



Functional characterisation of ANKH mutants in the pathology of CPPDD and CMD

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ABSTRACT

Mutations at the terminals domains of *ANKH* are associated with calcium pyrophosphate deposition disease (CPPDD) and mutations within the central domains are associated with craniometaphyseal dysplasia (CMD). This study characterised the functional pathological effects of ANKH mutants in extracellular matrix biochemistry causing CPPDD and CMD. We first generated four *ANKH* mutant overexpression constructs containing either CPPDD linked P5L and E490del or CMD linked S375del and G389R mutations. Transfecting these constructs into CH-8 articular chondrocyte and HEK293 cells the dynamic differential localisation was monitored by confocal microscopy and the interactions with autophagy marker LC3 was investigated. Next we determined extracellular inorganic pyrophosphate (ePPi), mineralization, ENPP1 activity, expression of *ENPP1*, *TNAP* and *PIT-1* to characterise mutant biochemical effects in the pathogenesis of CPPDD and CMD. Our data showed the P5L mutation delayed cell membrane localization but once the protein was recruited into the membrane it functioned to increase ePPi, mineralization and ENPP1 activity. The E490del mutated protein remained mostly cytoplasmic, forming punctate bodies that co-localized with LC3 and increased mineralization and ENPP1 activity with an initial but unsustained increase in *TNAP* and *PIT-1*. The S375del mutation rendered ANKH completely cytoplasmic and trended to decrease ePPi and increase mineralization. The G389R mutation delayed cell membrane localisation of ANKH, trended to decrease ePPi and increased mineralization and also co-localized with LC3. Our results demonstrate a link between pathological localisation of ANKH mutants with different degrees in severity of abnormal mineralization. Furthermore, mutant ANKH functions may be related to synthesis of defective proteins, PPi transport, ENPP1 activity and expression of *ENPP1*, *TNAP* and *PIT-1*.

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ABBREVIATIONS

ACTB – β actin

ALP – alkaline phosphatases

AMP – adenosine monophosphate

AMPK – Adenosine monophosphate-activated protein kinase

ANKH – progressive ankylosis protein homologue

AP – autophagosome

ARM – armadillo-like

ARS – Alizarin red S

Atg – autophagy related proteins

ATG16L1 – autophagy related protein 16 like 1

Atgs – autophagy related proteins

ATP – adenosine triphosphate

BAC – bacterial artificial chromosome

BCA – bicinchoninic acid assay

BCIP – 5-bromo-4-chloro-3'-indolylphosphate

BMSC – Bone marrow-derived stem cells

CALCR – calcitonin receptor

CaM – Calmodulin

cAMP – cyclic adenosine monophosphate

CDE – clathrin dependent endocytosis

cDNA – complimentary DNA

CFP – cyan fluorescent protein

CIE – clathrin independent endocytosis

CIS – cargo internalisation signal

CLASP – clathrin associated sorting protein

CMA – chaperone-mediated autophagy

CMD – craniometaphyseal dysplasia

CMV – cytomegalovirus

coll10a1 – collagen type 10 alpha 1

CPPDD – calcium pyrophosphate dihydrate deposition

Cq – quantitation cycle

CRISPR – Clustered Regularly Interspaced Short Palindromic Repeats

CTD – C-terminal domain

CTP – cytidine triphosphate

CTSK – cathepsin K

DEAE – diethylaminoethanol

diH₂O – deionised water

DMEM – Dulbecco’s modified eagle medium

DNase – deoxyribonuclease

dNTP – deoxynucleotide triphosphate

dT – deoxythymine

DTT – dithiothreitol

E.coli – Escherichia coli

EB – ethidium bromide

eCa – extracellular calcium

ECM – extracellular matrix

EDTA – ethylenediaminetetraacetic acid

eGFP – enhanced green fluorescent protein

ELK1 – ETS domain-containing protein Elk-1

ENPP1 – Ectonucleotide pyrophosphatase 1

ePPi – extracellular PPi

ER – endoplasmic reticulum

ERAD – ER-associated degradation

ERK1/2 – extracellular signal related kinase 1/2

EST – expressed sequence tag

FBS – fetal bovine serum

GABA – gamma-amino butyric acid

GalT – Galactosyltransferase

GAPDH – Glyceraldehyde 3-phosphate dehydrogenase

GCALP – germ cell alkaline phosphatases

GLUT1 – Glucose transporter 1

Glv-1 – Gibbon ape leukemia virus receptor 1

GTP – guanosine triphosphate

H+M-MLV-RT H+ – moloney murine leukemia virus reverse transcriptase

HA – hydroxyapatite

HAF – human adult fibroblasts

HCl – hydrochloric acid

HEK293 – human embryonic kidney cells

HIF1 α – hypoxia inducible factor 1 α

hiPSCs – human induced pluripotent stem cells

HRE – hypoxia responsive elements

HRP – horseradish peroxidase

IALP – intestinal alkaline phosphatases

IgG – immunoglobulin G

Ihh – Indian hedgehog

IL-1 β – interleukin 1 β

IL-6 – interleukin 6

IMS – industrial methylated spirit

iPPi – intracellular PPI

JNK – c-Jun N-terminal kinases

Da – Dalton

KLD – Kinase-Ligase-Dpnl

LB – Lysogeny Broth

LC3 – light chain 3

LSM – laser scanning microscope

MAPKs – mitogen associated protein kinases

MC3T3-E1 – mouse osteoblastic cells

MgCl₂ – magnesium chloride

MIT – microtubule interacting and transport

MMP – matrix metalloproteases

MOPS – 3-(N-morpholino) propanesulfonic acid

MRDA – recessive mental retardation, deafness and ankylosis syndrome

mRNA – messenger RNA

mTORC1 – mammalian target of rapamycin complex 1

MV – matrix vesicles

MYBB1a – Myb-binding protein 1a

NaOH – sodium hydroxide

NBT – nitro-blue tetrazolium

NF- κ B – nuclear factor kappa-light-chain-enhancer of activated B cells

NFATc1 – nuclear factor of activated T cells 1

NMR – nuclear magnetic resonance

NTP – nucleotides tri-phosphate

OA – osteoarthritis

p-JNK – phospho-JNK

PAGE – Polyacrylamide gel electrophoresis

PAGs – Polyacrylamide gels

PALP – placental alkaline phosphatases

PAS – pre-autophagosomal structure

PBS – phosphate buffered saline

PCR – polymerase chain reaction

PE – phosphatidylethanolamine

PFA – paraformaldehyde

Pi – inorganic phosphate

PiT-1 – inorganic phosphate transporter 1

PKC – protein kinase C

PKC α – protein kinase C alpha

PPi – inorganic pyrophosphate

PTHrP – parathyroid hormone-related protein

PVDF – polyvinylidene difluoride

RFP – red fluorescent protein

RNA – ribonucleic acid

RNase – ribonuclease

SDS – sodium dodecyl sulfate

SH-SY5Y – human neuroblastoma cell line

SIP – sphingosine 1-phosphate

siRNA – short interfering RNA

SNPs – single nucleotide polymorphisms

SOC – Super optimal broth with Catabolite repression

SPHK1 – sphingosine kinase 1

SRP – signal recognition particle

STK36 – serine threonine kinase

SV40 – simian virus 40

TBE – tris-buffered EDTA

TBST – tris-buffered saline containing tween20

TE – Tris-Cl EDTA

TGF- β – transforming growth factor beta

TGS – Tris-Glycine SDS buffer

TNAP – Tissue non-specific alkaline phosphatase

TNF- α – tumour necrosis factor alpha

TRAP – tartrate resistant acid phosphatase

TTP – thymidine triphosphate

ULK – unc-like kinase

ULKs – unc-51-like Kinases

UTP – uridine triphosphate

UTR – untranslated region

UV – ultraviolet

YFP – yellow fluorescent protein

Chapter 1

Introduction

1 – INTRODUCTION

1.1 – Bone Biology

1.1.1 – A brief history and importance of bone

Pre-historic humans used bone tools and weapons to elevate themselves above rivals for resources. The use of bone weapons as an alternative to the bare hand was the beginning of the end of altruistic selection for humankind. A precise blow using a bone “awl” offering increased range and deadly force would allow a mother to defend her offspring from stronger predators or rivals, whom would otherwise succeed. Evolutionary bone growth in humans is directly related to our planets gravitational force and we have adapted the perfect density, length and structure to accommodate us comfortably, on two legs, on earth. Evidently, evolution has favored bone as the minimal armour in protecting our most vital organ – the brain. Therefore, it is clear that bone has been the backbone of human superiority on earth and allowed us to colonize and expand as a species.

