa with the GST tag and 29.4kDa without the GST tag. The protein was expressed in C41 cells following a similar strategy to that used to produce the PH domain protein, the methodology for which is found in section 2.4.3 (Figure 75). As with the PH domain, C41 cultures were grown for 2 days at 20°C to improve the solubility of the fusion protein but unlike the PH domain the solubility remained high for the remainder of the purification with less visible protein precipitate coming out of solution at each step. Glutathione sepharose beads were used to separate the recombinant protein from the lysate, and the recombinant protein bound to the beads was eluted via cleavage of the GST tag using thrombin.

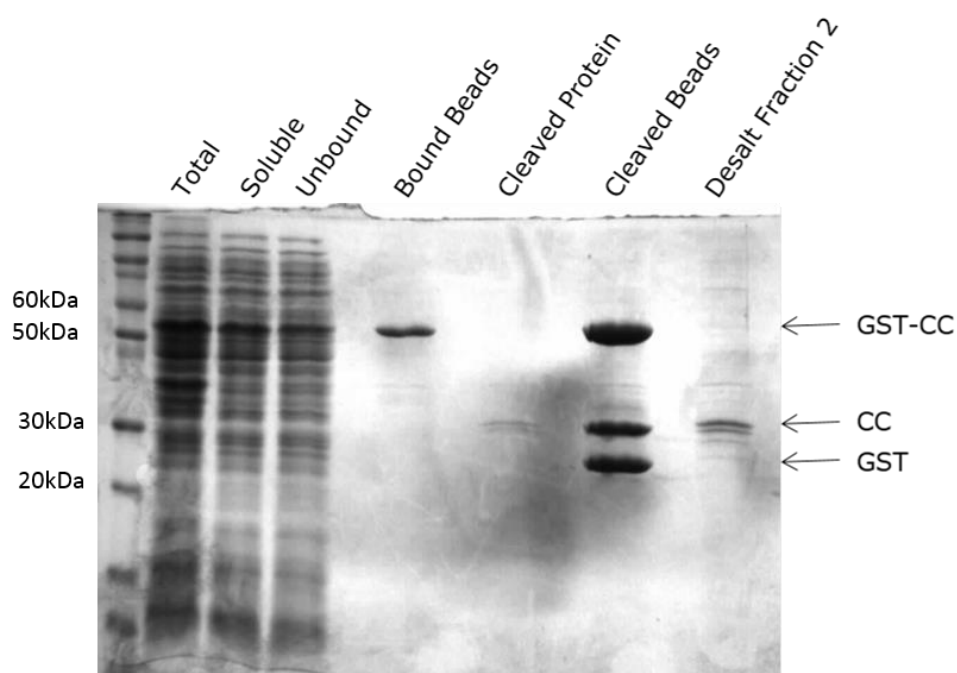


Figure 75 - SDS-PAGE gel of the purification of the coiled coil region of DEF6 (amino acids 313-550). Lanes indicate the total protein from cell lysis, the soluble fraction after centrifugation, the protein that remained unbound to the glutathione sepharose beads after incubation, the protein bound to the beads, the eluted protein after thrombin cleavage from the beads, the protein remaining on the beads, and the protein after desalting.

Like the PH domain, the GST- DHL₃₁₃₋₅₅₀ fusion protein was desalted without a gel filtration step due to the degradation seen attempting gel filtration of the PH domain. However, later work using analytical gel filtration on the protein indicated that it could withstand gel filtration. Future preparations should take this into account to increase the purity of the protein produced. The yield of purified GST-DHL₃₁₃₋₅₅₀ was typically around 2mg from a 1L culture.

6.2.3 Biophysical analysis of the region of DEF6 comprising amino acids 313-550 (DHL₃₁₃₋₅₅₀) is consistent with it adopting the predicted coiled-coil conformation.

To test whether the expressed protein DHL₃₁₃₋₅₅₀ forms a coiled coil, circular dichroism was utilized. The ratio between the ellipticity values at ~220nm due to the π - π^* transition and the π - π^* transition at ~205nm indicates differences between a single α -helix and interacting helices as found in coiled coils. This is due to the sensitivity of the π - π^* to this difference, whilst the π - π^* transition is responsive to total α -helical content allowing a measure to be made of conversion between the two states. The ratio of the ellipticity at 222nm and 208nm is commonly used to measure coiled coil formation^{227,228}, however, as described by Lau *et al*²²⁸ and Zhou *et al*²²⁹, in this work the ellipticity values at the local minima will be used rather than setting the wavelengths at 222nm and 208nm as a red shift is seen particularly in the π - π^* transition at 205nm upon conversion of helix to coiled coil. Values below 0.86 are alpha helical whilst those above 1 are coiled coil.

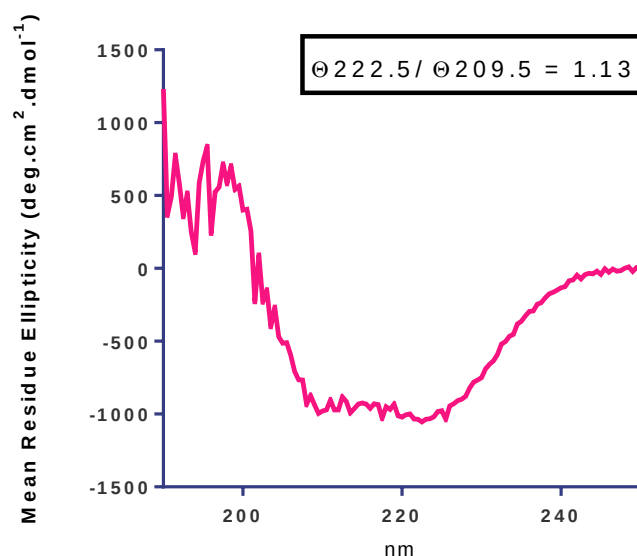


Figure 76 – The CD spectrum of the purified and cleaved 313-550 protein construct of DEF6 indicates a helical structure due to the minima near 222nm and 210 nm, and coiled coil formation due to the ratio of these minima. The protein was dissolved in 20mM potassium phosphate buffer at pH7.4 to a concentration of 0.1mg/mL and had its ellipticity measured using a Pi-Star-180 Spectrophotometer. Values were transformed from machine units to mean residue ellipticity using the formula in section 2.5.2. Minima were chosen to measure the coiled coil content for each protein construct. The ratio of ellipticity at the two local minima is shown in the box, indicating coiled coil formation.

The 313-550 protein gave a ratio at $\Theta_{222.5}/\Theta_{209.5}$ of 1.13, indicating the protein formed a coiled coil structure, confirming the prediction (Figure 76).

6.2.4 The use of proline mutations to break coiled coils

Insertion of proline residues into a coiled coil is a commonly used strategy for breaking the coiled coil²³⁰, and was used by Fiumara *et al.* to break coiled coil formation to assess the role of the glutamine/asparagine rich coiled coils in the aggregation of several coiled coil proteins⁷⁰. They found that the inserted proline residues caused short coiled coil peptides to form an unfolded structure (random coil), and caused Htt and CPEB (glutamine/asparagine rich proteins) to be located diffusely rather than in aggregates.

	abcdefg
321	LHKDL
	KQKRREQ
	REQRERR
	RAAKEEE
	LL R
	LQQLQEE
	KERKLQE
	LELLQEA
	Q R Q AERL
	LQEEEEER
	RRSQH
	R
	EL
	QQALEGQ
	LREAEQA
	RASMQAE
	MELKEEE
	AARQRQR
	I K E L E EM
	QQRQLQEA
	LQLEVKA
	RRDEESV
	RIAQTRL
	LEEEEEK
	L K Q L M QL
	KEEQERY
	IERAQQE
	KEELQQE
	MAQQSRS
	LQQAQQQ
	L EE V RQN
	RQRADED
	VEAAQRK
	LRQASTN
	VKHWNVQ
	MNRLMHP 553

Figure 77 - The amino acid sequence of the predicted coiled coil forming region of DEF6 with the amino acids to be replaced by proline residues highlighted in red. Adapted from Hey *et al*¹².

To investigate whether DEF6 coiled coil formation and aggregate formation could be disrupted, proline mutations were inserted into both the *a* and *d* amino acid positions of the heptad repeats in four different positions in the predicted DEF6 coiled coil as shown in Figure 77. This work was done in conjunction with Fiona Tooke, a final year undergraduate project student. The positions were chosen spaced apart to produce disruption in different regions of the predicted coiled coil structure.

The DEF6 amino acid sequence with the proposed proline insertions was resubmitted to Paircoil2 and to Coils to assess the predicted effect they would have on coiled coil formation.

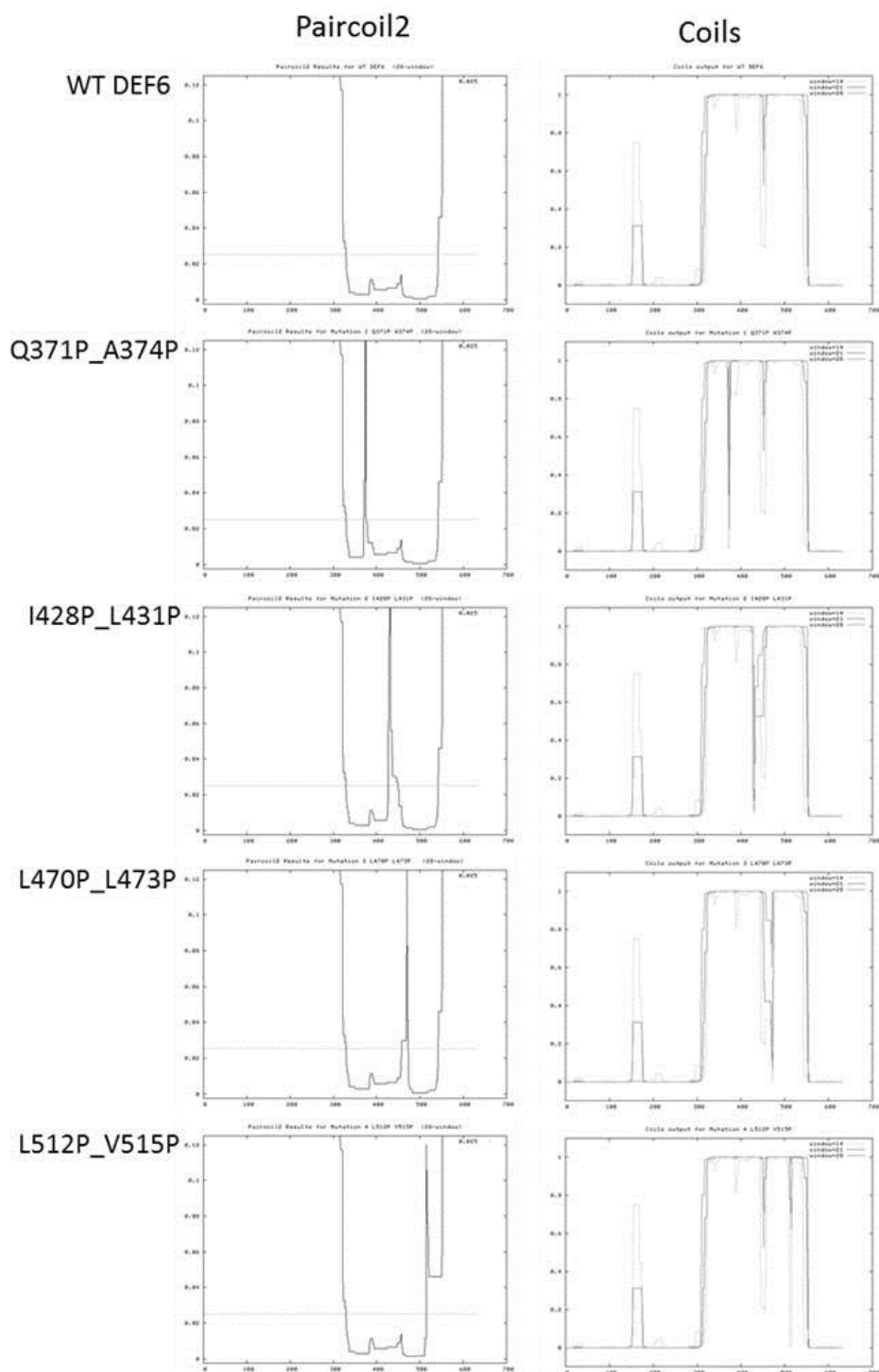


Figure 78 - Inserting proline residues into DEF6 is predicted to cause small breaks in the coiled coil

Upon resubmission of the sequence containing each set of proline mutations to Paircoil2 and Coils, the mutations were predicted to break the coiled coil, but not disrupt it entirely (Figure 78).

The 4 pairs of proline mutations were inserted into the GFP-DEF6 full length construct using the following mutagenic primers:

Q371P_A374P (CCm1)

Sense: 5'- GGA GGC GCC GCG GCA GCC CGA GCG G -3'
Encoding the sequence EAPRQPER amino acids 369-376

I428P_L431P (CCm2)

Sense: 5'- CAG CGG CAG CGC CCC AAG GAG CCG GAG GAG ATG CA -3'
Encoding the sequence QRQRPKEPEEM amino acids 424-434

L470P_L473P (CCm3)

Sense: 5'- GAG GAG GAA GAG AAG CCG AAG CAG CCG ATG CAG CTG AAG GAG -3'
Encoding the sequence EEEKPKQPMQLKE amino acids 465-478

L512P_V515P (CCm4)

Sense: 5'- CCA GCA GCA GCC GGA GGA GCC GCG GCA GAA CC -3'
Encoding the sequence QQQPEEPRQN amino acids 509-518

6.2.4.1 The effect of proline mutations in the coiled-coil region on the cellular localisation of DEF6

Mutated GFP-tagged, full-length DEF6 constructs were transfected into Cos7 cells to determine the effects of the mutations. Consistent with previous data, GFP alone is diffuse in both untreated and arsenite treated cos7 cells, whilst the full length wild type DEF6 GFP construct is distributed diffusely in untreated cells but forms granules in arsenite treated cells (Figure 79). The distribution of the GFP-tagged proteins were classified as being (1) diffusely localised, (2) granule forming, or (3) being present in rounded-up cells, indicative of cell death. This data is preliminary as the experiment was only repeated once.

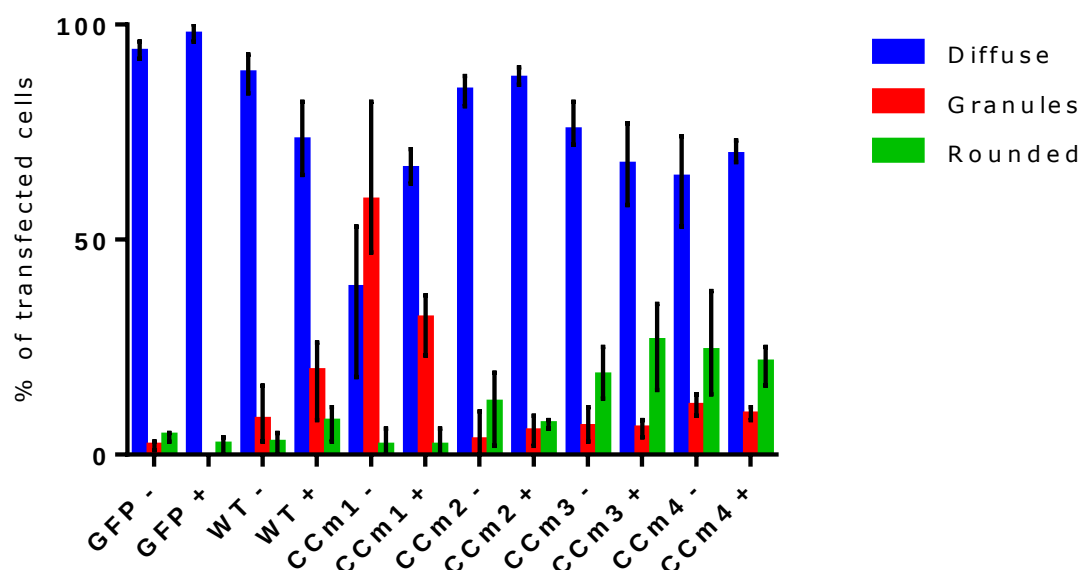
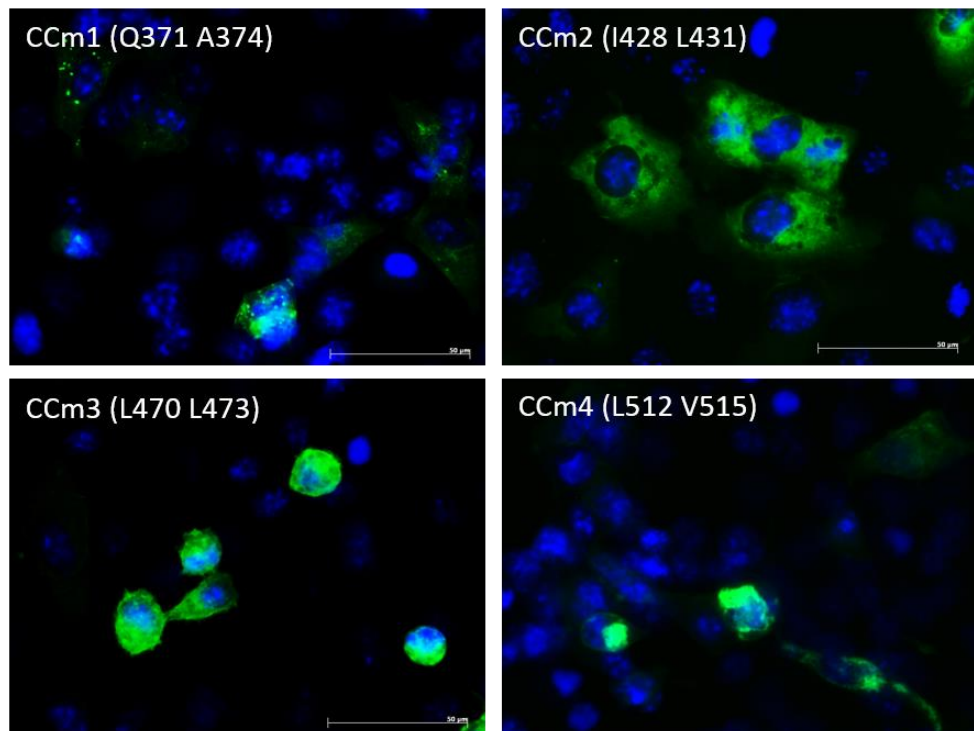


Figure 79 - Graph showing the percentage of cells demonstrating diffuse or granulated GFP-tagged protein in cells transfected with GFP-tagged DEF6 protein constructs both without (WT) and with (CCm1-4) proline mutations in the C-terminus. Cos7 cells were transfected with GFP-DEF6 containing proline mutants in 4 different positions in the C-terminus (CCm1 = Q371P_A374P, CCm2 = I428P_L431P, CCm3 = L470P_L473P, CCm4 = L512P_V515P). Cells were either left untreated (-) or treated with 1 μ M arsenite for 1 hour (+). Cells were fixed and an AxioVertS100 microscope was used to take widefield images of the fixed cells which were then counted and classified. Error bars show range of values.