1.1.2 – Initiation of ossification in fetal bone development

There are two fundamental aspects in fetal bone development – the initial patterning which then directs differentiation of bone through a process called ossification. Briefly, patterning occurs early (8 weeks) in embryonic development and involves all three of the main germ layers – endoderm, ectoderm and mesoderm. The fourth major population of cells, the neural crest cells also contribute to bone ossification (Franz-odendaal 2011). Regardless of origin, skeletogenesis is initiated via the migration of mesenchymal cells originated from these embryonic lineages to positions of future bone. These localised cells condense and pattern the shape and size of bones and can either differentiate into chondroblasts

(endochondral ossification) or osteoblasts (intramembranous ossification). Endochondral ossification forms the base and posterior part of the skull, the axial skeleton and appendicular skeleton, whilst intramembranous ossification forms the remaining skull, jaw and clavicle. The main difference between these two mechanisms is that endochondral ossification relies on a cartilage pre-cursor (anlage) to outline the future bone but intramembranous ossification can directly form bone (Berendsen and Olsen 2015) (Figure 1).

1.1.3 – Endochondral ossification in fetal bone development

Endochondral ossification is triggered when proliferating chondroblasts become hypertrophic within the growing cartilage anlage and differentiate into ‘hypertrophic chondrocytes’. Hypertrophic chondrocytes direct neighboring cells into becoming osteoblasts that then invade the cartilage anlage forming the primary ossification centre. However, recent work indicates that osteoclasts, blood vessel endothelial cells and hematopoietic cells also accompany these invading osteoblasts into the cartilage anlage and these osteoblasts may be pre-cursor osteoblasts (Maes et al. 2010). Nonetheless, as ossification progresses hypertrophic chondrocytes undergo apoptosis and leave behind a cartilage mold, that is calcified by the invading osteoblasts to form a true bone matrix (primary spongiosa). The bone matrix is comprised of two major components – organic (95% collagen Type 1) and inorganic (hydroxyapatite crystals, calcium, phosphorus and others). A secondary ossification centre at one or both longitudinal ends of the developing bone is formed when chondroblasts in the novel location become hypertrophic (Kronenberg 2003). Chondrocytes continue to proliferate between the primary spongiosa and secondary ossification centre – this region is termed the growth plate. Within the growth plate, chondrocytes are organized columnar into structural and functional zones via a negative feedback loop involving two master regulators – Indian hedgehog (Ihh) and

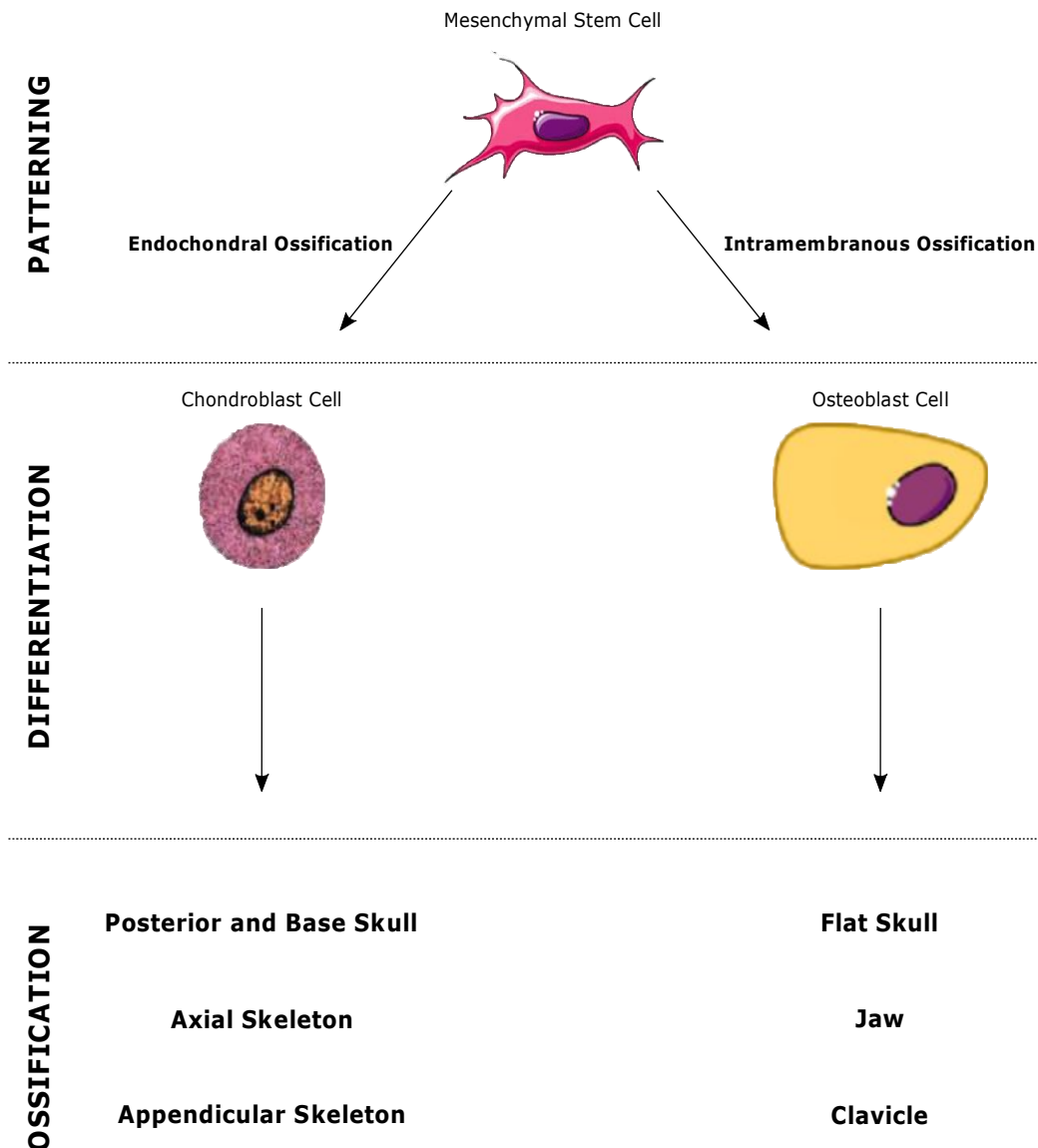


Figure 1 – Schematic representation of differing ossification pathways.

Mesenchymal stem cells (MSCs) are *patterned* into two distinct pathways of ossification – Endochondral (left) and Intramembranous (right). MSCs destined for endochondral ossification differentiate into chondroblasts, whilst MSCs destined for intramembranous ossification differentiate into osteoblasts. Endochondral ossification forms the posterior and base of the skull and the skeletal bones. Intramembranous ossification forms the flat bones of the skull, jaw and clavicle.

parathyroid hormone-related protein (PTHrP) (Kronenberg 2003, Maes et al. 2010) (Figure 2).

1.1.4 – Ossification zones during endochondral ossification

Chondroblasts at the secondary ossification centre express PTHrP, which acts to keep nearby chondrocytes proliferating. Recent work demonstrated chondrocytes becoming hypertrophic when sufficient PTHrP was not present and then started expressing Ihh (U-I. Chung et al. 1998, Ung Il Chung et al. 2001). Through an unknown mechanism Ihh acts to increase PTHrP expression and keep chondroblasts at secondary ossification centre proliferating. This creates graded expression of PTHrP - increased in cells near the secondary ossification centre, and Ihh - increased in cells near the primary spongiosa (Ohba 2016). This graded expression is responsible for the columnar structure of chondrocytes and creates five zones with unique cells and structures within the growth plate. At the primary spongiosa, osteoblasts are actively ossifying new bone (ossification zones). Slightly further from the primary spongiosa, hypertrophic chondrocytes are undergoing apoptosis and the cartilage mold is being invaded by osteoblasts to grow the bone (calcification zone). However, recent data suggests that not all hypertrophic chondrocytes within the calcification zone are fated for apoptosis and may differentiate into osteoblasts or osteoclasts (Yang et al. 2016). Roughly in the middle of the growth plate, between the primary spongiosa and secondary ossification centre, chondrocytes are becoming hypertrophic due to their distance from PTHrP expressing chondroblasts at the secondary ossification centre (hypertrophic zone). Near to the secondary ossification centre, chondrocytes experiencing sufficient PTHrP are actively proliferating and adding to the cartilage mold (proliferation zone). Lastly, at the secondary ossification centre, chondroblasts are maintaining PTHrP expression and orchestrating chondrocyte function within the growth plate (resting zone) (Kronenberg 2003, Ohba 2016) (Figure 2).