The effect of the proline mutation of conserved hydrophobic residues varied with their location within the C-terminus of DEF6. Cells expressing DEF6 proline mutant Q371P_A374P (CCm1) exhibited DEF6 granules that formed spontaneously in the majority of transfected cells (Figure 79, Figure 80A). Interestingly, granule formation was decreased in cells treated with arsenite to induce translational arrest (Figure 79, Figure 80B). The cells expressing CCm2 (I428_L431), CCm3 (L470_L473) and CCm4 (L512_V515) demonstrated diffuse localisation of the protein in both untreated and arsenite treated cells, with an increase in the number of rounded cells in CCm3 and CCm4 indicative of cell death (Figure 79, Figure 80).

A Untreated



B Treated with 1μM arsenite for 1 hour

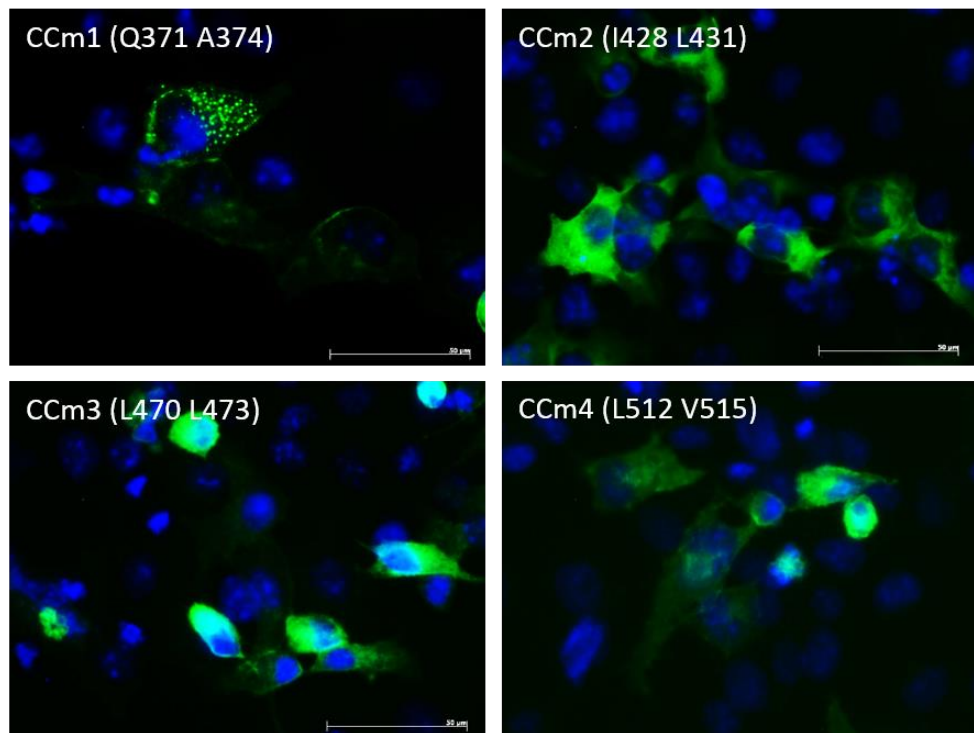


Figure 80 - Proline mutations in the C-terminus of DEF6 alter the morphology of the granules found in untreated Cos7 cells transfected with DEF6. Cos7 cells were transfected with GFP-DEF6 containing proline mutants in 4 different positions in the C-terminus (CCm1 = Q371P_A374P, CCm2 = I428P_L431P, CCm3 = L470P_L473P, CCm4 = L512P_V515P). Cells were either left untreated or treated with 1μM arsenite for 1 hour. Cells were fixed and stained with DAPI containing mountant (blue). An AxioVertS100 microscope was used to take widefield images of the fixed cells. Scale bar is 50μM.

These data suggest that small breaks in the coiled coil region towards its C-terminus may prevent the formation of granules, as suggested by the diffuse localisation of mutations 2-4 even after induction of translational arrest via arsenite treatment. This is consistent with the model of coiled-coil formation driving the aggregation of DEF6. That CCm1 (Q371P_A374P) increases aggregate formation in the absence of arsenite treatment suggests that it alters the conformation of the protein enough to lead to an open conformation, suggested earlier in this study, resulting in constitutive aggregate formation.

6.2.4.2 The effect of proline mutations on the biophysics of the coiled coil region

The proline mutations were inserted into the GST-313-550 protein construct to determine whether the changes in morphology of GFP-DEF6 were due to disruption of coiled coil structure. Each proline mutant construct was expressed in C41 cells and purified using the same strategy as the GST-313-550 protein (as in methods section 42) (Figure 81). Yields were slightly lower than for the GST-313-550 coiled coil protein without any proline insertions for most of the proline mutant protein constructs, with 1.5mg, 1.9mg and 1.4mg produced for each of CCm2, CCm3 and CCm4 respectively from 1L of growth. CCm1 however had a higher yield with 4mg produced. Based on the SDS-PAGE gels, this contained a larger amount of lower weight protein impurities than in the other protein purifications.

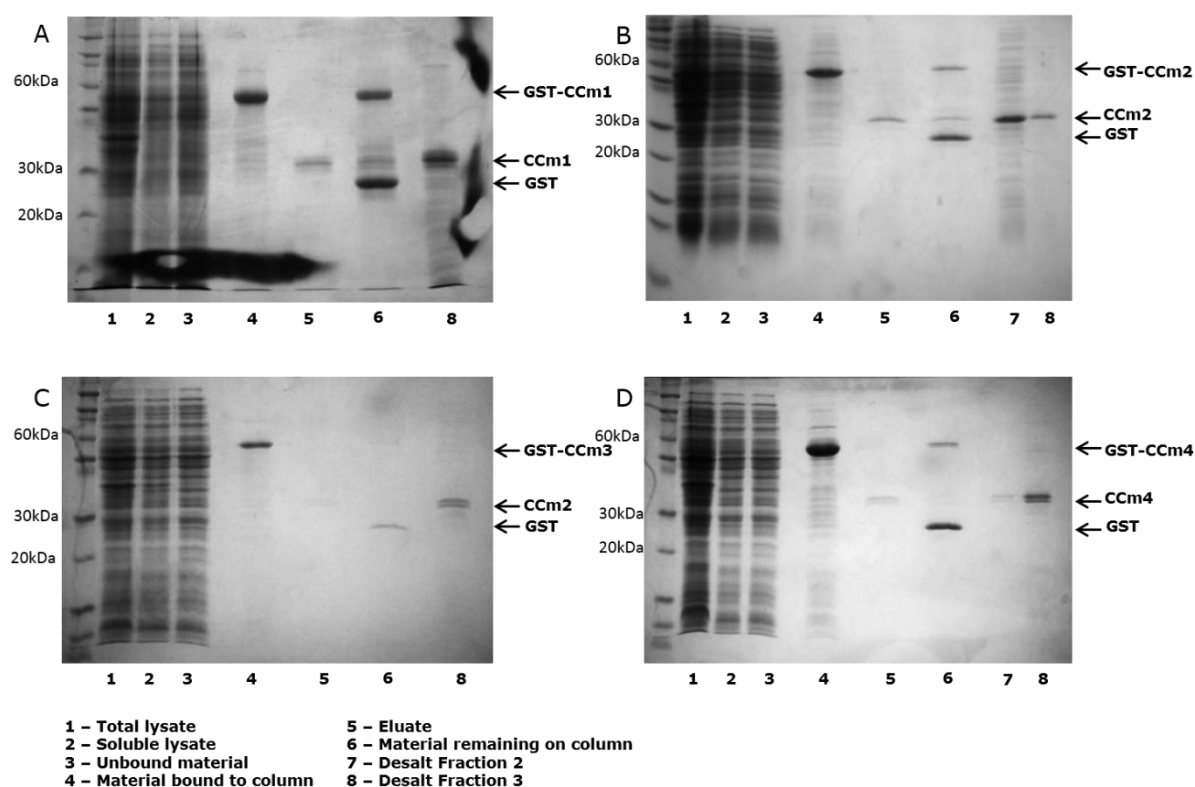


Figure 81 – Purification of the 313-550 DEF6 construct with proline mutations following the same purification protocol as with the PH domain (205-215) and the coiled coil protein (313-550 no mutations). (A) Q371P A374P (CCm1), (B) I428 L431 (CCm2), (C) L470P L473P (CCm3) and (D) L512P V515P (CCm4). Lanes indicate the total protein from cell lysis, the soluble fraction after centrifugation, the protein that remained unbound to the glutathione sepharose beads after incubation, the protein bound to the beads, the eluted protein after thrombin cleavage from the beads, the protein remaining on the beads, and the protein after desalting.

The circular dichroism spectrum of each protein was measured to determine the extent of coiled coil formation.

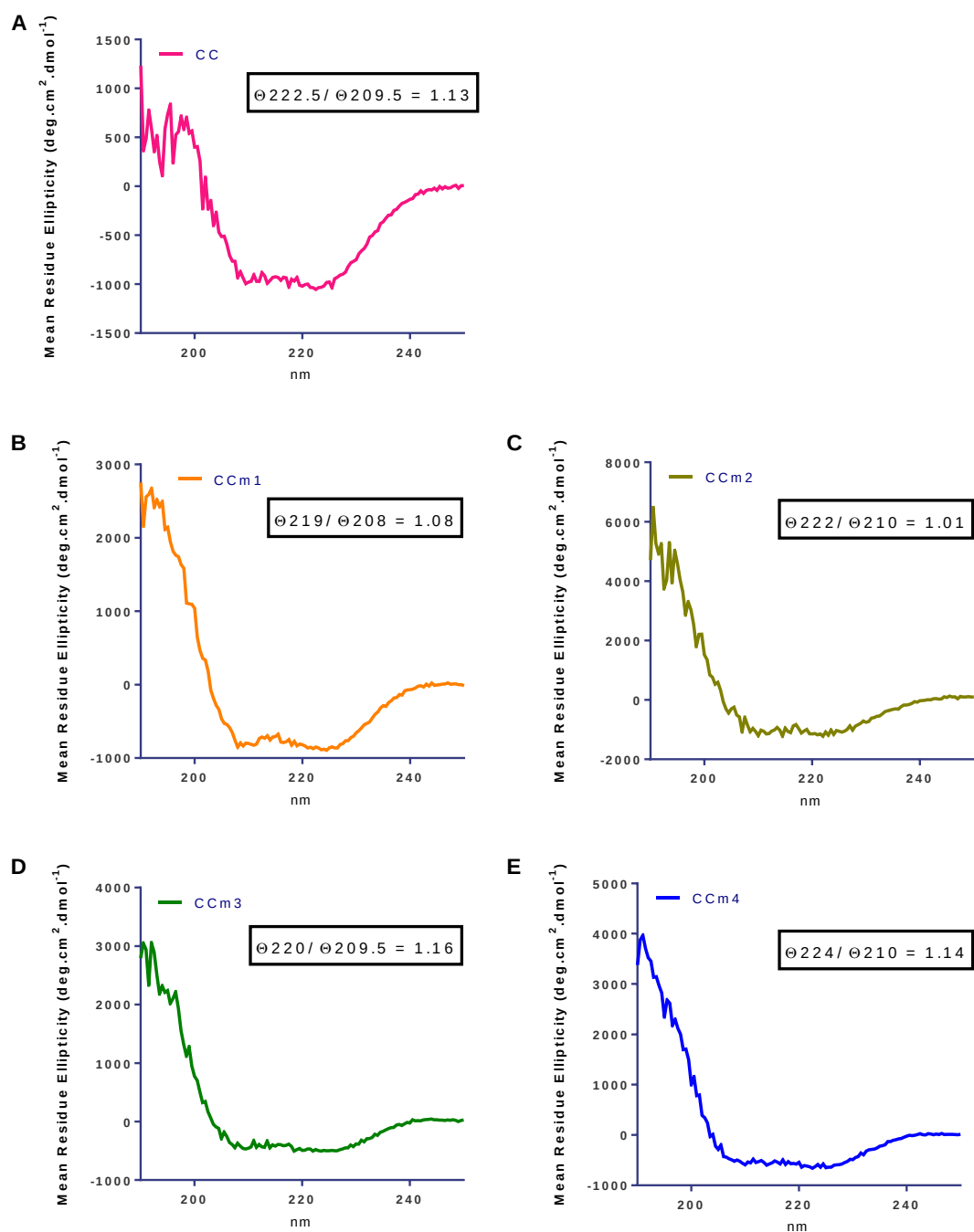


Figure 82 - All four mutants still form a coiled coil as measured by CD. WT DEF6 313-550 (A) and each mutant protein construct of DEF6 amino acids 313-550 (B) Q371P A374P (CCm1), (C) I428 L431 (CCm2), (D) L470P L473P (CCm3) and (E) L512P V515P (CCm4) was dissolved in 20mM potassium phosphate buffer at pH7.4 to a concentration of 0.1mg/mL and had its ellipticity measured using a Pi-Star-180 Spectrophotometer. Values were transformed from machine units to mean residue ellipticity using the formula in section 2.5.2. Minima were chosen to measure the coiled coil content for each protein construct.

The ratio of minima for all proteins constructs are within the value range indicating coiled coil formation (Figure 82). This suggests that the proline

insertions at the a and d positions of heptad repeats did not break the interaction between alpha helices that indicates coiled coil formation. The differences in cellular localisation seen with the GFP tagged proline mutants (Figure 80) may then be due to kinks in the coiled coil region preventing oligomer formation and aggregation rather than disruption of the coiled coil structure.

6.2.5 Proline mutations in recombinant DEF6 protein affect the size of oligomer formed.

To assess variation in the ability of the mutant DEF6 proteins to oligomerise, dynamic light scattering was used to determine whether any of the proline mutations led to a difference in the ability to oligomerise. Dynamic light scattering measures fluctuations of the scattering intensity of laser light passing through a solution of protein. These fluctuations are analysed by the instrument to give a velocity of Brownian motion of the protein, from which their size is calculated using the Stokes-Einstein relationship. Thus from the data, a size distribution can be found for the proteins in the solution. The data presented suggests the percentage of the total solution volume that contains particles of each size, giving populations of oligomers of different sizes.

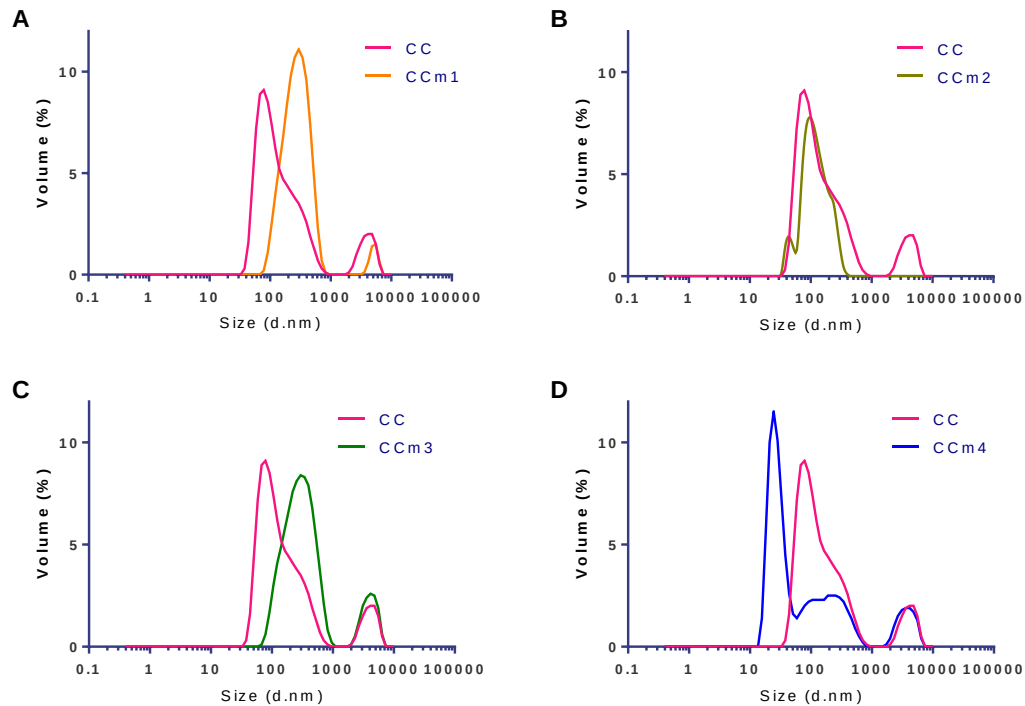


Figure 83 - Dynamic Light Scattering indicates changes in the size of oligomers upon insertion of proline mutants into the 313-550 protein. Each mutant protein construct of DEF6 amino acids 313-550 (A) Q371P A374P (CCm1), (B) I428 L431 (CCm2), (C) L470P L473P (CCm3) and (D) L512P V515P (CCm4) was dissolved in 20mM sodium phosphate buffer at pH7.4 with 150mM NaCl to a concentration of 1mg/mL and measured using a Malvern Zetasizer as well as the wild type CC protein (pink line on each trace).

The data from dynamic light scattering suggests the populations of different oligomers that the coiled coil constructs form (Figure 83). In the wild type coiled coil protein construct (amino acids 313-550) there are two main populations of approximately 78nm and 295nm in diameter, the first being the population with the highest volume. A small population is also visible at very high sizes, likely to be large aggregates. These can be cleared by centrifugation.

Both Q371P A374P (CCm1) and L470P L473P (CCm3) exhibit main populations of oligomers approximately 295nm in diameter with no peak at 78nm, indicating one population of larger oligomers than the wild type protein. For Q371P A374P (CCm1) this is consistent with the cellular data showing the aggregation of this mutant observed in cells transfected with the GFP-tagged mutant. L470P L473P (CCm3) also did not demonstrate aggregation of the GFP tagged mutant, instead

remaining diffuse in both untreated cells and those treated with arsenite to induce translational arrest. It appears CCm3 has a greater ability to form larger oligomers than the wild type 313-550 protein construct, but is unable to aggregate in cells. This could be due to the difference in the two constructs, in cells the proline mutant is in the full length protein, whilst in the solution studies with purified protein, only amino acids 313-550 are used. The mutation may have an increased ability to oligomerise when the domain is free, but be prevented from doing so by molecular forces within the full length protein, which also prevent its aggregation. Similarly, although I428 L431 (CCm2) showed lower levels of aggregation in the cells, the DLS data suggests similar oligomer formation to the wild type 313-550 protein construct.

In addition to low levels of the 2 main populations present with the wild type coiled coil protein, L512P V515P (CCm4) has its largest oligomeric population at 24.36nm in diameter, indicating less oligomerisation than the other protein constructs. This result is consistent with the lack of aggregate formation in Cos7 cells, indicating that the L512P V515P mutation is able to abrogate oligomer formation and thus prevent formation of DEF6 granules in cells.

6.2.6 The coiled coil region of DEF6 contains an amino acid sequence that matches the putative coiled coil trigger sequence

Coiled coil proteins commonly contain a short autonomous helical folding unit called the trigger sequence, which is conserved in a wide variety of coiled coil proteins²³¹. It is thought that their formation of helix forms a nucleation point for the formation of the extended alpha helical coiled coil. Whilst the trigger sequences do not dimerize as such, they are thought to interact, bringing two strands into the correct register for coiled coil dimerization, allowing the coil to “zip up”. This retains the requirement for the extended series of heptad repeats for proper dimerization.

Whilst it has been suggested that a consensus sequence for the trigger sequence is unlikely as the helix can be stabilized through a variety of intramolecular interactions²³¹, a 13 residue sequence with limited sequence variation that exists in many two stranded coiled coils but not in coiled coils of higher numbers of strands has been identified²³². The trigger sequence appears to have a stabilizing effect, and is required in many cases for proper coiled-coil oligomer formation. The consensus sequence is:

xxLExchxcxcx

Where x is any amino acid, c is any charged amino acid and h is any hydrophobic amino acid.

Searching the coiled coil region of DEF6, a sequence matching this consensus sequence is found at amino acids 442-454:

LQLEVKARRDEES

This falls in the middle of the coiled coil region of DEF6. This is within the region with a lower predicted coiled coil score, and contains fewer glutamine residues than other regions within the coiled coil region.

Primers were designed to delete the trigger sequence from DEF6. The region deleted was extended to 14 amino acids to keep the heptad repeats of the coiled coil in register. The primers were as follows:

Forward: CGAATCGCTCAGACCAGACTG

Reverse: GGCCTCCTGCAACCGCTG

Which cause the deletion of amino acids 442-455, LQLEVKARRDEESV.

The primers were used to delete the putative trigger sequence from both the full length GFP-DEF6 construct and from the GST-313-550 construct, referred to as GFP-DEF6 $\square$ trig or GST-CC $\square$ trig respectively.

6.2.6.1 Removing the trigger sequence from GFP-DEF6 increases the number of cells in which it is expressed exhibiting GFP-DEF6 granules

A plasmid expressing the GFP-DEF6 Δ trigger construct was transfected into Cos7 cells. As shown before, GFP alone is diffuse in both untreated and arsenite treated cos7 cells, whilst the full length wild type DEF6 GFP construct is diffuse in untreated cells but forms granules in arsenite treated cells (Figure 84). Cells expressing the GFP tagged protein were classified as demonstrating a diffuse localisation, forming granules, or being present in rounded up cells. This data is preliminary as the experiment was only performed once.

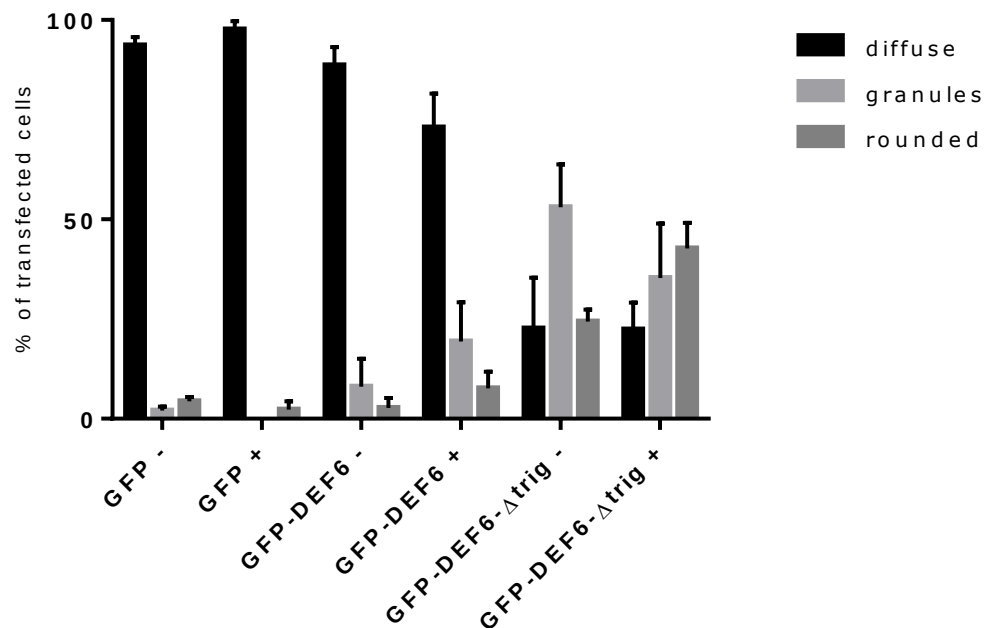


Figure 84 - Graph showing the percentage of cells demonstrating diffuse or granulated GFP-tagged protein in cells transfected with GFP-tagged DEF6 protein constructs with the putative trigger sequence (amino acids 442-455) deleted. Cos7 cells were transfected with GFP alone, GFP-DEF6 or GFP-DEF6 Δ trig. Cells were either left untreated (-) or treated with 1 μ M arsenite for 1 hour (+). Cells were fixed and an AxioVertS100 microscope was used to take widefield images of the fixed cells which were then counted and classified. Bars show standard deviation between the three areas in which cells were counted.

As can be seen in Figure 84 and Figure 85, deleting the trigger sequence has the opposite effect to that expected. In the full length protein, removing the trigger

sequence induces granule formation suggesting a higher propensity for aggregation and oligomerisation.

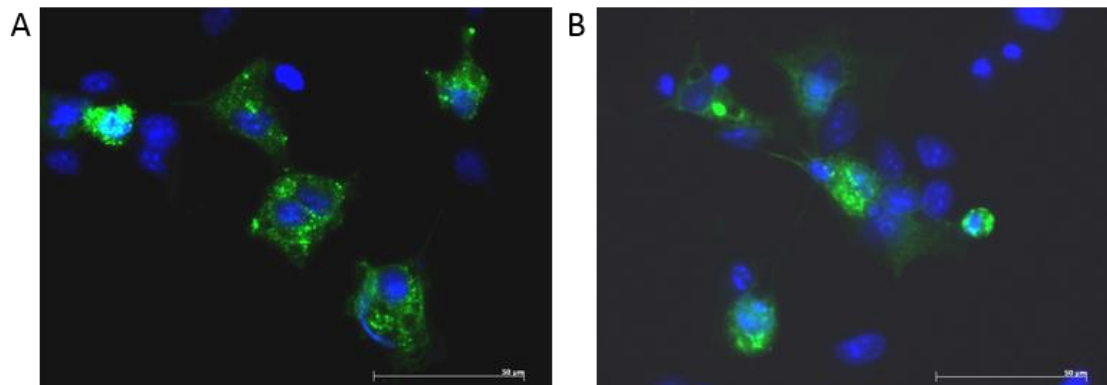


Figure 85 – Deleting the putative trigger sequence in the coiled coil region of DEF6 induces granule formation of GFP-DEF6Δtrig. Cos7 cells were transfected with GFP-DEF6Δtrig. Cells were either left untreated (-) or treated with 1μM arsenite for 1 hour (+). Cells were fixed and stained with DAPI containing mountant (blue). An AxioVertS100 microscope was used to take widefield images of the fixed cells. Scale bar is 50μM.

Potentially, this effect is due to the disruption of the C-terminal region, causing intramolecular effects that prevent foci formation such as the proposed closed conformation of DEF6 to be released, and thus promoting granule formation. To assess the effect of deleting the trigger sequence on the coiled coil region alone, the trigger sequence was deleted from the 313-550 protein construct.

6.2.6.2 Purification and characterisation of a protein construct containing the amino acids 313-550 with the trigger sequence removed

The sequence was successfully deleted out of the GST-313-550 construct, and the protein expressed in C41 cells using the same strategy used for the other 313-550 constructs (detailed methodology found in section 2.4.3) (Figure 86).

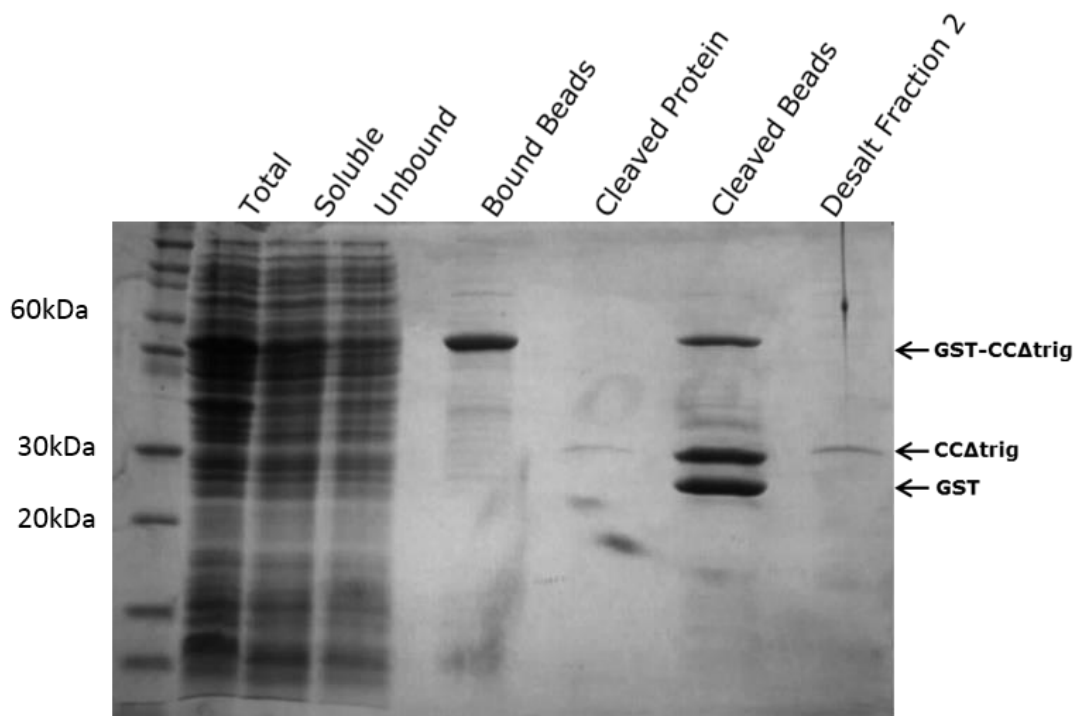


Figure 86 – Purification of GST-313-550 Δ trigger (GST-CC Δ trig) using the same protocol as for the coiled coil protein (313-550). Lanes indicate the total protein from cell lysis, the soluble fraction after centrifugation, the protein that remained unbound to the glutathione sepharose beads after incubation, the protein bound to the beads, the eluted protein after thrombin cleavage from the beads, the protein remaining on the beads, and the protein after desalting.

The circular dichroism spectra of each protein was measured to determine whether removing the trigger sequence has an effect on the ability of the protein construct to form a coiled coil structure.

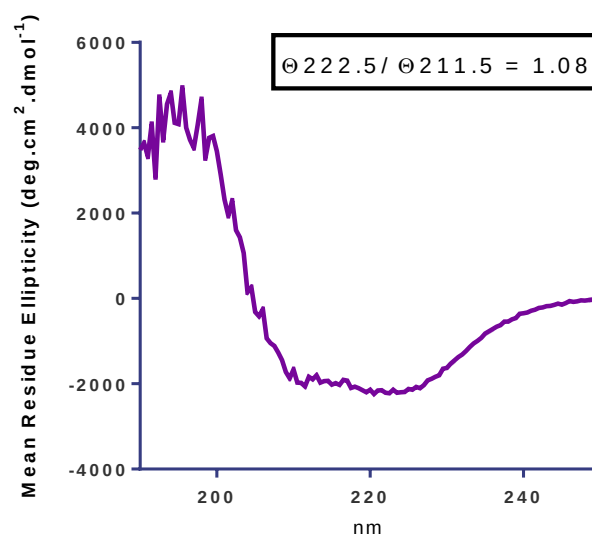


Figure 87 – Removing the putative trigger sequence does not prevent coiled coil formation. The 313-550 protein with its trigger sequence (442-455) removed was dissolved in 20mM potassium phosphate buffer at pH7.4 to a concentration of 0.1mg/mL and had its ellipticity measured using a Pi-Star-180 Spectrophotometer. Values were transformed from machine units to mean residue ellipticity using the formula in 2.6.2. Minima were chosen to measure the coiled coil content for each protein construct.

The circular dichroism data suggests that coiled coil formation has not been inhibited by the removal of the putative trigger sequence as the ratio of the minima remains above 1 (Figure 87).

As for the DEF6 protein containing the proline mutants, the light scattered by the purified protein was measured using dynamic light scattering to determine the size of oligomers produced by the protein.

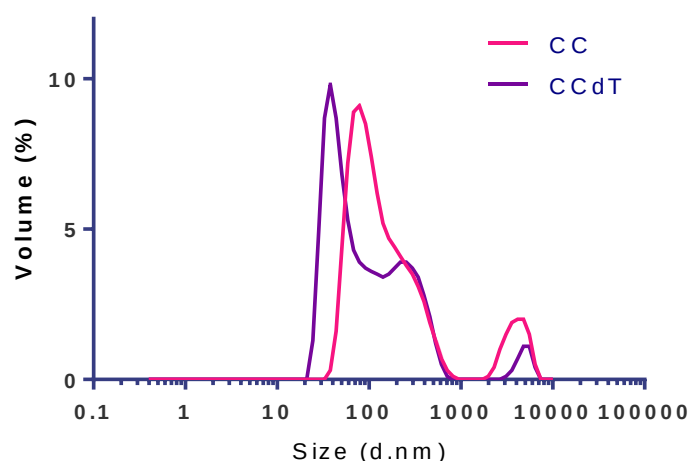


Figure 88 – Dynamic Light Scattering shows the formation of smaller oligomers upon removal of the trigger sequence. The 313-550 protein with its trigger sequence (442-455) removed was dissolved in 20mM sodium phosphate buffer at pH7.4 with 150mM NaCl to a concentration of 1mg/mL and measured along with the 313-550 protein construct using a Malvern Zetasizer.

The data from dynamic light scattering (Figure 88) suggests that the main population of oligomers formed by protein with the trigger sequence removed are approximately 43.82nm in diameter, smaller than the wild type coiled coil protein (Figure 88). There is a smaller population corresponding to the 295nm peak found in the other constructs, indicating the ability to aggregate to a low level, but a preference for a smaller oligomeric state.

Due to the decrease in oligomer size in the protein with the trigger sequence removed, the dynamic light scattering data suggests that the trigger sequence may be involved in facilitating the formation of larger oligomers, rather than the formation of the initial coiled coil, as this is still present as demonstrated by the circular dichroism data. Whilst it may decrease the formation of larger oligomers by the coiled coil stretch of the protein, this effect may not carry over to the full length protein as expressed in the Cos7 cells, as disruption of the coiled coil may promote further structural changes in the full length protein that then promote the wide scale formation of foci as seen in Figure 85.

6.3 Conclusions

Similar to other granule forming proteins a prion-like motif, enriched in glutamine and asparagine residues, is also present in the DHL domain of DEF6 between amino acids 310-550. In DEF6 however, this region forms a coiled coil structure, rather than the β -amyloid structure most often associated with prion-like proteins.

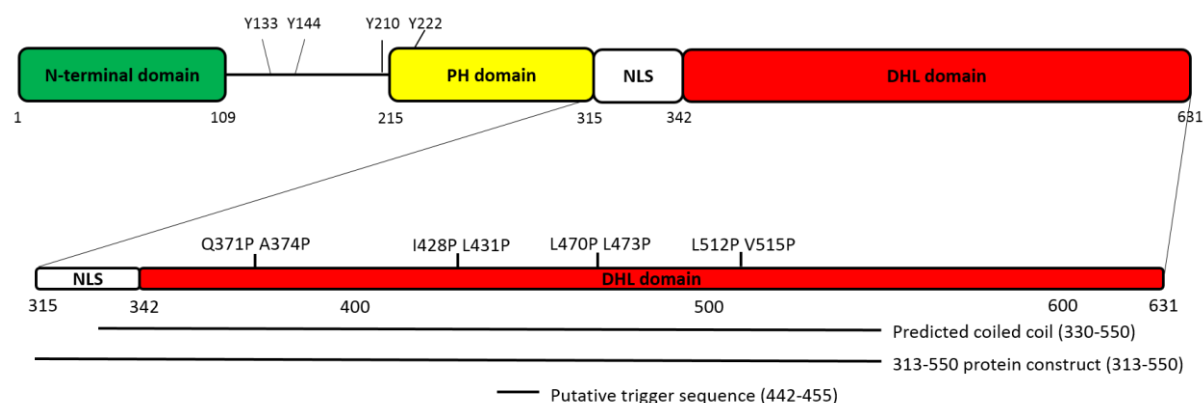


Figure 89 - Diagram indicating the location of the predicted coiled coil, the position of the proline mutations and the trigger sequence, and the amino acids covered by the protein construct.