Zones

Cells

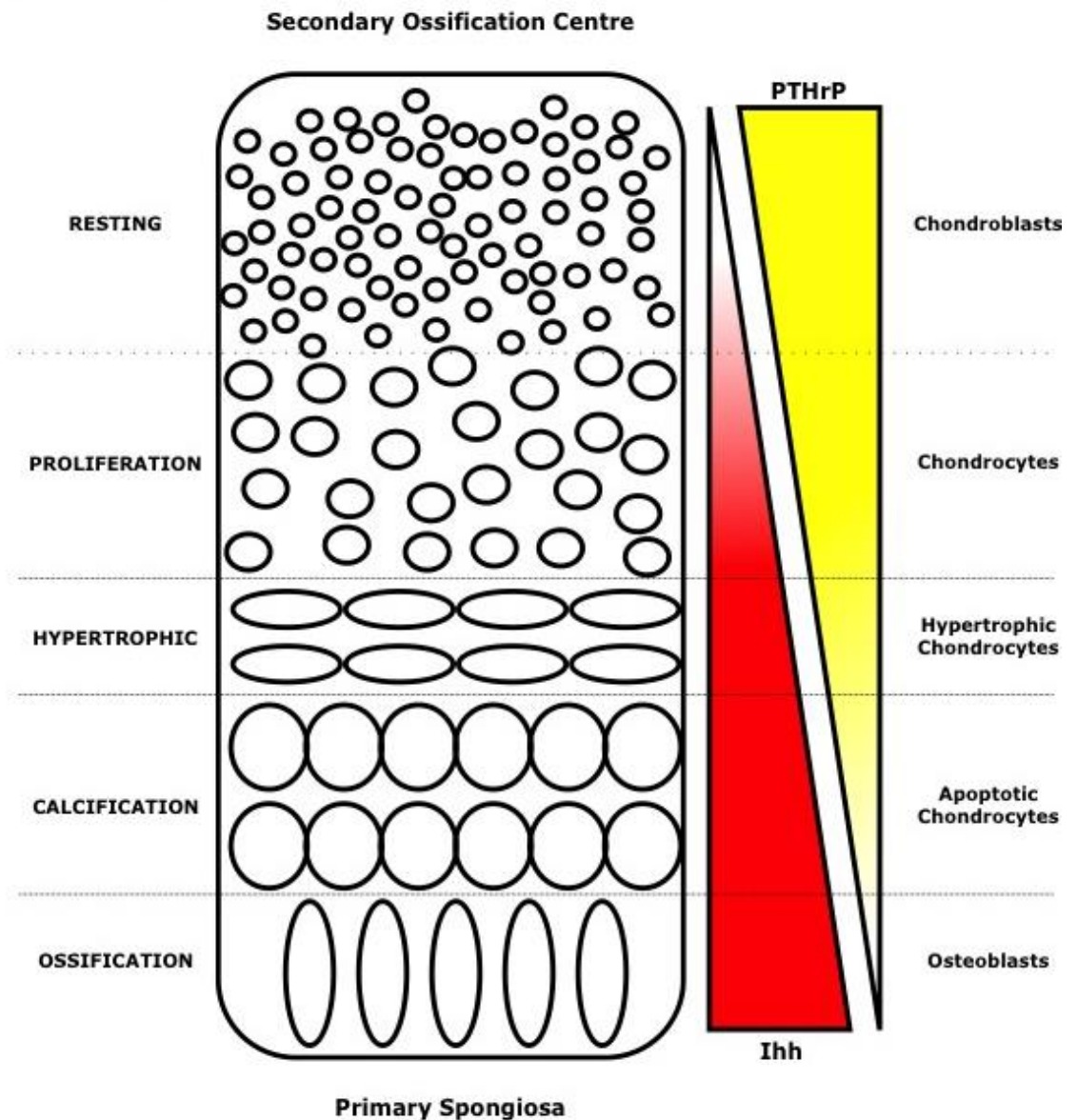


Figure 2 – Schematic representation of endochondral ossification.

Endochondral ossification is regulated by synergistic expression of parathyroid hormone (PTHrP) and Indian hedgehog (Ihh). This graded expression forms five distinct zones starting containing distinct cell types – Ossification zone where osteoblasts are actively forming new bones, Calcification zone where apoptotic chondrocytes are exiting and the cartilage mold is being invaded by osteoblasts, Hypertrophic zone contains chondrocytes beginning to express hypertrophic markers but still forming cartilage mold, Proliferative zone contains actively dividing chondrocytes ready to enter the hypertrophic zone and Resting zone contains chondroblasts that are expressing PTHrP to orchestrate chondrocyte differentiation.

1.1.5 – The process of intramembranous ossification during fetal bone development

In contrast to endochondral ossification that requires a cartilage mold to direct bone growth, intramembranous ossification forms the flat bones (skull, clavicle and jaw) via direct ossification of a tissue network. Intramembranous ossification is initiated when mesenchymal cells derived from the neural crest condense into compact nodules and this becomes the ossification centre (Franz-odendaal 2011). Cellular expansion around the ossification centre defines the early regions of the new bone. Within this region mesenchymal cells differentiate into osteoblasts that begin secreting osteoid – a type 1 collagen-containing matrix. The osteoid can deposit minerals such as calcium to create a spongy bone matrix. However, whether this is aided by the osteoblast or is a natural process is unknown (Percival and Richtsmeier 2013). Osteoblasts within these growing calcified structures can either leave or differentiate into osteoclasts – the classic bone cell. Osteoblasts able to leave arrange themselves along the outside edge of the growing osteoid and function to increase the density of the growing bone by adding new layers of osteoid (spicules). Osteoclasts within the bone function to maintain, remodel and add the blood vessels as required. These spicules grow larger with time and eventually merge at their calcification fronts with others to become the top and bottom plates (compact bone) (Berendsen and Olsen 2015, Franz-odendaal 2011, Percival and Richtsmeier 2013) (Figure 3).

1.1.6 – The role and function of the matrix vesicle during ossification

The osteoid matrix is able to deposit calcium-containing minerals to form new bone. However, the requirements of calcification often mean that surrounding cells have to maintain an appropriate concentration of the desired mineral to enable efficient ossification. Within the matrix, osteoblasts and hypertrophic chondrocytes (endochondral