It is possible to disrupt, to varying extents, the formation of foci in cells by inserting proline residues into the coiled coil region to form kinks in the coiled coil. The addition of proline residues at Q371 and A374 (CCm1) increased foci formation, whilst those at I428 L431 (CCm2), L470 L473 (CCm3) and L512 V515 (CCm4) decreased foci formation. These mutations alter the size of oligomer formed as demonstrated by the dynamic light scattering experiments, thus having a knock on effect on the ability of the protein to coalesce into foci. This further supports the identification of the C-terminus as the driver of DEF6 aggregation into foci. The putative trigger sequence identified appears to promote aggregation of the full length GFP tagged DEF6 when expressed in Cos7 cells, but dynamic light scattering reveals that it leads to the formation of smaller oligomers when it is removed from the 313-550 coiled coil protein construct. It does not decrease the formation of a coiled coil, suggesting its role may be in driving the formation of larger oligomers rather than the initial

interactions between strands to form a coiled coil. The increased aggregation in the cell data may be due to disruption of the closed form of DEF6, exposing sequences that drive aggregation.

The presence of the prion-like sequence and previous work in chapter 5 showing the aggregation of the DHL domain supports the theory that the DHL domain is responsible for aggregation of DEF6 into granules. The confirmation that the DHL domain can form a coiled coil structure provides a potential mechanism for DEF6 aggregation that is more reversible than the prion-like β -amyloid aggregation that glutamine/asparagine rich regions would normally suggest. This is a novel mechanism for aggregation with regards to P-body formation, but fits with observations in other aggregate-forming systems; most notably those studied by Fiumara *et al*⁷⁰.

6.3.1 Implications of a coiled coil in DEF6

Mediating oligomerization through coiled coil interactions between the C-terminus allows for a method of oligomerization to drive aggregation into DEF6 granules. As currently most known P-body aggregation is mediated via prion-like aggregation¹⁰², this presents an alternative mechanism for P-body protein aggregation. Additionally, due to differences in mechanism, this may explain the inability for the DHL domain of DEF6 to fully co-localise with other granules, as this would suggest that the mechanism of aggregation is not compatible with aggregation with other P-body proteins. This is supported by the data in the previous chapter (section 5.2.2) indicating that the C-terminus alone can aggregate but does not fully co-localise with other markers for P-bodies suggesting a lack of hetero-oligomerisation with other P-body forming proteins. This suggests a model by which DEF6 interacts with itself via coiled coil interactions in its C-terminus, and further interacts with either mRNA, or mRNA binding proteins via its N-terminal domain, bringing it into P-bodies.

Mediating oligomerisation is a known role of coiled coil domains, usually achieved with a flexible single chain coiled coil that binds in an anti-parallel manner²³³. Further work on determining the orientation of coiled coil oligomerisation in DEF6 will further improve our understanding of the packing of DEF6 into its granular formation.

It has been reported previously that the ratio of asparagine and glutamine residues to each other in glutamine/asparagine rich regions in prion-like proteins indicates the extent of both β -amyloid formation and toxicity of the protein²⁰⁹. Asparagine residues have been shown to increase the aggregation of proteins into β -amyloid aggregates, but to also decrease toxicity of the protein. This has led to the suggestion that aggregation prevents toxicity by locking up protein. Glutamine-rich proteins, unable to form β -amyloid and aggregate as readily, are more toxic to cells as a result. DEF6 is glutamine rich, but is still able to form aggregates, likely to be due to its ability to form coiled coil structures. This may help prevent DEF6 toxicity to cells, which is particularly relevant to the studies by the Altman group which indicate DEF6 promotes experimental autoimmune encephalomyelitis and experimental autoimmune uveitis^{8,9,58}. In this view of the role of DEF6 causing autoimmune disease, aggregation of DEF6 may be a protective mechanism, preventing the inappropriate lymphocyte response and cytokine production that leads to these diseases.

On the other hand, it can be argued that the studies by the Pernis group^{4,10,56} could also be explained by excessive DEF6 aggregation, but without free DEF6 being toxic to cells. In this view in which DEF6 protects against autoimmune disease, issues that either cause excess aggregation of DEF6 into granules or disrupt the normal rate of clearance of DEF6 granules would cause a decrease in free DEF6 in the cell, thus disrupting IRF4 control in a similar manner to that modelled in those studies by knocking out DEF6. Further, sequestration of DEF6 would have implication on its ability to interact CDC42, impairing the

spatiotemporal organization of the T cell leading to issues with engagement with the antigen presenting cell, and with the correct cellular response to activation^{5,145}.

These models however do not address whether the DEF6 foci found in T cells have a purpose outside a protective segregation of toxic DEF6 protein or as an accidental over aggregation. DEF6 granules co-localise with proteins found in P-bodies, which suggests a potential for a role in mRNA metabolism. There are several possibilities for its role here, as it may be involved in the segregation of mRNA, the breakdown of mRNA or potentially could be involved in releasing segregated mRNAs back into the cytoplasm for translation. There is currently no data on what implications DEF6 has on the fate of mRNAs in granules. It is also currently unknown whether DEF6 has different functions at different stages within the T cell. Data in this study has shown that DEF6 can form granules after activation of T cells (section 3.2.1), but following this process in real time should allow for more detailed knowledge of the timing of DEF6 aggregation in relation to activation.

One question arising from the data showing that the C-terminus is able to form a coiled coil is, what form the DHL domain takes when it is acting as a Rho-GEF? There are two possibilities: the DHL domain is only active in the closed, non coiled-coil form, or DEF6 can exhibit GEF activity in a coiled coil form, unlike other Rho-GEF DHL domains.

6.3.2 It is possible for GEF domains to adopt a coiled coil structure and retain activity?

Whilst the structure of Rho-GEF DHL domains is normally a “chaise longue” shaped extended structures of anti-parallel α -helices, several proteins that interact with the GTPase RhoA have been shown to or predicted to form coiled coils, which are often to be found within the Rho binding domains of these proteins²³⁴. It is thought that RhoA may in fact preferentially interact with coiled

coil domains as many of its interaction partners Rhotekin, rhophilin, citron kinase and kinectin are predicted to contain coiled coils²³⁵. The RhoA interacting serine/threonine kinases ROCK²³⁴ and PKN²³⁶ have both been shown to contain coiled coil domains. Additionally PKN is also able to bind Rac1, another protein that interacts with DEF6²³⁷.

ROCK1 (Rho-associated, coiled-coil containing protein kinase 1) has a role in promoting actin stability after it is activated by binding RhoA. It has features similar to DEF6, including an enrichment of glutamine and asparagine residues compared to the rest of the protein within the coiled coil region. Rock1 contains a similar richness in glutamine/asparagine residues to Def6 (20 glutamine or asparagine residues in its richest 80mer, with no proline residues in this stretch). Other similarities to DEF6 include its C-terminal PH domain, and a caspase cleavage site, which DEF6 has also been predicted to contain (at amino acid 106). Additionally, ROCK has been shown to be a part of stress granules⁹⁹. The sequestration of ROCK1 into stress granules inhibits apoptosis by preventing ROCK1-mediated JNK activation and JIP-3 phosphorylation; mirroring a model in which DEF6 sequestration could prevent DEF6 mediated control of either the transcription factor IRF4 or its control of actin filament formation via its GEF activity. Interestingly, only active ROCK1 is recruited to stress granules to inhibit apoptosis⁹⁹.

Rock1 is not a GEF, so whilst ROCK1 illustrates the ability of coiled coils to bind the switch regions of RhoA, it does not demonstrate that interactions between a coiled coil domain and the switch regions in a GTPase is sufficient for nucleotide exchange. Catalytic activity of a coiled coil to a GTPase has been demonstrated however in the exchange factor Sec2p, which activates the Rab GTPase Sec4p via a coiled coil region²³⁸⁻²⁴⁰. Sec2p forms a dimeric coiled coil, and the interface with Sec4p is across the two helices. Reorganisation of the coiled coil helices of Sec2p upon binding the GTPase promote reorganisation of the switch 1 region in

the GTPase, changing the positions of amino acids essential to binding GDP, thus causing it to loose affinity and dissociate. Whilst this suggests that it is feasible for DEF6 to possess a both GEF activity as a coiled coil and undergo aggregation through the same structures, the data in this study does not allow for a definite characterisation of whether the DHL domain of DEF6 is active in the coiled coil form. Further GEF activity studies using the proline mutants *in vivo*, and using the purified protein constructs *in vitro* could shed light on this. The two actions are clearly not mutually exclusive within a cell, as several of the cell images demonstrate both extensive filopodia formation above that seen in the untransfected or GFP-transfected cells, however there is likely to be a heterogenous population of DEF6 structures within the cell, and it could be that each form controls a different activity.

6.3.3 Coiled coil aggregation

Coiled coils have long been known to be involved in oligomerisation, for example in transcription factors or cytoskeletal proteins. Their ability to promote larger scale aggregation has also been demonstrated in several cases for example the aggregation of the yeast pyruvate decarboxylase Pdc1 into P-bodies in *S.Pombe* which is reliant upon a C-terminal coiled coil²¹⁹. Interestingly Pdc1 has been suggested to be a scaffold protein, interacting both with itself to drive aggregation, but also with other P-body components in a manner analogous to the decapping enhancer Edc4 in higher eukaryotes. Edc4 however does not aggregate via coiled coil interactions. As discussed in the previous chapter, the FRAP dynamics of DEF6 point to it potentially having a scaffold role in P-bodies due to its slow recovery rates. Its ability to recover to some extent however does suggest that the coiled coil interactions that help it to form aggregates are reversible.

This is seen in other coiled-coil containing proteins that form aggregates, for example in PABPN1 (a poly-A tail binding protein), which accumulates into

inclusions that are the hallmark of oculopharangeal muscular dystrophy²²⁰. Again, PABPN1 requires its coiled coil region to form aggregates, but is able to shuttle in and out of the inclusions, even when further induced to aggregate via the addition of extra pathogenic repeat sequences. This ability to shuttle in and out of inclusions has also been noted in poly-glutamine inclusions in a similar manner to the fast exchange of P-body and stress granule components with the cytoplasm, with an early demonstration of the ability of protein aggregates to exchange with the nucleoplasmic pool. This was demonstrated by the ability of poly glutamine ataxin1 to form inclusions with varying rates of exchange indicating inclusions are not just inert lumps of protein²⁴¹.

The ability of some polyQ and glutamine/asparagine rich proteins to exhibit shuttling in and out of aggregates suggests an aggregated form different to that of the extended β sheet amyloid form. It has been known for some time that native coiled coils can transform to amyloid fibrils upon changes in temperature or ionic strength²⁴², and that the nucleation step for these proteins may well be that of oligomerised coiled coils²¹⁰. However, there is a distinct possibility that the coiled coil form can be sufficient for amyloidogenesis and inclusion formation, and that conversion to a β sheet form is not necessary⁷⁰. This leads to the possibility of a more dynamic structure, and the ability to exchange in and out of the aggregates as shown by DEF6. As well as being more dynamic than an amyloid fibril form, the glutamine/asparagine rich coiled coil is likely also to be more dynamic than a standard coiled coil structure due to the high number of glutamine residues. By studying the N-terminal coiled coil domain and glutamine-rich domain of histone deacetylase 4, it has been determined that the glutamine residues allow for a highly diverse range of interactions within and between strands, with both hydrogen bonds and van der Waals interactions forming²⁴³. It was noted that this diversity was at the cost of stability, as the propensity for glutamines meant there were less residues involved in the

hydrophobic and electrostatic interactions normally found between chains of coiled coils. The use of glutamine residues in coiled coils thus allows for both a more reversible interaction due to the relative instability of the interchain interactions and for a wider diversity of possible interactions, which has interesting implication on the ability of DEF6 to both potentially interact with itself in multiple orientations, but also to allow a large variety of other proteins to interact with it in the P-bodies.

A further aspect that may increase the ability to reverse aggregation into the granular form readily may be linked to low complexity and intrinsically disordered regions. Prion-like glutamine/asparagine rich domains themselves form a subset of intrinsically disordered proteins, which can take on multiple structures rather than forming one fixed structure, demonstrate reversibility of interaction and can interact with many partners, as with the glutamine rich coiled coils mentioned earlier²¹¹. *In vitro* this aggregation of low complexity regions leads to the formation of hydrogel, which in cells allows the liquid droplet like behaviour of mRNP granules. This allows them to display features such as surface tension, and allows granules to selectively merge with one another. Additionally, intrinsically disordered regions have been noted to be highly likely to be present in the vicinity of coiled coils, and to be the key to the reversibility of aggregation seen by the proteins studied⁷⁰.

As previously mentioned in section 6.1.1 of this chapter, links have been shown between prion domain containing proteins and involvement in mRNA binding. These mRNA binding proteins although able to form aggregates similar to amyloid aggregates in line with their prion domains, were however more dynamic and the aggregates able to depolymerise²¹¹. Similarly, a link has been shown between proteins containing intrinsically disordered regions and mRNA binding²⁴⁴. The intrinsically disordered regions within the mRNA interactome differ from those found in the whole proteome by a preponderance for glycine,

arginine, lysine and tyrosine residues²⁴⁴. They also display a higher occurrence of repetitive amino acid motifs, and low complexity. It is thought that intrinsically disordered regions may form extended structures with multiple folded units linked with short connectors, leading these regions to act as polymorphic recognitions sites for multiple binding partners. This multivalency is one ingredient necessary for phase transitions, which could be controlled by ionic strength, or post translational modifications giving a mechanism for reversible aggregation into granules.

Low complexity regions have been linked to the ability of P-body proteins to display dynamic exchange with the cytoplasm by both Malinovska *et al*²¹¹ and Kedersha *et al*⁸⁸, who noted that the metastable conformations displayed by low complexity regions allowed reversible associations using different structural forms.

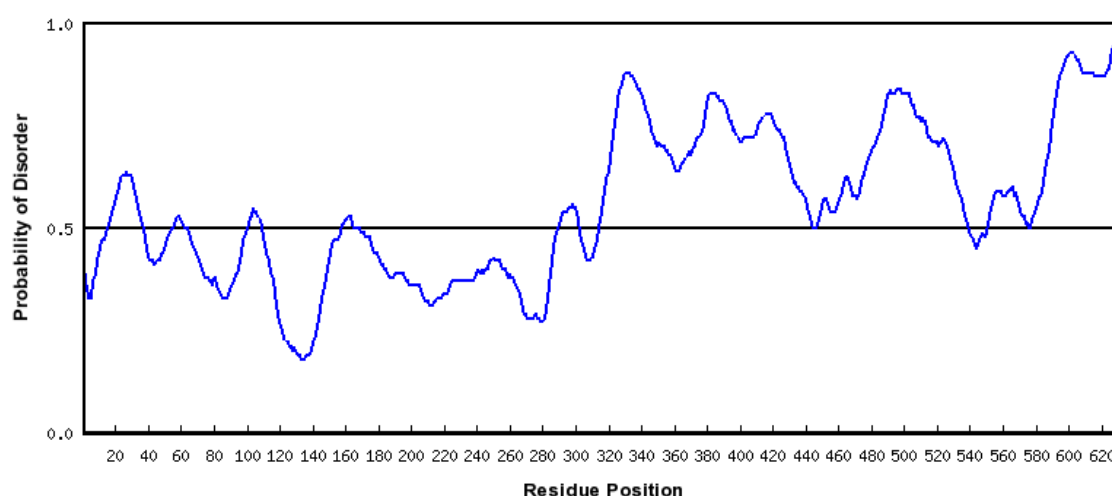


Figure 90 - Prediction for disordered regions in DEF6 using the RONN algorithm²⁴⁵ indicates an increased probability for disorder within the C-terminal half of the protein.

DEF6 is predicted to form a disordered region in its C-terminal half, with a particularly highly predicted region at the very C-terminus of the protein. Whilst this study has shown that most of the C-terminus in fact can form a coiled coil structure, this may reflect the ability of the C-terminus to switch between the closed and open coiled coil structures. Supporting the prediction of a high level

of disorder at the very C-terminal end of the protein is the solubility of the C-terminal protein constructs. Attempts to produce constructs containing the last 90 amino acids failed due to solubility and stability issues, whilst the coiled coil constructs which do not contain that region were stable and soluble, indicative of the coiled coil region forming a well folded stable construct, and the remaining C-terminus forming a disordered region that leads to unwanted aggregation and solubility issues, and is more open to degradation than a tightly folded domain.

The data in this chapter points to a system in which conversion of DEF6 to a coiled coil form allows reversible interactions leading to oligomerisation and finally aggregation into mRNP foci. The predicted disorder of the C-terminus of DEF6 provides support for the ability of it to shift between two forms, and the highly disordered C-terminal may be involved in this switching, both due to signals leading to the opening of DEF6 and conversion to the coiled coil form as discussed in section 4.3.4, but also once it is open, it may provide further interactions to promote the reversible aggregation. This ability to reversibly aggregate allows for control over the amount of DEF6 in granules, potentially allowing for control over its binding to elements such as IRF4 and CDC42. Small changes to the sequence of DEF6, for example by inserting proline residues have been shown to effect its ability to aggregate, thus providing evidence that it is possible for small cellular changes to alter the sequestration of DEF6, disrupting the control over its IRF4 and CDC42 binding. Whilst the data has not shown whether the coiled coil form and the active GEF form of the C-terminus of DEF6 can co-exist, it has provided important insight into the interactions DEF6 can perform that allow it to execute its role in P-bodies, which may have a consequence on its role in autoimmune disease.

7.Conclusions

7 Conclusions

The work described here has developed our understanding of the formation of granules by DEF6, showing that they are present in activated T cells, and not just an artefact of GFP tagged proteins expressed in Cos7 cells, and furthering our understanding of which domains contribute to both the aggregation of DEF6 into granules, and their association with other cytoplasmic foci. The dynamics of the DEF6 granules have also been explored, providing potential roles for these granules in cells and in the role DEF6 plays in the immune response, as well as allowing comparison between DEF6 granules and those formed by other proteins. Finally, the work on the PH domain has furthered the work by Fiona Hey, ruling out the nuclear localisation signal as necessary for Itk binding and phosphorylation of DEF6, and suggesting that Itk phosphorylation is not the only route to granule formation.