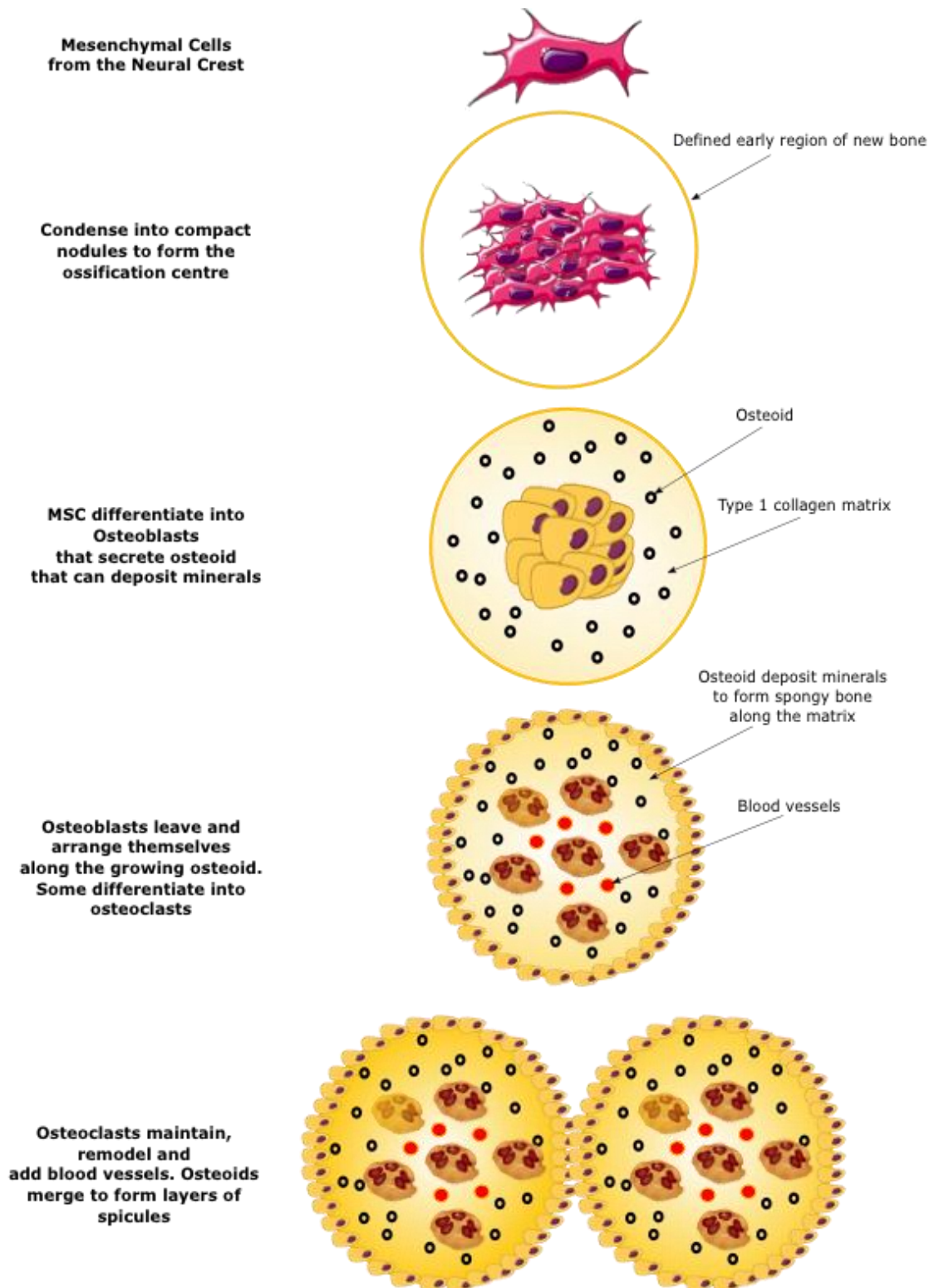


Figure 3 – Schematic representation of intramembranous ossification.

Mesenchymal stem cells (MSCs) from the neural crest condense into compact nodules to define the early region of new bone and ossification centre. They differentiate into osteoblasts that secrete osteoid to deposit minerals on the forming matrix. Some osteoblasts exit and align themselves along the defined region, whilst some differentiate into osteoclasts and maintain, remodel and add blood vessels to the forming spongy bone. Finally, several osteoids can merge to form layers of spicules, in the process of growing flat bone.

ossification) secrete matrix vesicles via budding of their cellular membrane. These matrix vesicles (20-200 nm) appear to be bound by a lipid bilayer and are thought to contain several key proteins responsible for maintaining proper mineral properties of the matrix (D'Angelo et al. 2001). Biogenesis of these matrix vesicles is a debated subject within the field and although a general consensus of a budding process is accepted, there is evidence that biogenesis of matrix vesicles and cell plasma membrane are independent of each other (Leach et al 1995). The matrix vesicle lipid bilayer also contains subtle differences, such that the matrix vesicle is enriched in acidic phospholipids (phosphatidylinositol and phosphatidylserine) and proteins required for mineralization. The importance of these subtle differences has been explored in several studies (Golub 2009) and the general conclusion is of a specialized membrane- and micro-domains containing denser concentrations of proteins are the site of the budding process.

1.1.7 – Proteins and their function within the matrix vesicle

The exact composition of the matrix vesicle is still a novel area of scientific research, however many important proteins and lipids have been identified. Tissue non-specific alkaline phosphatase (TNAP – HGNC:438) is the best studied, first described in 1924, and was predicted to be a different variety of already known “esterase” in the pancreas, spleen and liver (Robison and Soames 1924). Ectonucleotide pyrophosphatase 1 (ENPP1 – HGNC:3356) is a more recent discovery, with seminal studies conducted in 2002 and 2004 describing its important role (Harmey et al. 2004, Hessle et al. 2002). Both ENPP1 and TNAP are discussed in detail further. Several annexins (principally 2, 5 and 6) are associated with calcium homeostasis in matrix vesicles, as they can form hexameric structures within the high acidic lipid bilayer to facilitate calcium transport (Kirsch et al. 1997). Another transporter protein, sodium-dependent phosphate transporter 1 (PIT-1 – HGNC:10946) is also present within the matrix vesicle lipid bilayer. PIT-1 is also

discussed in detail further. Several enzymes such as matrix metalloproteases (MMP2, 9 and 13), aminopeptidases, carboxypeptidases and proteoglycanases are present within the lipid bilayer. These act to degrade the osteoid matrix and proteins that inhibit mineralization whilst increasing the availability of prerequisite minerals (Balcerzak et al 2006, D'Angelo et al 2001, Glaser and Conrad 1981, Massey-Harroche et al 1998, Licia N Y Wu et al 2002, Wuthier 1975).

1.1.8 – The process of mineralization within the extracellular matrix

The term matrix encompasses any space outside the cell where mineralization is actively occurring and herein is referred to as the extracellular matrix (ECM). Osteoblasts, chondrocytes and osteocytes that are processing ossification maintain a suitable ECM for mineralization via budding of matrix vesicles (MV) and express a highly conserved inorganic pyrophosphate transporter, the human homolog of mouse progressive ankylosis – ANKH (HGNC:15492). Within the ECM a synergistic relationship between ANKH, ENPP1, TNAP and PIT-1 exists to control mineralization (Williams 2015). Mineralization of either the cartilage mold (endochondral ossification) or osteoid matrix (intramembranous ossification) requires crystallization of a calcium containing salt – hydroxyapatite (HA). Initiation of HA crystallization (nucleation), an extremely important event in bone mineralization has been experimentally shown to occur within the lumen of MVs that have accumulated sufficient calcium and inorganic phosphate (Pi) (Hsu and Clarke Anderson 1996, K. A. Johnson et al. 2000, Kirsch et al. 2000, Licia N.Y. Wu et al. 2003). However, nucleation of HA has also been observed within the ECM (Anderson et al. 2004). Once MVs have nucleated HA, breakdown of the MV lipid bilayer (membrane) releases HA crystal into the ECM where mineralization is propagated from this template. The ECM contains appropriate concentrations of calcium and Pi to form crystalline arrays and deposit HA (70% of bone weight in humans) (Golub 2009).

1.1.9 – The synergistic relationship of ANKH, ENPP1, TNAP and PIT-1 in the maintenance of ECM

The synergistic relationship of ANKH, ENPP1, TNAP and PIT-1 acts to maintain the appropriate concentrations of calcium and Pi in the ECM. ANKH is discussed in much greater detail below but briefly, is responsible for the transport of inorganic pyrophosphate (PPi) from intracellular locations to the ECM of bone cells (Figure 4).

1.1.9.1 – ENPP1

ENPP1 is a ~130,000 Da membrane bound nucleoside triphosphate phosphohydrolase that hydrolyses tri-nucleotides into PPi (Belli and Goding 1994, Nam et al 2011) and generates the majority of the free PPi in the ECM (K. A. Johnson et al 2000, Kristen Johnson, Moffa, et al 1999, Kristen Johnson, Vaingankar, et al 1999). The catalytic domain of ENPP1 utilizes a zinc co-factor to preferentially bind and hydrolyze adenosine triphosphate (ATP), but can also hydrolyse guanosine triphosphate (GTP), cytidine triphosphate (CTP), thymidine triphosphate (TTP) and uridine triphosphate (UTP) to their respective monophosphates, and also 3', 5'-cAMP (cyclic adenosine monophosphate) to AMP. ENPP1 successively hydrolyses the 5' nucleotide from the 3' hydroxyl terminal of these tri-phosphates and generates the PPi required in mineralization (Nam et al 2011).