7.1 DEF6 granules form in activated T cells

DEF6 localises to the immune synapse in Jurkat T cells activated by presentation of SEE by Raji B Cells. In these cells, DEF6 granules can be seen in the cytoplasm, demonstrating that endogenous DEF6 is able to form the foci studied in Cos7 cells. The presence of P-bodies in activated T cells was also confirmed, with both DDX6 and Xrn1 forming granules in activated Jurkats. Due to the sources of the antibodies, the localisation of DEF6 could not be directly compared with that of Xrn1 or DDX6, but Xrn1 granules appeared to be localised towards the immune synapse side of the T cell. Due to heterogeneity of protein composition of granules, and the overlap between different types of granules, despite being P-body markers DEF6 would not be expected to always fully co-localise with all granules formed by P-body markers, as it may only be present in P-bodies at certain stages during their development, or in specific subtypes.

If DEF6 has a role in regulation of mRNA translation, as is suggested by the association of DEF6 granules with granules formed by P-body forming proteins,

the existence of endogenous granules in activated T cells could suggest a role in regulating the immune response beyond that attributed to IRF4 binding or its GEF activity. The production of cytokine protein has been demonstrated to be decoupled from the production of cytokine mRNA. Transcription of cytokine mRNA occurs within hours of T cell activation, but cytokine protein levels are not detected until sometime after, indicating a decoupling between the two processes, and a level of translational control¹¹¹. It is not currently known what the identity of any mRNA that may be associated with DEF6 granules are, but any regulation of translation would add another level of spatiotemporal organisation by DEF6 to complement its spatiotemporal organisation in T cells via its GEF activity on CDC42⁵.

The spatial organisation of translation to near the immune synapse was observed by the localisation of PABP and eIF4E to areas of the T cells corresponding to the dSMAC. Although this was not as obvious a polarisation as that of DEF6, it appears that translation is likely to be happening on the immune synapse side of the cell. These proteins have been shown in neurones to localise translation to subsynaptic regions allowing for spatial control of protein production. What role DEF6 has in this process, and particularly what role the granular form of DEF6 has is unclear. DEF6 granules were observed throughout the cytoplasm, and its link with the pool of DEF6 at the immune synapse is not clear.

7.2 DEF6 recruitment to cell junctions, in particular the immune synapse may be driven by active transport along microtubules and patterned via PIP₃ binding

P-bodies have been shown to interact with both the actin and microtubule cytoskeleton, with different effects on their movement and localisation. Interaction with the actin cytoskeleton leads to granules that move within small areas due to corraling by the actin, whilst interaction with the microtubule network is believed to allow for long distance travel via active transport. The

dynamics of DEF6 movement suggest an ability to undergo active transport, linked to the microtubule network. This would provide a mechanism for transport to the immune synapse. Previously, this translocation has been linked to phosphorylation by Lck³⁵, and to binding of PIP₃. PIP₃ has later been shown to be unnecessary for the translocation event³⁴, but may be involved in organising the location of DEF6 once at the immune synapse to allow for spatiotemporal organisation of the actin cytoskeleton in a manner similar to that of DOCK1¹⁴⁵. This spatiotemporal patterning necessary for CDC42 activation at the immune synapse is also dependent on the activity of Itk, which phosphorylates DEF6 at tyrosines 210 and 222⁵.

7.3 Itk phosphorylation of DEF6 is not the only route to DEF6 granule formation

The formation of DEF6 granules has previously been suggested to be driven by Itk phosphorylation of residues Y210 and Y222¹². However, data following the production of granules over time suggests that whilst Itk destabilizes the protein enough to cause aggregation, formation of small more ordered foci is likely to be regulated via a separate route linked to the arrest of translation that can be induced by arsenite treatment or the integrated stress response prompted by T cell activation. Destabilization of the protein to form aggregates similar to those formed by mimicking Itk phosphorylation can also be prompted by removal of the C-terminal end of DEF6, supporting an auto inhibitory interaction mediated by the C-terminus that holds DEF6 in a closed, non-aggregating structure. An open and closed model for DEF6 has previously been proposed for DEF6 for the activation of its GEF activity³. Whether the open or closed form is responsible for GEF activity is unknown. Both an alpha-solenoid structure as proposed for the closed form, and a coiled coil structure as proposed for the open form would be able to bind GTPases, as shown by beta-importin²⁰⁰ and Sec2p²⁴⁰ respectively. The open coiled coiled form is proposed to be the form required for aggregation into granules in line with the removal of the C-terminus. The signal that prompts

this switch is unknown, but there are candidate sites for phosphorylation on the C-terminus of DEF6 that could provide one. How these signals could lead to structural changes that open the protein and allow coiled coil interactions to take place is unknown.

7.4 The positively charged patch formed by the nuclear localisation signal in DEF6 is not necessary for Itk binding or phosphorylation of DEF6

Continuing the work of Fiona Hey in identifying a binding patch for Itk on DEF6, protein constructs were made of the PH domain with and without the nuclear localisation signal which has been suggested as the binding site due to it forming a patch of contiguous positive charge. The purification protocol was successful in producing protein that demonstrated biophysical characteristics expected of PH domain protein, although it was not stable enough to use for structural studies by NMR. Using the protein in functional studies with recombinant active Itk demonstrated that protein lacking the contiguous positively charged patch provided by the nuclear localisation signal both bound and was phosphorylated successfully by Itk, indicating that this is not the site of Itk binding. The protein purification method used to produce PH domain protein can be used in the future to further this work in collaboration with Amy Andreotti and her collection of Itk mutants⁶⁸.

7.5 The N-terminus facilitates co-localisation with DCP1 granules

Studying the granule formation and co-localisation with DCP1 of both the DHL domain and the N-terminal domain has shown that whilst both form granules, only the N-terminal domain is able to fully co-localise with DCP1. This suggests that it is the N-terminus that is responsible for the co-localisation of the full length DEF6 with DCP1, whilst the C-terminus is likely to have a role in driving aggregation due to its Q/N rich sequence in line with other aggregating sequences. Interestingly, the DHL domain partially co-localises with DCP1, clustering around DCP1 granules and occasionally forming separate granules

encapsulated by DCP1. Granules have suggested to form through liquid-liquid unmixing interactions, with the constituent proteins forming interactions with each other that are strong enough to form separate domains within the cytoplasm without need of a membrane. In mixtures of multiple types of polymer more than two phases are possible, and it is possible that whilst the granule forming protein can interact to form a phase separate to the cytoplasm, the DHL domain may be forming separate interactions that are not compatible with those formed by other granule forming proteins, forming a further separate phase and thus the N-terminus is necessary for co-localisation of DEF6 with P-body forming proteins.

7.6 DEF6 exchanges between the cytoplasm and granules with a similar rate to proteins with a structural role in P-bodies and stress granules

Further characterisation of the granules was made through study of the dynamics of movement in and out of GFP-DEF6 granules via FRAP, and live imaging experiments. FRAP suggested that DEF6 exchanges between the granules and the cytoplasm at a rate comparable to that of other P-body proteins that are not involved in mRNA tethering. These proteins shuttle in and out quickly, whereas DEF6 granules recovered with a similar rate to proteins involved in the scaffolding of the granules. This provides some evidence for the role DEF6 has in the foci it forms, in that it is likely to provide a stabilising role for the granules, rather than be involved directly in mRNA binding and shuttling for translational regulatory purposes.

7.7 DEF6 granules can move in the cytoplasm with dynamics that suggest active transport

Further indications of the role of DEF6 were derived from the live imaging experiments. These showed that DEF6 granules are mobile within the cytoplasm, although they move at a relatively slow rate. The N-terminus alone moved faster, and visited larger areas of the cytoplasm. MSD analysis of the tracks of

each type of granule suggested that they could move via active transport, providing evidence for a potential link between DEF6 granules and the microtubule network. This could provide a mechanism for the translocation of DEF6 to the immune synapse in T cells after activation, and may suggest a role for the DEF6 granules in providing a method of transport to the immune synapse to set up the effects it has there. These have previously been shown to be linked to its role as a Rho-GEF, in providing organisation to the actin ring at the immune synapse. Differences in the MSD traces between the full length DEF6 and the N-terminal domain suggest that the N-terminus may be more strongly associated with the microtubule network as it was unable to diffuse freely within the cytoplasm. The full length DEF6 may be able to control its association with the microtubule network as it demonstrated both diffusion and active transport, potentially controlled via the phosphorylation events that have been shown to control DEF6 translocation to the immune synapse.

7.8 The C-terminus of DEF6 is able to form a coiled coil structure through which oligomerisation into granules is likely to happen

The DHL domain has previously been predicted to form a coiled coil structure due to its amino acid sequence. This prediction has been verified via production of protein spanning the predicted coiled coil region, which has been measured via circular dichroism to form a coiled coil structure. Attempts to break the coiled coil structure using proline mutants following a method set out by Fiumara *et al*⁷⁰ had varied results. Whilst none of the mutations were able to prevent coiled coil formation, they had varying effects on the ability of the protein to form oligomers, and on the ability of GFP tagged protein in Cos7 cells to form foci. Similarly, the predicted trigger sequence for the coiled coil stretch was not necessary for coiled coil formation, and removal of it did not prevent granule formation. It is likely that the mutants that decreased oligomer formation and granule formation were enough to prevent interactions between multiple coiled coils due to the likely bends induced in the structure, but were not enough to

prevent coiled coil formation due to the length of the coiled coil forming region. The mutation that increased oligomer and granule formation was the most N-terminal of the mutations, and may have altered the full length structure enough to break the inhibitory interactions that keep the protein closed and in its diffuse non granule forming structure. This work links the observation made by Fiumara *et al*⁷⁰ that glutamine/asparagine rich regions of proteins can form coiled coils, to the mechanism of aggregation of the glutamine/asparagine rich proteins found in P-bodies and stress granules. Whilst many of these may aggregate in a prion-like beta sheet manner, coiled coils provide a further mechanism of aggregation that may be more reversible than prion-like aggregation. The balance between functional and pathogenic aggregation is fine, and coiled coil interactions may allow DEF6 to reversibly associate into granules in a manner that does not normally disrupt cellular function. However, the potential for pathogenic aggregation still exists, and due to its links to autoimmune disease and potential links to the control of mRNA translation, pathogenic aggregation of DEF6 could prove to be a further mechanism of unwanted immune dysregulation.

8.Appendix

8 **Appendix**

8.1 **Coding DEF6 nucleotide Sequence**

```
1 atggccctgc gcaaggaact gctcaagtcc atctggtacg cctttaccgc gctggacgtg
61 gagaagagtg gcaaagtctc caagtcccag ctcaaggtgc tgtcccacaa cctgtacacg
121 gtcttgacac tcccccatga ccccgaggcc ctggaggaac acttccgaga tgatgatgac
181 ggccctgtgt ccagccaggg atacatgcc tacctcaaca agtacatcct ggacaaggtg
241 gaggaggggg cttttgttaa agagcacttt gatgagctgt gctggacgct gacggccaag
301 aagaactatc gggcagatag caacgggaac agtatgctct ccaatcagga tgccttcgcg
361 ctctggtgcc tcttcaactt cctgtctgag gacaagtacc ctctgatcat ggttctctgat
421 gaggtggaat acctgctgaa aaaggtactc agcagcatga gcttgagggt gagcttgggt
481 gagctggagg agcttctggc ccaggaggcc caggtggccc agaccaccgg ggggctcagc
541 gtctggcagt tcttgaggct cttcaattcg ggccgctgcc tgcggggcgt gggccgggac
601 accctcagca tggccatcca cgaggtctac caggagctca tccaagatgt cctgaagcag
661 ggctacctgt ggaagcgagg gcacctgaga aggaactggg ccgaacgctg gttccagctg
721 cagcccagct gcctctgcta ctttgggagt gaagagtgca aagagaaaag gggcattatc
781 ccgctggatg cacactgctg cgtggagggt ctgccagacc gcgacggaaa gcgctgcatg
841 ttctgtgtga agacagccaa ccgcacgtat gagatgagcg cctcagacac gcgccagcgc
901 caggagtgga cagctgccat ccagatggcg atccggctgc aggcgagggt gaagacgtcc
961 ctacacaagg acctgaagca gaaacggcgc gagcagcggg agcagcggga gggcgccggg
1021 gcggccaagg aagaggagct gctgcggctg cagcagctgc aggaggagaa ggagcggaag
1081 ctgcaggagc tggagctgct gcaggaggcg cagcggcagg ccgagcggct gctgcaggag
1141 gaggaggaac ggcgccgcag ccagcaccgc gagctgcagc aggcgctcga gggccaactg
1201 cgcgaggcgg agcaggcccg ggctccatg caggctgaga tggagctgaa ggaggaggag
1261 gctgcccggc agcggcagcg catcaaggag ctggaggaga tgcagcagcg gttgcaggag
1321 gccctgcaac tagaggtgaa agctcggcga gatgaagaat ctgtgcgaat cgctcagacc
```

1381 agactgctgg aagaggagga agagaagctg aagcagttga tgcagctgaa ggaggagcag
 1441 gagcgctaca tcgaacgggc gcagcaggag aaggaagagc tgcagcagga gatggcacag
 1501 cagagccgct ccctgcagca ggcccagcag cagctggagg aggtgcggca gaaccggcag
 1561 agggctgacg aggatgtgga ggctgccag agaaaactgc gccaggccag caccaacgtg
 1621 aaacactgga atgtccagat gaaccggctg atgcatccaa ttgagcctgg agataagcgt
 1681 ccggtcacca gcagctcctt ctcaggcttc cagccccctc tgcttgccca ccgtgactcc
 1741 tccctaaagc gcctgaccgc ctggggatcc cagggaaca ggacccccctc gccaacagc
 1801 aatgagcagc agaagtcctt caatgggtgg gatgaggctc ctgccccggc ttccaccct
 1861 caggaagata aactggatcc agcaccagaa aattaga

8.2 DEF6 amino acid sequence

1 MALRKELLKS IWYAFTALDV EKSGKVSQSQ LKVLSHNLYT VLHIPHPVA LEEHFRDDDD
 61 GPVSSQGYMP YLNKYILDKV EEGAFVKEHF DELCWTLTAK KNYRADSNGN SMLSNQDAFR
 121 LWCLFNFLSE DKYPLIMVPD EVEYLLKKVL SSMSLEVSLG ELEELLAQEA QVAQTTGGLS
 181 VWQFLELFNS GRCLRGVGRD TLSMAIHEVY QELIQDVLKQ GYLWKRGLR RNWAERWFQL
 241 QPSCLCYFGS EECKEKGII PLDAHCCVEV LPDRDGKRCM FCVKTANRTY EMSASDTRQR
 301 QEWTAAIQMA IRLQAEKTS LHKDLQKRR EQREQRERRR AAKEEELLRL QQLQEEKERK
 361 LQELELLQEA QRQAERLLQE EEERRRSQHR ELQQALEGQL REAEQARASM QAEMELKEEE
 421 AARQRRIKE LEEMQQLQE ALQLEVKARR DEESVRIAQT RLLEEEEEKL KQLMQLKEEQ
 481 ERYIERAQQE KEELQQEMAQ QSRSLQQAQQ QLEEVQRNRQ RADEDVEAAQ RKLRQASTNV
 541 KHWNVQMNRL MHPIEPGDKR PVTSSSFSGF QPPLLAHRDS SLKRLTRWGS QGNRTPSPNS
 601 NEQQKSLNGG DEAPAPASTP QEDKLDPAPE N

8.3 Sequencing results from cloning

8.3.1 GST-205-342 (GST-PH- (+NLS))

```
DEF6          VWQFLELFNSGRCLRGVGRDTLSMAIHEVYQELIQDVLKQGYLWKRGHLLRRNWAERWFQL 240
seq          ---WQATFGGGDHPKSDLVPRGSAIHEVYQELIQDVLKQGYLWKRGHLLRRNWAERWFQL 215
              :   *..*           . . . *****
DEF6          QPSCLCYFGSEECCKEKGRIIPLDAHCCVEVLPDRDGKRCMFCVKTANRTYEMSASDTRQR 300
seq          QPSCLCYFGSEECCKEKGRIIPLDAHCCVEVLPDRDGKRCMFCVKTATRTYEMSASDTRQR 275
              *****
DEF6          QEWTAAIQMAIRLQAEGKTSLHKDLKQKRREQREQRERRRAAKEEELLRLQQLQEEKERK 360
seq          QEWTAAIQMAIRLQAEGKTSLHKDLKQKRREQREQRERRRAAK----- 318
              *****
```

8.3.2 GST-205-342 Y210E Y222E

```
DEF6          VWQFLELFNSGRCLRGVGRDTLSMAIHEVYQELIQDVLKQGYLWKRGHLLRRNWAERWFQL 240
seq          ---WQATFGGGDHPKSDLVPRGSAIHEVYQELIQDVLKQGYLWKRGHLLRRNWAERWFQL 222
              :   *..*           . . . *****
DEF6          QPSCLCYFGSEECCKEKGRIIPLDAHCCVEVLPDRDGKRCMFCVKTANRTYEMSASDTRQR 300
seq          QPSCLCYFGSEECCKEKGRIIPLDAHCCVEVLPDRDGKRCMFCVKTATRTYEMSASDTRQR 282
              *****
DEF6          QEWTAAIQMAIRLQAEGKTSLHKDLKQKRREQREQRERRRAAKEEELLRLQQLQEEKERK 360
seq          QEWTAAIQMAIRLQAEGKTSLHKDLKQKRREQREQRERRRAAK----- 325
              *****
```

8.3.3 GST-205-315 (GST-PH- (-NLS))

```
DEF6          SVWQFLELFNSGRCLRGVGRDTLSMAIHEVYQELIQDVLKQGYLWKRGHLLRRNWAERWFQ 239
seq          ---WQATFGGGDHPKSDLVPRGSAIHEVYQELIQDVLKQGYLWKRGHLLRRNWAERWFQ 225
              :   *..*           . . . *****
DEF6          LQPSCLCYFGSEECCKEKGRIIPLDAHCCVEVLPDRDGKRCMFCVKTANRTYEMSASDTRQ 299
seq          LQPSCLCYFGSEECCKEKGRIIPLDAHCCVEVLPDRDGKRCMFCVKTATRTYEMSASDTRQ 285
              *****
DEF6          RQEWTAAIQMAIRLQAEGKTSLHKDLKQKRREQREQRERRRAAKEEELLRLQQLQEEKER 359
seq          RQEWTAAIQMAIRLQA----- 301
              *****
```

8.3.4 GST-313-550 (GST-CC)

```
DEF6          QEWTAAIQMAIRLQAEGKTSLHKDLKQKRREQREQRERRRAAKEEELLRLQQLQEEKERK 360
seq          -----LQAEGKTSLHKDLKQKRREQREQRERRRAAKEEELLRLQQLQEEKERK 73
              *****
DEF6          LQELELLQEAQRQAERLLQEEEEERRRSQHRELQQALEGQLREAEQARASMQAEMELKEEE 420
seq          LQELELLQEAQRQAERLLQEEEEERRRSQHRELQQALEGQLREAEQARASMQAEMELKEEE 133
              *****
DEF6          AARQRQRIKELEEMQQLQALQLEVKARRDEESVRIAQTFLLEEEEEKCLKQLMQLKEEQ 480
```

```

seq      AARQRQRIKELEEMQQRLQEALQLEVKARRDEESVRIAQTRLLEEEEEKCLKQLMQLKEEQ 193
*****

DEF6     ERYIERAQQEKEELQQEMAQQSRSLQQAQQQLEEVQRNRQRADEDVEAAQRKLRQASTNV 540
seq      ERYIERAQQEKEELQQEMAQQSRSLQQAQQQLEEVQRNRQRADEDVEAAQRKLRQASTNV 253
*****

DEF6     KHWNVQMNRLMHPIEPGDKRPVTSSSFSGFQPPLLAHRDSSLKRLTRWGSQGNRTPSPNS 600
seq      KHWNVQMNRL---EFKGEFHIGRCSPG---DPLVLERP-----HRGSSISPYSESYR 300
*****      * .: : .* . ** : .* * .* : *

DEF6     NEQQKSLNGGDEAPAPASTPQEDKLDPAPE 631
seq      AAHWPSFYNVRDWKGPLSLPNLIALEHIPP- 330
          : * : . : * * * : * : *

```

8.3.5 GST-CCm1

Q371P A374P

```

seq      -----LQAEGKTSLHKDLKQKRREQREQRERRRAAKEEELLRLQQLQEEKERK 144
def6     QEWTAAIQMAIRLQAEGKTSLHKDLKQKRREQREQRERRRAAKEEELLRLQQLQEEKERK 360
*****

seq      LQELELLQEAPRQPERLLQEEEEERRRSQHRELQQALEGQLREAEQARASMQAEMELKEEE 204
def6     LQELELLQEAQRQAERLLQEEEEERRRSQHRELQQALEGQLREAEQARASMQAEMELKEEE 420
*****      *.*****

seq      AARQRQRIKELEEMQQRLQEALQLEVKARRDEESVRIAQTRLLEEEEEKCLKQLMQLKEEQ 264
def6     AARQRQRIKELEEMQQRLQEALQLEVKARRDEESVRIAQTRLLEEEEEKCLKQLMQLKEEQ 480
*****

seq      ERYIERAQQEKEELQQEMAQQSRSLQQAQQQLEEVQRNRQRADEDVEAAQRKLRQASTNV 324
def6     ERYIERAQQEKEELQQEMAQQSRSLQQAQQQLEEVQRNRQRADEDVEAAQRKLRQASTNV 540
*****

seq      KHWNVQMNRL-----EFPGRLER--PHRD-LTICLARFVK----- 356
def6     KHWNVQMNRLMHPIEPGDKRPVTSSSFSGFQPPLLAHRDSSLKRLTRWGSQGNRTPSPNS 600
*****      .*. * .*** *: *: .

```

8.3.6 GST-CCm2

I428P L431P

```

seq      -----LQAEGKTSLHKDLKQKRREQREQRERRRAAKEEELLRLQQLQEEKER 180
def6     RQEWTAAIQMAIRLQAEGKTSLHKDLKQKRREQREQRERRRAAKEEELLRLQQLQEEKER 359
*****

seq      KLQELELLQEAQRQAERLLQEEEEERRRSQHRELQQALEGQLREAEQARASMQAEMELKEE 240
def6     KLQELELLQEAQRQAERLLQEEEEERRRSQHRELQQALEGQLREAEQARASMQAEMELKEE 419
*****

seq      EAARQRQRPKEPEEMQQRLQEALQLEVKARRDEESVRIAQTRLLEEEEEKCLKQLMQLKEE 300
def6     EAARQRQRIKELEEMQQRLQEALQLEVKARRDEESVRIAQTRLLEEEEEKCLKQLMQLKEE 479

```

```

***** ** *****
seq      QERYIERAQQEKEELQQEMAQQSRSLQQAQQQLLEEVQRNRQRADEDVEAAQRKLRQANTN 360
def6     QERYIERAQQEKEELQQEMAQQSRSLQQAQQQLLEEVQRNRQRADEDVEAAQRKLRQASTN 539
          *****

```

```

seq      VKHWNVQMNRL-----EFPGRLER--PHRD----LTICLALTVR----- 393
def6     VKHWNVQMNRLMHPIEPGDKRPVTSSSFSGFQPPLLAHRDSSLKRLTRWGSQGNRTSPN 599
          *****

```

8.3.7 GST-CCm3

L470P L473P

```

seq      -----LQAEGKTSLHKDLKQKRREQREQRERRRAAKEEELLRLQQLQEEKERK
76
def6     QEWTAAIQMAIRLQAEGKTSLHKDLKQKRREQREQRERRRAAKEEELLRLQQLQEEKERK
360

```

```

seq      LQELELLQEAQRQAERLLQEEEEERRRSQHRELQQALEGQLREAEQARASMQAEMELKEEE
136
def6     LQELELLQEAQRQAERLLQEEEEERRRSQHRELQQALEGQLREAEQARASMQAEMELKEEE
420

```

```

seq      AARQRQRIKELEEMQQRLQEALQLEVKARRDEESVRIAQTRLLEEEEEKPKQPMQLKEEQ
196
def6     AARQRQRIKELEEMQQRLQEALQLEVKARRDEESVRIAQTRLLEEEEEKLMQLKEEQ
480

```

***** ** *****

```

seq      ERYIERAQQEKEELQQEMAQQSRSLQQAQQQLLEEVQRNRQRADEDVEAAQRKLRQASTNV
256
def6     ERYIERAQQEKEELQQEMAQQSRSLQQAQQQLLEEVQRNRQRADEDVEAAQRKLRQASTNV
540

```

```

seq      KHWNVQMNRL-----EFPGRLER--PHRD-LTICLGAFSV----- 288
def6     KHWNVQMNRLMHPIEPGDKRPVTSSSFSGFQPPLLAHRDSSLKRLTRWGSQGNRTSPNS 600
          *****

```

8.3.8 GST-CCm4

L512P V515P

```

def6     QEWTAAIQMAIRLQAEGKTSLHKDLKQKRREQREQRERRRAAKEEELLRLQQLQEEKERK 360
seq      -----LQAEGKTSLHKDLKQKRREQREQRERRRAAKEEELLRLQQLQEEKERK 94

```

```

def6     LQELELLQEAQRQAERLLQEEEEERRRSQHRELQQALEGQLREAEQARASMQAEMELKEEE 420
seq      LQELELLQEAQRQAERLLQEEEEERRRSQHRELQQALEGQLREAEQARASMQAEMELKEEE 154

```

```

def6     AARQRQRIKELEEMQQRLQEALQLEVKARRDEESVRIAQTRLLEEEEEKLMQLKEEQ 480
seq      AARQRQRIKELEEMQQRLQEALQLEVKARRDEESVRIAQTRLLEEEEEKLMQLKEEQ 214

```

```

*****

def6      ERYIERAQQEKEELQQEMAQQSRSLQQAQQQLEEVRQNRQRADEDVEAAQRKLRQASTNV 540
seq       ERYIERAQQEKEELQQEMAQQSRSLQQAQQQEEPRQNRQRADEDVEAAQRKLRQASTNV 274
*****

def6      KHWNVQMNRLMHPIEPGDKRPVTSSSFSGFQPPLLAHRDSSLKRLTRWGSQGNRTPSPNS 600
seq       KHWNVQMNRL----- 284
*****

```

8.3.9 GST-CCAttrigger

```

seq       -----LQAEGKTSLHKDLKQKRREQREQRERRRAAKEEELLRLQQLQEEKERK 156
def6      QEWTAAIQMAIRLQAEGKTSLHKDLKQKRREQREQRERRRAAKEEELLRLQQLQEEKERK 360
*****

seq       LQELELLQEAQRQAERLLQEEEEERRRSQHRELQQALEGQLREAEQARASMQAEMELKEEE 216
def6      LQELELLQEAQRQAERLLQEEEEERRRSQHRELQQALEGQLREAEQARASMQAEMELKEEE 420
*****

seq       AARQQRRIKELEEMQQRLQEA-----RIAQTRLLEEEEEKQQLMQLKEEQ 262
def6      AARQQRRIKELEEMQQRLQEALQLEVKARRDEESVRIAQTRLLEEEEEKQQLMQLKEEQ 480
*****

seq       ERYIERAQQEKEELQQEMAQQSRSLQQAQQQLEEVRQNRQRADEDVEAAQRKLRQASTNV 322
def6      ERYIERAQQEKEELQQEMAQQSRSLQQAQQQLEEVRQNRQRADEDVEAAQRKLRQASTNV 540
*****

seq       KHWNVQMNRL----- 332
def6      KHWNVQMNRLMHPIEPGDKRPVTSSSFSGFQPPLLAHRDSSLKRLTRWGSQGNRTPSPNS 600
*****

```

8.3.10 GFP-DEF6-m1

Q371P A374P

```

seq       -----DWAERWFQL 9
def6      VWQFLELFNSGRCLRGVGRDTLSMAIHEVYQELIQDVLKQGYLWKRGHLLRRNWAERWFQL 240
*****

seq       QPSCLCYFGSEECKEKGRIIPLDAHCCVEVLPDRDGKRCMFCVKTATRTYEMSASDTRQR 69
def6      QPSCLCYFGSEECKEKGRIIPLDAHCCVEVLPDRDGKRCMFCVKTANRTYEMSASDTRQR 300
*****

seq       QEWTAAIQMAIRLQAEGKTSLHKDLKQKRREQREQRERRRAAKEEELLRLQQLQEEKERK 129
def6      QEWTAAIQMAIRLQAEGKTSLHKDLKQKRREQREQRERRRAAKEEELLRLQQLQEEKERK 360
*****

seq       LQELELLQEAPRQPERLQLQEEEEERRRSQHRELQQALEGQLREAEQARASMQAEMELKEEE 189

```

```

def6      LQELELLQEAQRQAERLLQEEEEERRRSQHRELQQALEGQLREAEQARASMQAEMELKEEE 420
          ***** ** . *****

seq       AARQQRRIKELEEMQQRLQEALQLEVKARRDEESVRIAQTRLLEEEEEKCLKQLMQLKEEQ 249
def6     AARQQRRIKELEEMQQRLQEALQLEVKARRDEESVRIAQTRLLEEEEEKCLKQLMQLKEEQ 480
          *****

seq       ERYIERAQXEKEELQXEMAQQXRSLQQAQQQLEEXVRQNRQRADEDVEAAQRKLRQASTNV 309
def6     ERYIERAQXEKEELQXEMAQQXRSLQQAQQQLEEVQRNRQRADEDVEAAQRKLRQASTNV 540
          *****

seq       KHWNVQMX----- 317
def6     KHWNVQMNRLMHPIEPGDKRPVTSSSFSGFQPPLLAHRDSSLKRLTRWGSQGNRTPSPNS 600
          *****

seq       -----XADASN----- 323
def6     NEQQKSLNGGDEAPAPASTPQEDKLD PAPEN 631

```

8.3.11 GFP-DEF6-m2

I428P L431P

```

seq       -----XXXXXXXXXERREQREQRERRRAAKEEELLRLQQLQEEKERK 38
def6     QEWTAAIQMAIRLQAEGKTSLHKDLKQKRREQREQRERRRAAKEEELLRLQQLQEEKERK 360
          : *****

seq       LQELELLQEAQRQAERLLQEEEEERRRSQHRELQQALEGQLREAEQARASMQAEMELKEEE 98
def6     LQELELLQEAQRQAERLLQEEEEERRRSQHRELQQALEGQLREAEQARASMQAEMELKEEE 420
          *****

seq       AARQQRRIKEPEEMQQRLQEALQLEVKARRDEESVRIAQTRLLEEEEEKCLKQLMQLKEEQ 158
def6     AARQQRRIKELEEMQQRLQEALQLEVKARRDEESVRIAQTRLLEEEEEKCLKQLMQLKEEQ 480
          *****

seq       ERYIERAQXEKEELQXEMAQQXRSLQQAQQQLEEXVRQNRQRADEDVEAAQRKLRQASTNV 218
def6     ERYIERAQXEKEELQXEMAQQXRSLQQAQQQLEEVQRNRQRADEDVEAAQRKLRQASTNV 540
          *****

seq       KHWNVQMNRLMHPIEPGDKRPVTSSSFSGFQPPLLAHRDSSLKRLTRWGSQGNRTPSPNS 278
def6     KHWNVQMNRLMHPIEPGDKRPVTSSSFSGFQPPLLAHRDSSLKRLTRWGSQGNRTPSPNS 600
          *****

seq       NEQQKSLNGGDEAPAPASTPQEDKLD PAPEN-PLLXPCSSQCHIHQDXXTAXPVXDPSXY 337
def6     NEQQKSLNGGDEAPAPASTPQEDKLD PAPEN----- 631
          *****

```

8.3.12 GFP-DEF6-m3

L470P L473P

```

seq       -----XXXXXXXXXERREQREQRERRRAAKEEELLRLQQLQEEKERK 43
def6     QEWTAAIQMAIRLQAEGKTSLHKDLKQKRREQREQRERRRAAKEEELLRLQQLQEEKERK 360

```

```

: *****
seq      LQELELLQEAQRQAERLLQEEERRRSQHRELQQALEGQLREAEQARASMQAEMELKEEE 103
def6     LQELELLQEAQRQAERLLQEEERRRSQHRELQQALEGQLREAEQARASMQAEMELKEEE 420
          *****

seq      AARQRRIKELEEMQQRQLQEALQLEVKARRDEESVRIAQTRLLEEEEEKPKQFMQLKEEQ 163
def6     AARQRRIKELEEMQQRQLQEALQLEVKARRDEESVRIAQTRLLEEEEEKPKQFMQLKEEQ 480
          *****

seq      ERYIERAQQEKEELQQEMAQQSRSLSLQQAQQQLLEEVQRNRQRADEDVEAAQRKLRQASTNV 223
def6     ERYIERAQQEKEELQQEMAQQSRSLSLQQAQQQLLEEVQRNRQRADEDVEAAQRKLRQASTNV 540
          *****

seq      KHWNVQMNRLMHPPIEPGDKRPVTSSSFSGFQPPLLAHRDSSLKRLTRWGSQGNRTSPNS 283
def6     KHWNVQMNRLMHPPIEPGDKRPVTSSSFSGFQPPLLAHRDSSLKRLTRWGSQGNRTSPNS 600
          *****

seq      NEQQKSLNGGDEAPAPASTPQEDKLD PAPEN-PLLAPCSSQCHIHQDLXXSXPVGD PSSY 342
def6     NEQQKSLNGGDEAPAPASTPQEDKLD PAPEN----- 631
          *****

```

8.3.13 GFP-DEF6-m4

L512P V515

```

seq      -----XXXXXAERREQREQRRRAAKEEELLRLQQLQEEKERK 39
def6     QEWTAAIQMAIRLQAEGKTSLHKDLKQKRREQREQRRRAAKEEELLRLQQLQEEKERK 360
          : *****

seq      LQELELLQEAQRQAERLLQEEERRRSQHRELQQALEGQLREAEQARASMQAEMELKEEE 99
def6     LQELELLQEAQRQAERLLQEEERRRSQHRELQQALEGQLREAEQARASMQAEMELKEEE 420
          *****

seq      AARQRRIKELEEMQQRQLQEALQLEVKARRDEESVRIAQTRLLEEEEEKPKQFMQLKEEQ 159
def6     AARQRRIKELEEMQQRQLQEALQLEVKARRDEESVRIAQTRLLEEEEEKPKQFMQLKEEQ 480
          *****

seq      ERYIERAQQEKEELQQEMAQQSRSLSLQQAQQQPEEPQRNRQRADEDVEAAQRKLRQASTNV 219
def6     ERYIERAQQEKEELQQEMAQQSRSLSLQQAQQQLEEVRQRNRQRADEDVEAAQRKLRQASTNV 540
          *****

seq      KHWNVQMNRLMHPPIEPGDKRPVTSSSFSGFQPPLLAHRDSSLKRLTRWGSQGNRTSPNS 279
def6     KHWNVQMNRLMHPPIEPGDKRPVTSSSFSGFQPPLLAHRDSSLKRLTRWGSQGNRTSPNS 600
          *****

seq      NEQQKSLNGGDEAPAPASTPQEDKLD PAPEN-PLLAPCSSQCHIHQDLXTAXLWVIPALT 338
def6     NEQQKSLNGGDEAPAPASTPQEDKLD PAPEN----- 631
          *****

```

8.3.14 GFP-N-term

Stop codon inserted after amino acid 109

Met A L R K E L L K S I W Y A F T A L D V E K S G K V S K S Q L K V L S H
N L Y T V L H I P H D P V A L E E H F R D D D D G P V S S Q G Y M P Y L N

K Y I L D K V E E G V F V K E H F D E L C W T L T A K K N Y R A D S N

Stop

```
sequence      XXLGMDELYKSGRTQISSSSFEFCRRYPAMALRKELLKSIWYAFTALDVEKSGKVSQSQ 60
def6          -----MALRKELLKSIWYAFTALDVEKSGKVSQSQ 30
                *****
```

```
sequence      LKVLSHNLYTVLHIPHDPVALEEHRDDDDGVPSSQGYMPYLNKYILDKVEEGVFVKEHF 120
def6          LKVLSHNLYTVLHIPHDPVALEEHRDDDDGVPSSQGYMPYLNKYILDKVEEGAFVKEHF 90
                *****.
```

```
sequence      DELCWTLTAKKNYRADSNSMLSNQDAFRLWCLFNFLSEDKYPLIMVPDEVEYLLKKVL 179
def6          DELCWTLTAKKNYRADSNSMLSNQDAFRLWCLFNFLSEDKYPLIMVPDEVEYLLKKVL 150
                *****
```