1.1.9.2 – TNAP

There are four members of the alkaline phosphatase (ALP) family – intestinal, placental, germ cell and tissue non-specific (IALP, PALP, GCALP and TNAP, respectively). The most extensively studied is TNAP and provides the majority of evidence for ALP family function. With few exceptions, ALPs are homodimeric enzymes with each catalytic site

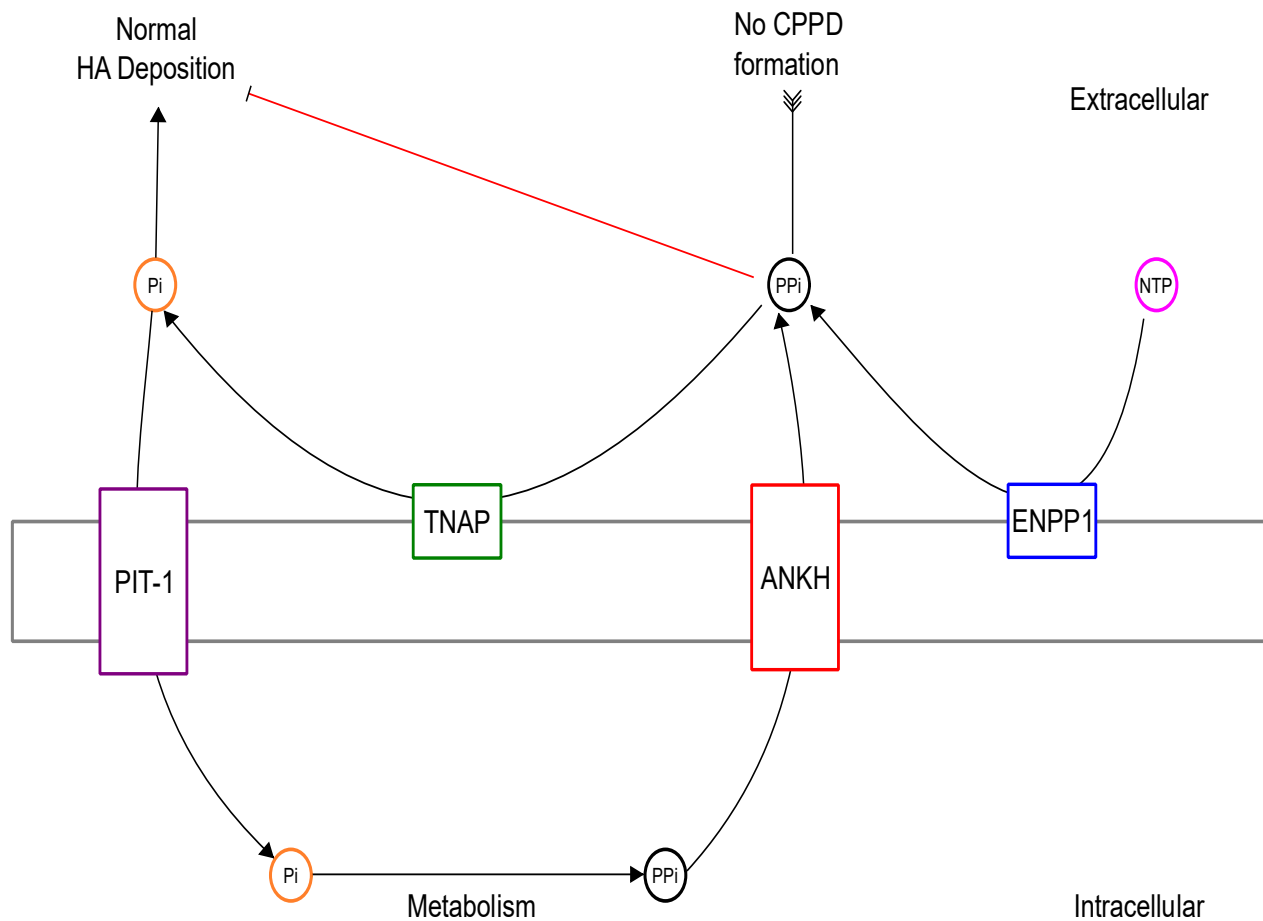


Figure 4 – Schematic of ANKH, ENPP1, TNAP and PIT-1 regulation of normal extracellular matrix.

ANKH transports PPi from intracellular regions into the extracellular matrix (ECM). ENPP1 catalyzes NTPs into PPi in the ECM. TNAP catalyzes PPi into Pi in the ECM. PIT-1 transports Pi from the ECM to inside the cell.

containing three metal cofactors – a single magnesium and two zinc atoms. TNAP is predicted to have a molecular weight of 57,000 Da and catalyzes PPi to generate Pi required in bone mineralization. As high concentrations of PPi are a potent inhibitor of HA nucleation and crystallization, TNAP serves a dual function in promoting bone mineralization – reducing PPi and increasing Pi (Millán 2006).

1.1.9.3 – PIT-1

A member of the type III Glvr-1 gene family, PIT-1 (also known as SLC20A1 and GLVR1) is a 73,000 Da sodium-phosphate symporter that functions to move phosphate into the lumen of the MV and cytoplasm of bone cells. There is sufficient evidence to relate PIT-1 function to ossification of bone, however, novel data has highlighted potential roles in liver development, vascular calcification and cellular proliferation (Beck et al 2009, 2010, Giachelli 2009, Li et al. 2006). PIT-1 expression has also been shown to be modulated by the levels of Pi and hence was predicted to be sensory and regulatory controller of mineralization (Dashen Wang et al 2001).

1.2 – The inorganic pyrophosphate transporter ANKH

ANKH is a highly conserved transmembrane PPi transporter that increases the concentrations of PPi in the ECM. During ossification, PPi is essential in regulating the deposition of HA and acts as a potent inhibitor. However, PPi is also a pre-requisite for HA crystallization as the majority of Pi in the ECM is derived from the hydrolysis of PPi. ANKH is currently associated with three distinct diseases – craniometaphyseal dysplasia (CMD), calcium pyrophosphate dihydrate deposition disease (CPPDD) and recessive mental retardation, deafness and ankylosis syndrome (MRDA). These diseases are discussed in section 1.3.

1.2.1 – Seminal studies on *ANKH*

1.2.1.1 – “*The role of the Mouse ank Gene in Control of Tissue calcification and arthritis*”

– Ho, Johnson and Kingsley, 2000

Almost 20 years ago, mice carrying the *ank* mutation were routinely used as a model of arthritis. They displayed a progressive and destructive form of arthritis accompanied by abnormal “flat-footed gait”, decreased mobility due to abnormal HA deposition in synovial and articular regions and joint fusion leading to “complete rigidity and death around 6 months of age”. In this seminal study, Ho et al. (2000) created bacterial artificial chromosome (BAC) clones and “shotgun” sequenced for mutations within chromosome 15 for a candidate gene responsible for mineralization and a single G to T substitution was found within the gene termed *ank* for progressive ankylosis. This mutation caused a nonsense mutation in protein ANK that was confirmed by sequence comparison to wild type mice via the loss of a *HinfI* restriction site. Ho et al. predicted the *ank* gene to encode a 492 amino acid protein with a molecular weight of 54,300 Da.

To characterize protein expression and subcellular localisation, Ho et al. first cloned *ank* into a mammalian expression vector (pCMV-ANK). Transfection into monkey kidney cells (COS-7) revealed the majority of expression at the cell plasma membrane and further suggested ANK to be a membrane protein. Northern blot analysis of adult mice revealed expression of ANK in several tissues including heart, brain, liver, spleen, lung, muscle and kidney. In-situ hybridization revealed abundant expression of ANK in articular cartilage of joints in the shoulder, elbow, wrist and digits of adult mice. Further sequence analyses predicted the protein would potentially contain three N-linked glycosylation sites and multiple putative phosphorylation sites. Implementing the Kyte-Doolittle algorithm

revealed 9-12 hydrophobic stretches, each containing ~20 residues and hence, they concluded ANK to be multipass transmembrane protein.

Implementing EST clones of zebrafish, rats, mice, cows and humans and using exon/intron structure of mouse *ank* Ho et al. established the first human ortholog of *ank* (*ANKH*). They noted that human ANKH is almost identical to mouse ANK, with only nine amino acid substitutions out of 492 amino acids. Mapping to the human genome revealed sequence matches on chromosome 5p, to a region previously associated with several human disorders that mimicked the mouse phenotype. Previous work on these disorders had revealed increased intracellular PPI (iPPi) in cultured skin fibroblasts and lymphoblasts. Therefore Ho et al. established primary skin fibroblasts cultures from wild type and mutant *ank* mice and noted a two-fold increase of iPPi in mutant *ank* fibroblasts compared to wild type. As “PPi acts as a potent inhibitor of HA deposition in the ECM”, they tested mutant *ank* affect on extracellular PPI (ePPi) and noted a three- to five-fold decrease in ePPi of mutant *ank* compared to wild type. Furthermore, both of these phenotypes were rescued when mutant *ank* fibroblasts were transfected with pCMV-ANK overexpression vector, indicating the mutated *ank* gene to be responsible for the disease phenotype observed in *ank* mice.

Lastly, Ho *et al.*, reasoned that due to the “opposite effects of ANK on PPI levels inside and outside the cell”, ANK may function as a PPI transporter. To test this theory they treated pCMV-ANK transfected COS-7 cells with 2.5 mM probenecid and measured no decrease in iPPi and hence suggested that ANK is “functioning through a probenecid-sensitive anion transport mechanism”. Therefore, undeniably Ho et al. offered the first clear insight into ANK function to regulate PPI and cemented ANK importance in mineralization during ossification.

1.2.1.2 – “*Heterozygous mutations in ANKH, the human ortholog of the mouse progressive ankylosis gene, result in craniometaphyseal dysplasia*” – Nurnberg et al. 2001

Nürnberg et al. 2001 was the first group to describe a clear link between specific *ANKH* mutations and CMD. However, CMD and its associated *ANKH* mutants are discussed in detail below and only the Nurnberg et al. description of *ANKH* topology is included within this section. Nurnberg et al. re-evaluated the proposed model of Ho et al. using a newer helix prediction program – TMpred and proposed an “alternative and somewhat more detailed model”. TMpred predicted 12 membrane-spanning helices compared to the 9-12 predicted by Ho et al. and an alternating inside/out orientation. Furthermore, they evaluated hydrophobicity and proposed that only every second helix contributes to the channel function and hence described a “six membered parallel assembly” topology of *ANKH*. Therefore, there would be six inner helices in a circular structure that are encompassed by the remaining six helices forming an inner transport channel and outer stabilizing structure. They also noted that the inner helices maintain a positive potential and that would be significant in transport of a negatively charged PPi molecule. However, as there is no crystal structure for *ANKH* the exact orientation of the helices and their number is still uncertain.

1.2.2 – Regulation of *ANKH* expression

ANKH regulation is a developing aspect of the field however several studies have demonstrated transforming growth factor beta (TGF- β) superfamily to regulate PPi concentrations within the ECM. Sohn et al. (2002) performed several experiments to develop a profile of ANK expression in mouse embryos and they primarily observed ubiquitous expression in ossification centres and related bone structures, however, they also observed ANK expression in non-mineralizing regions such as tendons and embryonic

joints. Furthermore, they tested whether TGF- β 1 regulates ANK expression within the metatarsal bones grown in organ culture. Treating cultures with either 10ng TGF- β 1/ml or left untreated for 5 days they measured expression of ANK mRNA in expected locations in control cultures (ossification centres of hypertrophic cells). However, in treated cells they measured increased expression of ANK mRNA (3.64 fold) in both hypertrophic and pre-hypertrophic regions. Another study also reported that TGF- β 1 is able to regulate PPi but only under normal ANK and ENPP1 expression (K. Johnson and Terkeltaub 2004). TGF- β is also reported to increase ANK mRNA in rat chondrocytes in a calcium dependent manner utilizing the protein kinase C (PKC) pathway (Netter et al 2004). The same group also reported a decrease in ANK mRNA of rat chondrocytes when treated with TNF- α and IL-1 β . This was further evident in studies evaluating influx of calcium on extracellular signal related kinase 1/2 (ERK1/2) and protein kinase C alpha (PKC α) signaling cascades that resulted in increased ANK expression (Cailotto et al. 2007, Oca et al. 2010). Oca et al. (2010) also observed that PIT-1 was required for TGF- β -mediated regulation of ANK expression. More recent work also demonstrated that TGF- β regulation of ANK required activation of the Ras/Raf/ERK signaling cascade in rat articular chondrocytes and that this was independent of Smad (Cailotto et al. 2011).

The presence of putative hypoxia responsive elements (HREs) upstream of ANK gene promoters provided direction to study the effect of oxygen on ANK expression. Zaka et al. (2009) showed a decrease in ANK expression when rat chondrocytes were cultured in hypoxic conditions. Furthermore, they showed that hypoxia inducible factor 1a (HIF1) would bind to the ANK HRE elements under normal conditions but would not bind under hypoxic conditions. The authors postulated that under hypoxic conditions a competitive molecule could bind to the ANK HRE and displace HIF1, however this molecule has not been identified (Zaka et al. 2009).

Recently, Nasi et al. (2016) described the role of interleukin-6 (IL-6) as a modulator on ANK expression. Initially, they exposed chondrocytes cultured from C57BL/6J mice to CPPD and HA crystals and measured increased secretion of IL-6 in HA treated cultures. Postulating a positive role for IL-6 in mineralization, they treated chondrocytes with IL-6 over 7 days and measured increased mRNA expression of several genes including *Ank* and *PIT-1* and also increased mineralization (perhaps this was CPPD rather than HA). This highlights a circular regulatory system in ANK mediated mineralization, where the end product (HA) can regulate its own formation.

JNKs (c-Jun N-terminal kinases) are known regulators of cellular apoptosis in chondrocytes, however Xu et al. (2013a, 2013b) recently described a novel regulatory role for ANK. When chondrocytes isolated from six-week old Sprague-Dawley rats or human patients were serially passaged for natural degeneration, there was negative correlation in expression and activity of ANK with p-JNK (activated JNK). When JNK activation was inhibited, ANK expression and activity was increased and the authors concluded that natural degeneration in rat and human chondrocytes was due to decreased ANK function modulated by JNK activation (Xu et al. 2013a, 2013b).

1.2.3 – Protein interactions with ANKH

Wang et al. (2008) have reported two interactions of ANKH mutants with PIT-1 and TNAP. Firstly, they reported that the E490del (RCV000005507) CPPDD associated mutant would down regulate expression and activity of TNAP in rat chondrocytes (Wang et al. 2008). Secondly, they reported that the M48T (RCV000005505) CPPDD mutant would not co-immunoprecipitate with PIT-1, whilst wild type ANKH would (Wang et al. 2009). They concluded that although ANKH function, in part, is related to its PPi transport

functions it was evident that the complex interplay of ANKH with both TNAP and PIT-1 would be a promising direction for future research.

Recently, Minashima et al. (2014) treated articular chondrocytes and femoral head explants from *ank/ank* mice and wild type mice with the inflammatory cytokine interleukin 1 beta (IL1- β) due to previous observations (K. Johnson and Terkeltaub 2004, Masuda and Hirose 2002, Wang et al. 2005) of ANKH overexpression in osteoarthritis (OA) cartilage and postulated whether ANK may interact or modulate function and activity of proteins that regulate catabolic events in OA chondrocytes. In wild type ANK mice chondrocytes, using yeast two-hybrid and co-immunoprecipitation methodology, Minashima et al. identified two proteins that interacted with ANK – sphingosine kinase 1 (SPHK1 - interacted with the N-terminal cytoplasmic region of ANK) and Myb-binding protein 1a (MYBB1a - interacted with a C-terminal cytoplasmic loop). Both SPHK1 and MYBB1a are regulators of nucleation factor kappa-light-chain-enhancer of activated B cells (NF- κ B), a protein responsible for several catabolic pathways in cells involved in ossification (Oeckinghaus and Ghosh 2009). Nucleation of MYBB1a inhibits NF- κ B, whilst SPHK1 enhances NF- κ B activity via its functional modulator sphingosine 1-phosphate (SIP) (Spiegel and Milstien 2011).

In *ank/ank* mice Minashima et al. (2014) reported increased nucleation of MYBB1a and decreased SPHK1 activity and repression of NF- κ B due to ablation of the ANK/MYBB1a and ANK/SPHK1 interaction. This was confirmed when they transfected *ank/ank* chondrocytes with ANKH mutants P5L (CPPD associated, RCV000005508) and F376del (CMD associated) – P5L decreased nucleation of MYBB1a but did not rescue SPHK1 activity, F376del increased SPHK1 activity but did not decrease nucleation of MYBB1a. Transfection of either mutant ANKH only partially rescued NF- κ B activity in the *ank/ank* mice chondrocytes and Minashima et al. (2014) concluded that “the interactions of ANK

with other proteins are altered or lost in chondrocalcinosis or CMD mutations of ANK and these altered or lost interactions may contribute to the disease phenotypes besides the altered PPi transport abilities of these mutations (pp 859, Minashima et al. 2014). These findings also rationalized ANKH function in ossification is not limited to simply maintaining PPi ratios in the ECM and that ANKH regulates and modulates several intracellular pathways via NF- κ B signaling.