8.3.15 GFP-DEF6-Δ-trigger

```
seq           -----RGKTSYTR---LSRTARSSGSSGSGAGAKKRAALQQEQEEKERK 89
def6          QEWTAAIQMAIRLQAEGKTSLHKDLKQKRREQREQRRRAAKEEELLRLQQEQEEKERK 360
                .**** : . * : . . * . : . *****
```

```
seq           LQELELLQEAQRQAERLLQEEEEERRRSQHRELQQALEGQLREAEQARASMQAEMELKEEE 149
def6          LQELELLQEAQRQAERLLQEEEEERRRSQHRELQQALEGQLREAEQARASMQAEMELKEEE 420
                *****
```

```
seq           AARQRRIKELEEMQQRLEQA-----RIAQTRLLEEEEEKLKQLMQLKEEQ 195
def6          AARQRRIKELEEMQQRLEQA LQLEVKARRDEESVRIAQTRLLEEEEEKLKQLMQLKEEQ 480
                *****
```

```
seq           ERYIERAQQEKEELQQEMAQQSRSLSLQQAQQQLEEVQRNRQRADEDVEAAQRKLQASTNV 255
def6          ERYIERAQQEKEELQQEMAQQSRSLSLQQAQQQLEEVQRNRQRADEDVEAAQRKLQASTNV 540
                *****
```

```
seq           KHWNVQMNRLMHPIEPGDKRPVTSSSFSGFQPPLLAHRDSSLKRLTRWGSQGNRTPSPNS 315
def6          KHWNVQMNRLMHPIEPGDKRPVTSSSFSGFQPPLLAHRDSSLKRLTRWGSQGNRTPSPNS 600
                *****
```

```
seq           NEQQKSLNGGDEAPAPASTPQEDKLDPAPE 346
def6          NEQQKSLNGGDEAPAPASTPQEDKLDPAPE 631
                *****
```

8.4 **DDX6 forms granules in Jurkat cells**

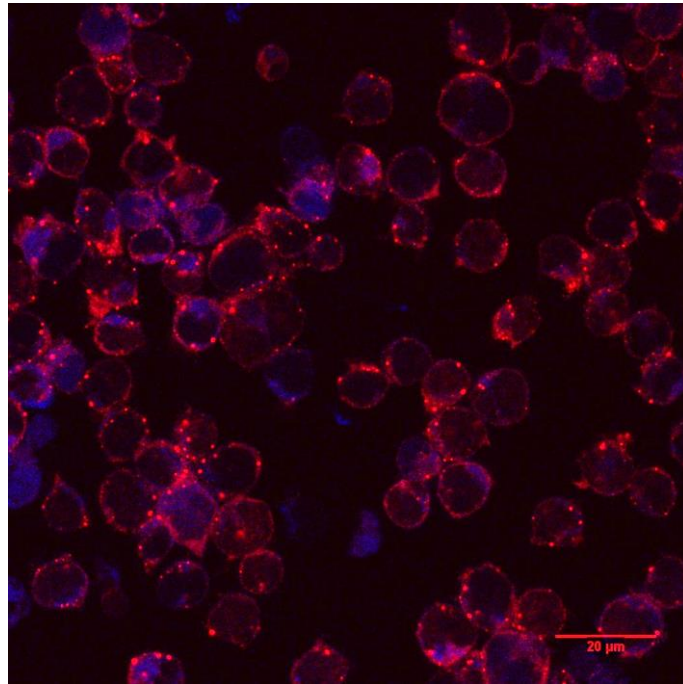


Figure 91 - DDX6 forms granules in Jurkat cells. Jurkat cells were stained with calcein (blue) and probed for DDX6 (red) using antibody. A single Z stack was botained using a Zeis LSM710 confocal microscope.

8.5 **Xrn1 forms granules in Jurkat cells**

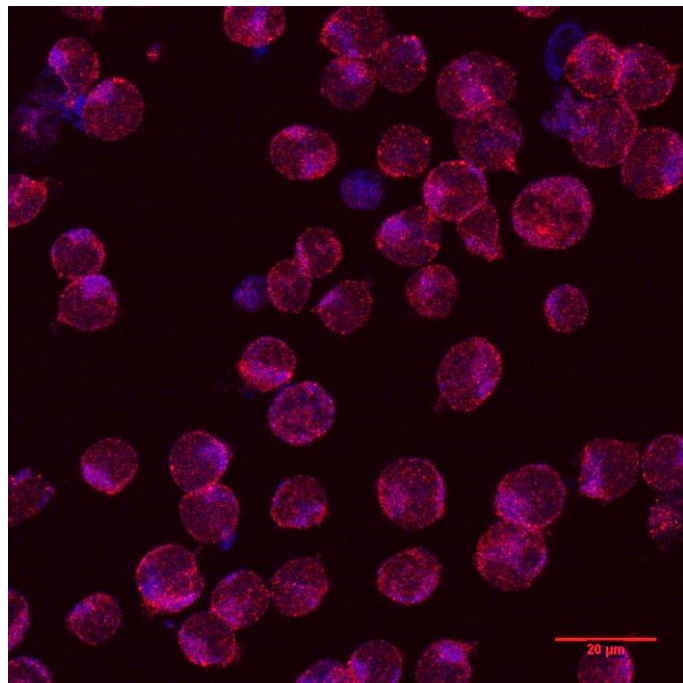


Figure 92 – Xrn1 forms granules in Jurkat cells. Jurkat cells were stained with calcein (blue) and probed for Xrn1 (red) using antibody. A single Z stack was botained using a Zeis LSM710 confocal microscope.

8.6 ConSurf Multiple Sequence Alignment

```

AGAP012332_AGambiae  - - - - - M A T L L R N V T N I C H A F N A L Q Q N - R D G F V S K S K L K V L T A N I
DEF6_MMusculus       - - - - - M A L R K E L L K I W Y A F T A L D V E - K S G K V S K S Q L K V L S H N L
LOC585280_SPurpuratus - - - - - M A R E C V P R L W H A F D A L D N D - K N G S A P K T E L K V L T A H I
TRIADDRAFT_TAdhearens M T T T I H N T S Q L K N L P D V Q T R A L L Y A F S V I D D E S N D G K A N H S Q I S V I C Q S I
DEF6_XLaevis         - - - - - M S L R T E L L K I W Y A F T S L D T E - K S G K V S K S Q L K V L S H N L
DEF6_GGallus         - - - - - M D L R A E L L K I W Y A F T A L D V E - K S G K V S K S Q L K V L S H N L
DEF6_DRerio          - - - - - M D L R A E L L K I W Y A F T A L D V E - K S G K V S K S Q L K V L S H N L
SWAP70_HSapiens      - - - - - M G S L K E L L K I W H A F T A L D Q D - H S G K V S K S Q L K V L S H N L
DEF6_HSapiens        - - - - - M A L R K E L L K I W Y A F T A L D V E - K S G K V S K S Q L K V L S H N L

```

```

AGAP012332_AGambiae  A T L L D L Y - G V E R G L D H F R - - S T A T L N F E H Y R Y Y L A Q K V F A G V S N E L P L A
DEF6_MMusculus       Y T V L N I P - H D P V A L E E H F R D D D D G P V S S Q G Y M P Y L N K Y I L D - - - - - K V E
LOC585280_SPurpuratus G N S L G A E N S N A I A K Q L M E T F Q D S P A I S F D D Y R H F L N K E F L - - - - - V
TRIADDRAFT_TAdhearens E S E F R T G - - F F F D R L Y N Y A D D Q K R Y D F L S L K K Y L E E C L D - - - - - R
DEF6_XLaevis         Y T V L C I P - H D P V A L E D H F R D D D D G P V S S Q G Y M P Y L N K Y I L D - - - - - K A V
DEF6_GGallus         Y T V L C I P - H D P V A L E E H F R D D D D G P V S S Q G Y M P Y L N K Y I L D - - - - - K V E
DEF6_DRerio          Y T V L K I P - H E A A A L E E H F R D D D D G P V S S Q G Y M P Y L N K Y I L D - - - - - K V V
SWAP70_HSapiens      C T V L K V F - H D S V A L E E H F R D D D D G P V S N Q G Y M P Y L N R F I L E - - - - - K V Q
DEF6_HSapiens        Y T V L H I P - H D P V A L E E H F R D D D D G P V S S Q G Y M P Y L N K Y I L D - - - - - K V E

```

```

AGAP012332_AGambiae  A L R N Y E T K I D E V C W L V C R M N - P P A N D Q Y Y T E E A V T K L F R I F C L V A D L S T D
DEF6_MMusculus       E G A F V K E H F D E L C W T L T A K K N Y R A D G I G S S P L S N Q D A F R L W C L F N F P S E D
LOC585280_SPurpuratus L E D M D L V K L E N Y S W M I C G S R Y S K N T - - K K V L D H T E I Y K M N R I F N F F A E P
TRIADDRAFT_TAdhearens I P R F D D S K F Y K V C W F Y R N H D S D E M - - V T K L K A Q Q L F K L W Q I C N V I N D Q
DEF6_XLaevis         E G T F V K E S L H E D C W T L T A K K N R H V - - Q T A L S N D A F H L W C L F N F P S E D
DEF6_GGallus         E G A F V K E N F D E L C W T L T A K K N Y R P D R N G S V V S H Q D A F K L W C L F N F P S E D
DEF6_DRerio          E G T F V K E N V D E L C W T L T A K K N R E E - - Q K S I L P A D A F R L W C L F I F P S E D
SWAP70_HSapiens      D - N F D K I E F N A M C W T L C V K K N L T E N - - P L L I T E R D A F K I W V I F N F P S E D
DEF6_HSapiens        E G A F V K E H F D E L C W T L T A K K N R A D S N G S M L S N Q D A F R L W C L F N F P S E D

```

```

AGAP012332_AGambiae  A S E P D L Y T V K I H P M E A L I I I K H L L N A L G L N Y D G D G K Y D Y L R T S E E S - - -
DEF6_MMusculus       K - - - - Y P L I M V P D E V E Y L L K K L L G S L S L E M G L G E L E E L L A Q D A Q S A Q T A
LOC585280_SPurpuratus G - - - - V F P A I L D P G E A N I M F E K L V V A M G T K W N L E E P Q S K V A V L Q D - - - -
TRIADDRAFT_TAdhearens T - - - - N C T T I A M V D L V P V F Q K I A R T L G V Q W N A D E F Y L H P - - - - -
DEF6_XLaevis         S - - - - Y P V I M V A D E V Q Y L L Q K L S I A L H E V T E V E L G D V L S S L R P - - - -
DEF6_GGallus         K - - - - Y P L I M V P D E V E Y L L K K I G T A M N V E L N S G E L D D C L S Q E F P Q - - - G Q
DEF6_DRerio          R - - - - Y P L V M I P D E V E Y L L K K F C M A M S V E L N Y V E L E D F I S Q D S V Q - - - Q
SWAP70_HSapiens      K - - - - Y P L I I V S E E I E Y L L K K L T E A M G G G W Q Q E Q F E N Y K I N F D D S - - - K
DEF6_HSapiens        K - - - - Y P L I M V P D E V E Y L L K K V L S S M S L E V S L G E L E E L L A Q E A Q V A Q T T

```

```

AGAP012332_AGambiae  - - L E F G E L L E M L R F K N Y D K V N O Y R E S I C E A I G D M Y Q T L I R D V I K K G Y L V R
DEF6_MMusculus       V G L S V W Q P L E L F N S G R C L R - V G R D S L S M A I Q E V Y Q E L I Q D V L K K G Y L W K
LOC585280_SPurpuratus - - L R F T N C L Q F P E Q H Y T G N V - - D S M C V K E G I S E V Y N N M I E V E K M G H L K K
TRIADDRAFT_TAdhearens R R A D F N K V I D E F G C G L P V S V - - D E L I E E I V C N L Y D F Y V L N I I K V G F L K K
DEF6_XLaevis         - V V T V W E F L Q V M T S P K F L R S - M S T E T L S I A I Q D L Y E E V I Q D V L K K G Y L L K
DEF6_GGallus         Q G L T V W Q P L D M V N S G R L L R Q - I E Q E A I S M A V E E V Y Q E V I S D V L K K G Y L W K
DEF6_DRerio          W Q F T V W T P L E M M N S C K L T R Q - I A K E T V S M A I E E V Y R E I V D V L K S G Y L W K
SWAP70_HSapiens      W G L S A W E L I E L I G N Q F S K Q - M D R Q T V S M A I N E V F N E L I L D V L K K G Y M K K
DEF6_HSapiens        Q G L S V W Q P L E L F N S G R C L R - - V O R D T L S M A I H E V Y Q S L I Q D V L K K G Y L W K

```

```

AGAP012332_AGambiae  R G Y V L P T F R E Y F F V L Q P C E L S Y Y K H P A D R E V C G T I V L D S S F M V K P A G N P S
DEF6_MMusculus       R G H L R R N W A E R W F Q L Q P S S A C Y F G S E E C K E K R G T I P L D A H C C V E V L P D - -
LOC585280_SPurpuratus K G H K V T N W K D R W F V L K P G S M A Y F V E K T L K E Q K G E I V I T T K W S V E V L P D - -
TRIADDRAFT_TAdhearens R G H R M T S I R D R Y F I L K H N V L E Y Y T E N - L R E K R G S I A I N Q Y T T V E S K P A - -
DEF6_XLaevis         K A N L R N T W T E R W F I L K P S S G Y Y L S E E C K E R K G I I P I D K D C G V E I L P D - -
DEF6_GGallus         K G Q L R R N W S E R W F M L K P S A S Y Y M S E E R K E K K G S I P L D K K C C V E V L P D - -
DEF6_DRerio          K G Q L R R N W T E R W F T L R P S T A L Y P T S E D K D H K G N I Q L D G N C G V E V L P D - -
SWAP70_HSapiens      K G H R R K N W T E R W F V L K P N I S Y Y V S E D L K D K K G D I L L D E M C C V E S L P D - -
DEF6_HSapiens        R G H L R R N W A E R W F Q L Q P S C A C Y F G S E E C K E K R G I I P L D A B C C V E V L P D - -

```

AGAP012332_AGambiae
 DEF6_MMusculus
 LOC585280_SPurpuratus
 TRIADDRAFT_TAdhearens
 DEF6_XLaevis
 DEF6_GGallus
 DEF6_DRerio
 SWAP70_HSapiens
 DEF6_HSapiens

S G K Q E K V Q R F T L V S G D - - - - - R T F E L T P D H K T R L Q W I A A L Q L A I T Y S
 - - R E G K R C M F C V K T A S - - - - - R T F E M A S D T R Q R Q E W T A A I Q T A I R L Q
 - - Q K G N K N L L V V Y S E D T S Q V N T K K R Y E L A S D P R N R Q E W V T A I E N V L K R M
 - - - E G K Y W F A V T D N T G - - - - - I V D I Y A V S Y R L K I D W I Y A I Q T A I A A N
 - - R D G R C M F C V K T S - - - - - K T F E M A S D T K H R Q E W V T A I Q T A I R L Q
 - - R D G R C M F C V K T S - - - - - R T F E M A S D T R Q R Q E W T A A I Q T A I R L Q
 - - R D G R C M F C L K T L T - - - - - K T F E L A S D T K Q R Q E W T A I Q T A I R L Y
 - - K D G K C L F L V K C F D - - - - - K T F E I A S D K K K Q E W I Q A I H S T I H L L
 - - R D G R C M F C V K T A N - - - - - R T F E M A S D T R Q R Q E W T A A I Q M A I R L Q

AGAP012332_AGambiae
 DEF6_MMusculus
 LOC585280_SPurpuratus
 TRIADDRAFT_TAdhearens
 DEF6_XLaevis
 DEF6_GGallus
 DEF6_DRerio
 SWAP70_HSapiens
 DEF6_HSapiens

- T G R E G P Q R D T V N R R K Q Q R - - A E Q R R R - - - - - - - - - - - - - - -
 A E G K T S L H K D L K Q K R R E Q R - - Q R E R R R A A K E E E L R L Q Q L Q E E K E R K L Q E
 E C G E S P H V Q E L E R R R D E R R - - K K E S A E E I D R L R Q L Q V E N E R Q R Q R L A E M
 Q K K K L P A Q I H I E L D R E M A R G A L A L Q L K E I Q A K Y K A L L T V Q T D L A M E S V A
 Q S G S L S L H R E L Q M R R R E Q R - - L E E Q R R A A P E L E M Q R L A E L Q N E K E R Q Q E
 A E G K K S L H K D L K Q K R R E Q R - - Q R E Q R K A A K E E E T Q R L K Q L Q E E K E R K L Q E
 V E G K T S L H K D L K L K R R D Q R - - Q R E R R R A A K E Q E L Q R L R A L Q E E R E R K M A E
 K L G S P P H K E A R Q R K - - - - - L K K N Q L A E Q E E L R Q M K E L Q A A N E S K Q E
 A E G K T S L H K D L K Q K R R E Q R - - Q R E R R R A A K E E E L R L Q Q L Q E E K E R K L Q E

AGAP012332_AGambiae
 DEF6_MMusculus
 LOC585280_SPurpuratus
 TRIADDRAFT_TAdhearens
 DEF6_XLaevis
 DEF6_GGallus
 DEF6_DRerio
 SWAP70_HSapiens
 DEF6_HSapiens