1.2.4 – The role of ANKH in bone cell differentiation

Chondrocytes, osteoblasts and osteoclasts require specific expression of pro-differentiation factors during their genesis in the process of ossification. Historically, ANKH function in ossification was largely limited to its function at the cell membrane as a PPi transporter and the fact that *ank* mice are normal at birth gave no merit to study ANKH function during ossification. However, as increasing evidence of ANKH intracellular function independent of PPi homeostasis was determined, researchers postulated a function of ANKH in cellular differentiation of bone cells. Johnson et al. (2008) implicated increased expression of *Vanin-1* to increased *ank/ank* mice mesenchymal cells' rate of differentiation. Vanin-1 regulates ossification via its modulation of glutathione, a regulator of chondrogenesis (Johnson et al. 2008). They related the increased differentiation of *ank/ank* mesenchymal cells into chondrocytes was due to depletion of glutathione within the mesenchymal cells resulting from increased Vanin-1. Johnson et al. (2008) offered no mechanistic evidence of ANKH interaction with Vanin-1 and therefore, it is likely that increased Vanin-1 could result from one of the many consequential phenotypes seen in *ank/ank* mice rather than to ANKH directly. Perhaps, answering whether *ank/ank* mice undergo endochondral ossification faster would be an interesting approach to validate this phenomenon.

Initial work by Zaka et al. (2006) demonstrated increased expression of *coll10a1*; a marker of hypertrophic chondrocytes, in transduced rat ATDC5 cells stably overexpressing ANK. However, they found no difference in expression of other hypertrophic markers – *osteopontin*, and *runx2* compared to null-vector transduced ATDC5 cells (Zaka et al. 2006). Wang et al. (2008) obtained somewhat similar results reporting that overexpression of wild type ANKH generally “disrupted” expression of hypertrophic markers of chondrogenic differentiation in ATDC5. However, when overexpressing CPPDD associated mutant E490del they noted a “delayed” expression profile (pp 25, Wang et al. 2008). Further work by Kim et al. (2010a) reported that bone marrow-derived stem cells (BMSC) and clavalial osteoblasts derived from *ank/ank* mice were slower to differentiate due to decreased expression of several genes. Kim et al. also reported a decrease in the “number and surface area” of osteoclasts in *ank/ank* mice. Overexpression of ANKH and CMD associated mutant F376del into pre-osteoblastic MC3T3-E1 cells increased their rate of differentiation. As they also measured no PPi transport for the F376del mutant they concluded by relating ANKH function in osteoblastic and osteoclastic differentiation to be mediated by interactions not reliant on only PPi concentrations and that ANKH is a positive regulator of these events (Minashima et al. 2010). A similar observation by Kim et al. (2010b) was made in chick tibial growth plate chondrocytes where overexpression of ANK also increased expression of hypertrophic differentiation markers – *Apase*, *runx2* and *type X collagen* (Kim et al. 2010b). Chen et al. (2011) supported these findings in a mouse model of the CMD associated F377del ANKH mutant where they measured decreased expression of pro-differentiation markers and impaired osteoblastogenesis and osteoclastogenesis (Chen et al. 2011).

Interestingly, it is evident that some contradicting results within this area of ANKH function are present. Kim et al. (2010a, 2010b) and Chen et al. (2011) have shown increased ANKH to increase bone cell maturation and loss of ANKH to decrease bone cell

maturation. However, this would contradict Johnson et al. (2008) as they reported increased differentiation potential in *ank/ank* mice. Perhaps, the two different biological systems are reflective of these contradictions but neither offered mechanistic evidence of ANKH function as a direct link to altered bone cell maturation.

A recent proof of concept study by Chen et al. (2017) introduced the F377del mutation into human induced pluripotent stem cells (hiPSCs) and corrected an existing S375del from an affected donor. They observed decreased differentiation of hiPSCs expressing CMD mutants into osteoclasts compared to wild type ANKH expressing hiPSCs. The mutant expressing hiPSCs also reabsorbed less bone chips compared to wild type. F377del expressing hiPSCs was more severe in attenuating differentiation compared to S37del expressing hiPSCs. However, S375del expressing hiPSCs also significantly decreased expression of osteoclast differentiation markers - *TRAP*, *CTSK*, *CALCR* and *NFATc1* (Chen et al. 2017).

1.3 – Diseases associated with ANKH

ANKH mutations cause two distinct conditions - calcium pyrophosphate dihydrate deposition disease (CPPDD [MIM118600]) and craniometaphyseal dysplasia (CMD [MIM123000]) with association to recessive mental retardation, deafness and ankylosis syndrome (MRDA) (Morava et al. 2011, Nürnberg et al. 2001, Williams et al. 2002). CPPDD typically presents with destructive arthritis, including chondrocalcinosis in about 50% of cases; it may mimic rheumatoid arthritis, gout or osteoarthritis and is the commonest form of inflammatory monoarthritis in the elderly, occurring in up to 40% of those over 65 years of age. Among individuals undergoing total hip replacement for the clinical diagnosis of osteoarthritis, about 50-60% patients had evidence of CPPDD (Abhishek and Doherty 2011, Zhang and Brown 2005). In contrast, CMD is a rare disorder

characterised by hyperostosis/sclerosis of the skull and abnormal modeling of the long bones. Individuals with severe forms of CMD can have reduced life expectancy as a result of compression of the foramen magnum (Nürnberg et al. 2001). CMD is caused by decreased ePPi, which allows increased HA deposition and altered bone modeling via chondrogenesis, osteoblastogenesis and osteoclastogenesis (Minashima et al. 2010). Patients affected by the recessive MRDA syndrome present a particularly severe phenotype of early onset ankylosis, mental retardation, deafness and possible hypophosphatemia (Morava et al. 2011). There is currently no specific treatment for CPPDD and CMD (NICE Guidelines – Accessed 12/08/18).

1.3.1 – CMD

A family study over five generations by Taylor and Sprague is widely regarded as the first clinical study of CMD. Published in 1989, the study described abnormal bone features presented by a two month old male infant being investigated for a nasal obstruction. Further investigation of the family revealed five affected members over four generations (including the infant and one unaffected male in generation five). At the time of publication the proband was aged nine and displayed classic CMD features - paranasal bossing, prominent nasal bridge, ocular hypertelorism and right facial palsy. Taylor and Sprague concluded their study by stating – “Greater understanding of the pathophysiology of this disorder is required before it can be adequately treated. Identification of the abnormal gene and the factors affecting its expression hold the key to future management” (pg 88, Taylor and Sprague 1989).

In a letter to the editor, Nürnberg et al. (2001) followed on from the successful identification of the *Ank* gene in mouse by Ho et al. (2000) a year earlier and conducted genetic analyses of *ANKH* gene in 9 unrelated families. They discovered single nucleotide

polymorphisms (SNPs) in 8 of these families resulting in four missense mutations, three single amino acid deletions and one presumed single amino acid insertion due to the formation of a new splice acceptor in intron nine. These were – W292R (Family 1), C331R (Family 2), S375del (Family 3), F377del (Families 4 and 5), P380insA (Family 6) and G389R (Families 7 and 8) (Nürnberg et al. 2001). Reichenberg et al. (2001) only 3 months later published a similar study using similar methods and described CMD in 5 families with autosomal dominant inheritance and 5 families with sporadic inheritance. They reported 2 families (C and F) with F377del, 2 families (D and S) with S375del and family G with P380insA (Reichenberger et al. 2001). These were the first studies to clearly define mutations within the *ANKH* gene to be causal in patients with CMD and interestingly all are located on predicted cytosolic sections of ANKH within the transmembrane regions.