- - - - - - - - - - - - - - - - E E E E Q L R S Q H L R R L E E T H V Q L E R E K Q A R L A E S Q
 L E L L Q E A Q R Q A R R L Q E E E E R S Q E K E L Q Q A L E G Q L R E A E Q A R A S M Q A E
 E R L R D E A A S K A E E E C A D R M A N E R E E E E N R R R E L E E R A K T Q A E I D A L K A A R
 R A V A E V N L I E A E E Q R E A E K Q L H A E R E I R L K A E E E N Q E H K Q R L D S H A K E I
 L E Q L R E A Q K K S E E I M L Q E Q B H R E Q Q E E M K R Q L E C Q L R E A E E A R A S M Q A E
 L E L L K E A Q R Q A R I L L Q E E E Q R R Q Q E E M Q R T L E I Q L Q E A E Q A R A S M Q A E
 L E L L K E A Q R Q A Q A M L E Q D E Q R R R Q Q E E Q L H Q A L E I Q L K E A E E A R A S M Q A E
 L E A V R K K E E A A S S A A E E E K R L Q T Q V E L Q A R F S T E L E R E K L I R Q Q M E E Q
 L E L L Q E A Q R Q A R R L Q E E E E R R S Q H R E L Q Q A L E G Q L R E A E Q A R A S M Q A E

AGAP012332_AGambiae
 DEF6_MMusculus
 LOC585280_SPurpuratus
 TRIADDRAFT_TAdhearens
 DEF6_XLaevis
 DEF6_GGallus
 DEF6_DRerio
 SWAP70_HSapiens
 DEF6_HSapiens

A N Q Y E A V A R E D S R R V A E L E D V K L C L E K M L E D E I Q A K R D E E I V N A L Q A R V L
 M L L K K K E A A R Q R Q R I A E L E E M Q E R L Q E A L Q L E V K A R R D E E A V R L A Q T R L L
 D V A R K A E E L A M R M E R E L E B A K N R V R E M E D R A R T Q E I D Q L T I A R D E A A
 Q A L Q E A N V E K Q A K L A R E Q K K S E E L L Q K M L V N E E M L R R M E A E K T D L E Q A V A
 M Q L K E T E A V Q Q K Q R I Q E L E Q L Q D H L Q E A L A Q E I T A R A R D E E A F R H A Q S K L L
 M V L K E E E A E R Q R K R I M E L E E M Q E R L Q E A L Q Q E V K A R R D E E A V R Y A Q A R L L
 M A L K E A E A E K Q R T R I R E L E A M Q Q R L E D A L Q Q E I K A R Q D E E A F R Y A Q A R L L
 V A Q K S S E L E Q Y L Q R V R E L E D M Y L K L Q E A L E D E R Q A R Q D E E T V R K L Q A R L L
 M L K E E E A A R Q R Q R I K E L E E M Q Q R L Q E A L Q L E V K A R R D E E A V R I A Q T R L L

AGAP012332_AGambiae
 DEF6_MMusculus
 LOC585280_SPurpuratus
 TRIADDRAFT_TAdhearens
 DEF6_XLaevis
 DEF6_GGallus
 DEF6_DRerio
 SWAP70_HSapiens
 DEF6_HSapiens

A E E - - - E K K E E L R Q L Q A E Q K S L L E Q E R Q K R M E F E H R Q Q E N E Q R L E A E G
 E E E - - - E K L K Q L M H L K E E Q E R Y I S R A Q Q E K Q E L Q Q E M A L Q S R E L Q H A Q Q
 R K A - - - E E L A L R M A K E E E R E N R R R E E E H R E K T Q Q S I D A L R A A E E A E R
 E N Q G L L K I K T E E L K K I Q E E Q E S N L R E E K E L K E K L E V I T D A K E K - - A K S F L
 M Q E - - - E K L R V L E R M R E E Q T Q Y V E R A Q K E K Q E L Q Q E M A V T S K E L Q E A Q K
 A E E - - - E K L K Q L M K L K E E Q E E V I I K T Q L E K Q V L K Q E M E N K N K C L E E A Q K
 A E E - - - E K M K A L M G L R E E Q E E V I Q R A Q R E K Q E L R Q E M S K S R A L E A Q R
 E E E - - - S K R A E L E K M H L E Q Q A I Q T T S A E K Q E L E N Q R V L K E Q A L Q E A M E
 E E E - - - E K L K Q L M Q L K E E Q E R Y I S R A Q Q E K E E L Q Q E M A Q Q S R E L Q Q A Q Q

AGAP012332_AGambiae
 DEF6_MMusculus
 LOC585280_SPurpuratus
 TRIADDRAFT_TAdhearens
 DEF6_XLaevis
 DEF6_GGallus
 DEF6_DRerio
 SWAP70_HSapiens
 DEF6_HSapiens

M L R Q L E E E R K R L D E L K L A R Q K I Q L S E D N K G I V E - - - A K L Q A M S P T Y R T S
 Q L E E V R Q N R Q R A D E D V E A A Q R K L R Q A S T N V K H W N - - - V Q M N R L M H P I E P G
 R A E G - - - A R K L I E R Q R E E E R R K L E E E E R Q A R - - - - E L E E L K K A Y E D A
 Q L A N L E E Y K N G N T L L K K T E N D I Q G L K T E G T H L N - - - T K I K D L R N K I S S V
 Q L E D V R E N R E R A D R D V Q V A Q N K L R Q A S T N V R H W N - - - I Q M N E L M H P I S P G
 Q L E E V R Y N R Q R V D Q D V M A A Q R K L R Q A S T N V K H W N - - - V Q M N R L M H P I E P G
 Q L E E T R A W R Y R V D Q D V V A A Q R K L R Q A S T N V K H W N - - - V Q M N R L M H P I G P G
 Q L E Q L E R K Q A L E Q E E E V K K K L E M A T N E T K S W K D K V A H E G L I R L I E P G
 Q L E E V R Q N R Q R A D E D V E A A Q R K L R Q A S T N V K H W N - - - V Q M N R L M H P I E P G

| |
|---------------------|
| AGAP012332_AGambiae | A | G | E | F | M | I | R | T | T | S | S | F | V | A | D | R | P | V | L | V | A | G | T | R | S | H | R | F | S | - |
|---------------------|

8.8 **Arsenite Treatment to promote granule formation**

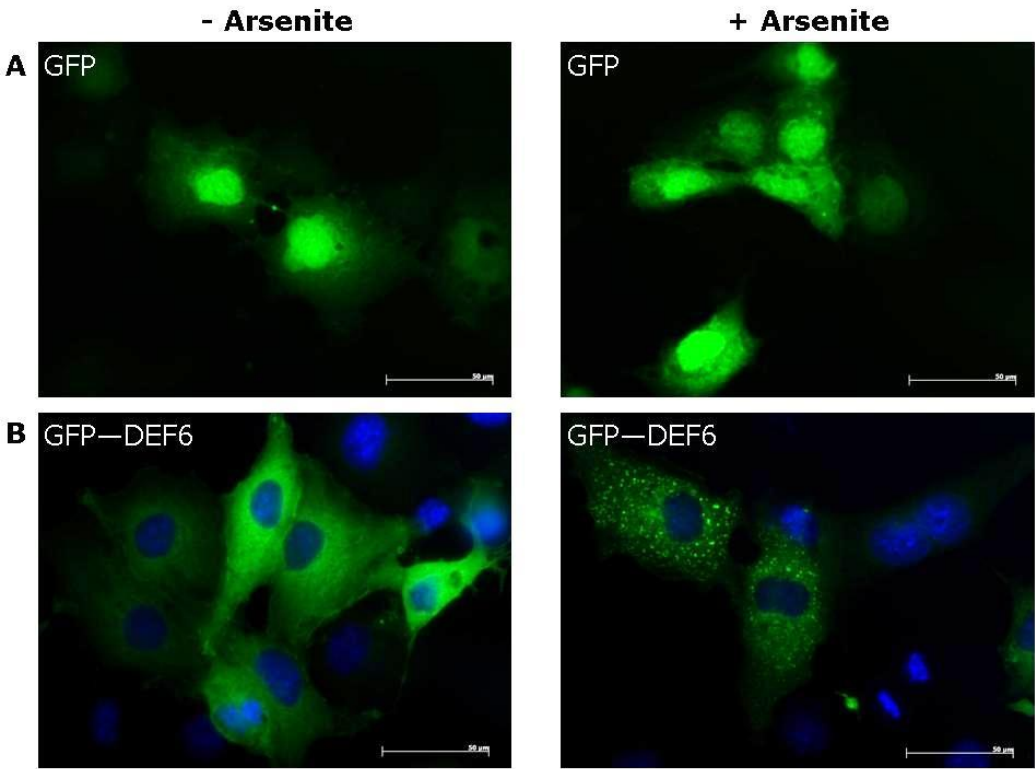


Figure 93 - Arsenite induces granule formation in GFP-DEF6 but not in GFP alone. Cos7 cells were transfected with GFP or GFP-DEF6 and treated with 1uM sodium arsenite for 1 hour 24 hours post transfection.

8.9 **Glutamine/Asparagine and Proline residues in GFP**

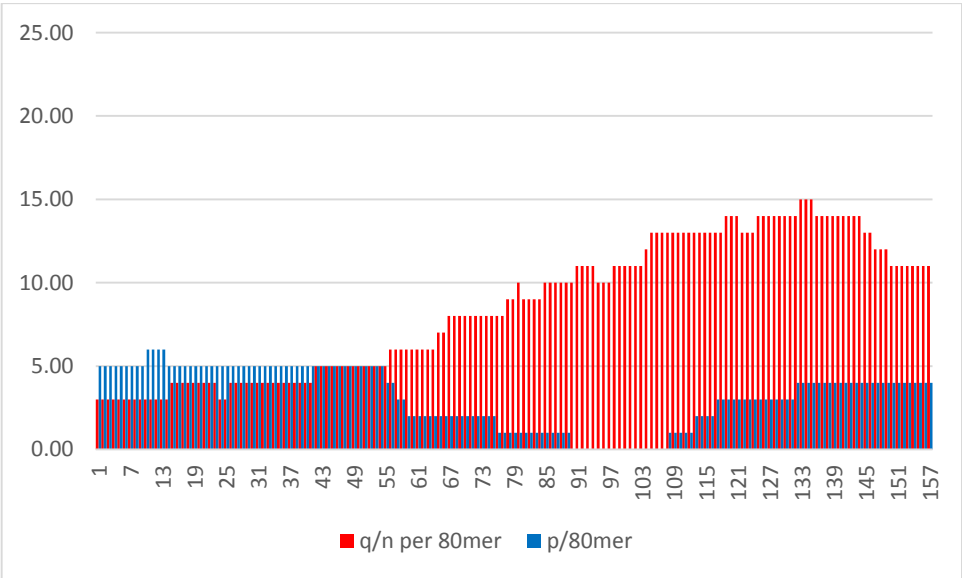
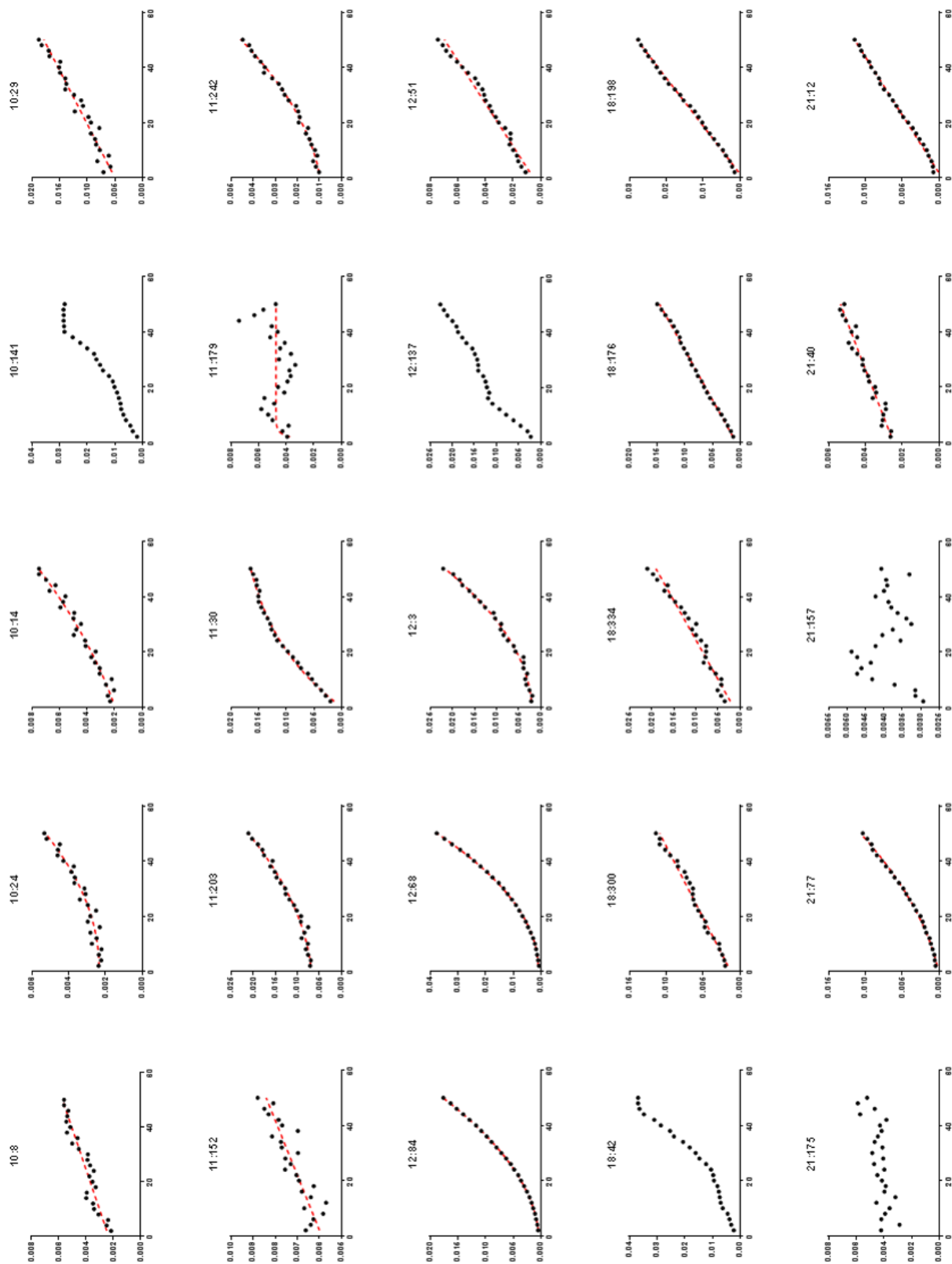


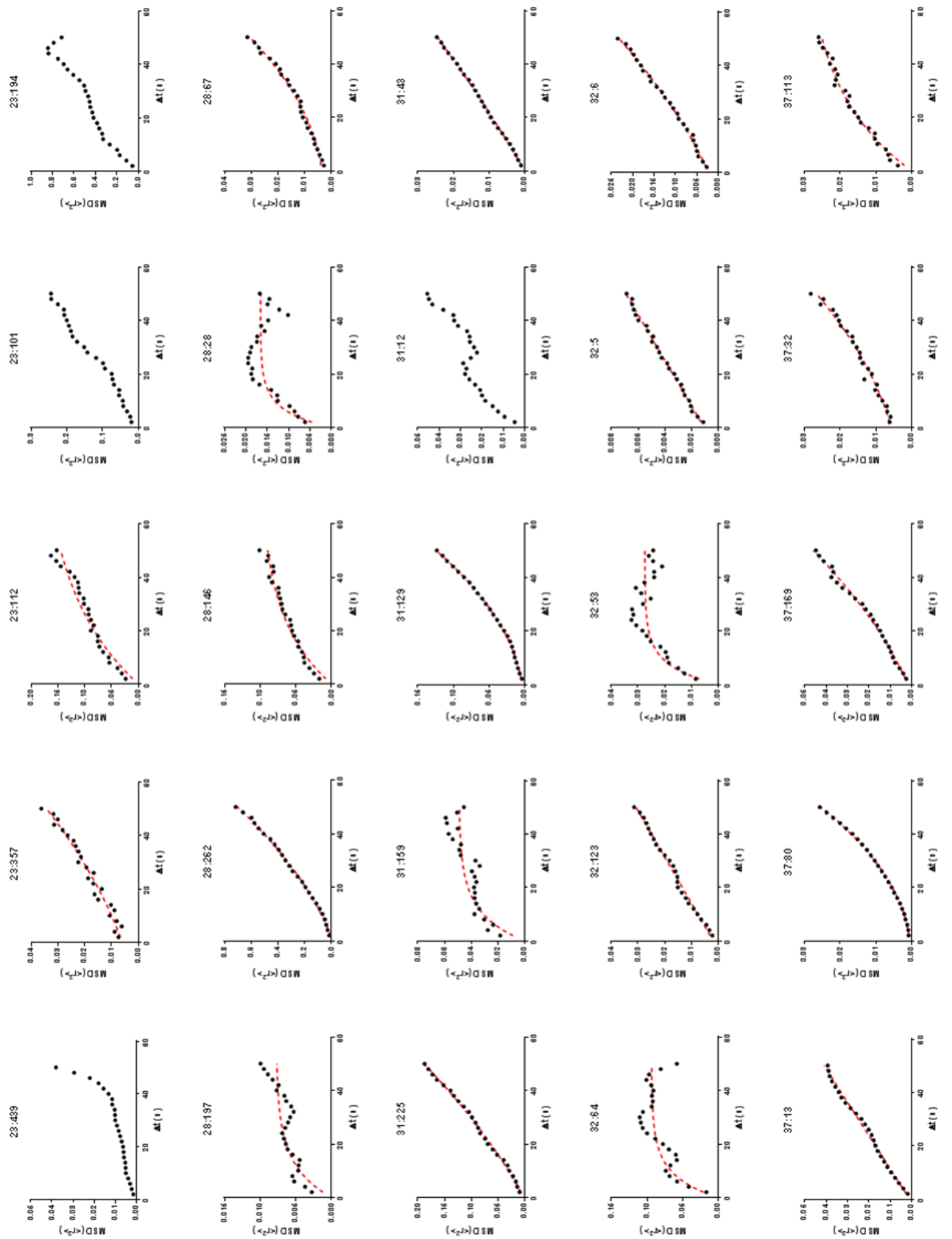
Figure 94 - A comparison of the number of glutamine/asparagine residues (red) and proline residues (blue) in GFP. The number of each type of residue per 80mer in the sequence of GFP was calculated and plotted against residue number to show regions rich or lacking in either glutamine and asparagine or proline residues.

8.10 MSD traces

8.10.1 GFP-DEF6



8.10.2 GFP-N-terminus



9 **References**

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