The next descriptions of novel CMD associated ANKH mutants were by Kornak et al. in 2010. They described a clinical phenotype of macrocephaly, hyperthyroidism, skull hyperostosis, skull sclerosis, cranial nerve compression (patients 1 and 2), hearing loss, paranasal bossing, metaphyseal flaring, and metaphyseal translucency (patients 1 and 2). These were associated with mutations within the *ANKH* gene – L334R, C339R and L391P (Kornak et al. 2010). In the same year Zajac et al. (2010) described a complex mutation consisting of two substitutions and one large deletion in a sporadic case of CMD – Y290Q and W292-E294del (Zajac et al. 2010). The patient displayed classic CMD features as described by Taylor and Sprague. Lastly, the most recent addition to the growing list of CMD associated ANKH mutants was by Dutra et al. in 2012, they reported – V285-Y290del (patient 1) and T393-K396del (patient 2). Both patients had severe phenotypes but the authors noted that the sizes of the deletions did not correlate with specific CMD phenotypes (Dutra et al. 2012) (Figure 5).

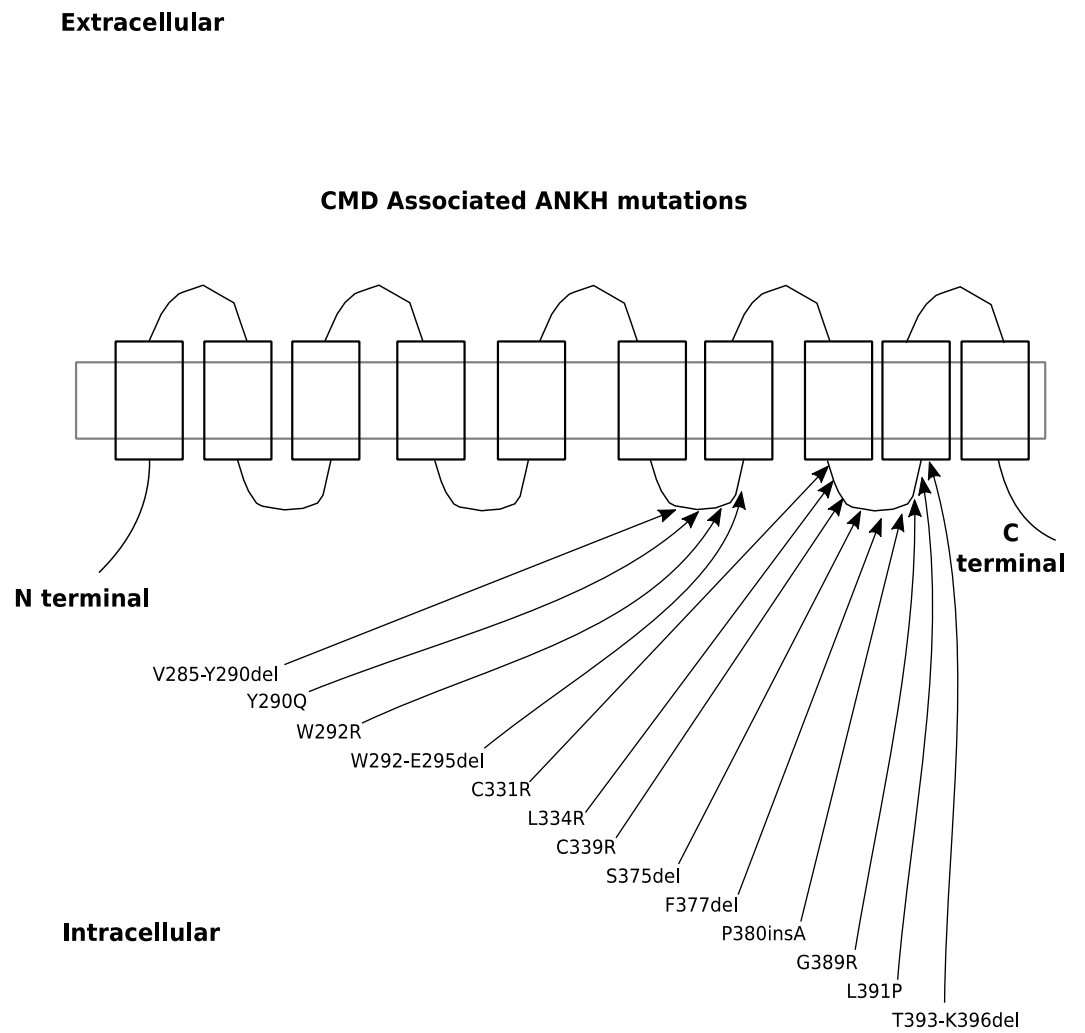


Figure 5 – Schematic representation of CMD associated mutant locations on ANKH.

1.3.1.1 – Molecular mechanism of CMD associated ANKH mutants

There is limited evidence regarding mechanistic effects of CMD associated ANKH mutant and much of the literature has focused on description of the clinical phenotype with little or no mechanistic determination. One study by Gurley et al. (2006) injected frog oocytes with mutants C331R and G389R and measured reduction of ANK mediated PPi transport, and the authors gave inclination to reduced membrane expression of the C331R mutant as a potential mechanistic phenotype responsible for the decrease in ePPi (Gurley et al. 2006). Zajac et al. (2010) when reporting the complex CMD mutation Y290Q and W292-E294del observed decreased ANKH expression at the cell membrane in COS-7 cells but the molecular mechanisms were not reported (Zajac et al. 2010).

1.3.2 – CPPDD

Designated chondrocalcinosis articularis by Aschoff et al. in 1966 the disorder was manifested clinically by episodic inflammation of the joints and calcification of the hyaline and fibrous cartilage. Reginato et al. in 1970 observed novel cases among natives of the Chiloe Island group that displayed a novel phenotype - ankylosis of joints (Reginato et al. 1970). Rodriguez-Valverde et al. (1980) conducted a major study of first-degree relatives of 46 cases in Spain and found 5 affected families. These families all showed calcified cartilage under X-ray examination (Rodriguez-Valverde et al. 1980). In a further study Rodriguez-Valverde et al. in 1988 found support for autosomal dominant inheritance (Rodriguez-Valverde et al 1988). In 1991, Doherty et al. reported familial CPPDD due to abnormal CPPD crystal deposition in 5 unrelated English kindred. The largest pedigree also suffered from benign fits in early life. The pattern of inheritance was further suggested to be autosomal dominant with 100% penetrance (Doherty et al. 1991). Later, in 1995, Hughes et al. further described the clinical phenotype in this large English family, which

included recurrent seizures and acute pseudogout with CPPD deposition in joints. Most importantly, the authors screened the genome of this large English family, with affected members over four generations, and mapped the responsible gene to chromosome 5p (Hughes et al. 1995). Andrew et al. in 1999, supported this mapping in a study of 2 families (French and Argentinian) and mapped the CPPDD locus to 5p15 (Andrew et al. 1999).

The first study to directly relate CPPDD to ANKH was conducted by Pendleton et al. in 2002 in which they screened affected family members from the Hughes et al. and Andrew et al. studies and found 3 mutations within the *ANKH* gene – M48T (French family), -11C-T (English family) and E490del (sporadic English patient) (Pendleton et al. 2002). The M48T mutation was predicted to be in the first transmembrane domain, whilst the -11C-T mutation creates a new ATG start codon and adds four additional residues to the ANKH protein and E490del was predicted to be in the cytoplasmic C-terminal region. Next, Williams et al. described the P5L mutant in 2002 (Williams et al. 2002), for the Argentinian family from Andrew et al. and this was quickly followed by Williams et al. in 2003 that described the P5T mutation in two American families (Williams et al. 2003). They also commented on position 5 in the ANKH protein being a “hot spot for mutation”. Predictably, another point mutation at residue 5 has been described by Gruber et al. in 2012 – P5S (Gruber et al. 2012). Apart from the usual mineralization of cartilage and joints, the patient presented with mineralization of the vertebrate disc, which is rare for sporadic cases but has been described in familial CPPDD. A novel polymorphism upstream on the *ANKH* gene within its promoter region termed -4G-A was shown to increase expression of ANKH protein and increase ePPi in CH-8 cells (immortalized chondrocytes). The same study also described molecular function in CH-8 cells of three previously identified ANKH mutants – P5L (increased expression of ANKH and type X collagen), M48T (no change in ePPi or genetic expression) and E490del (increased ePPi

and ANKH expression) (Zhang et al. 2005). Lastly, it was reported that the previously identified CMD associated G389R mutant was co-segregating in females diagnosed with early-onset CPPDD and although a chance association should not be excluded, this association highlights the complex nature of ANKH function in both CPPDD and CMD (Baynam et al. 2009) (Figure 6).

1.3.2.1 – Molecular mechanisms of CPPDD associated ANKH mutants

Pendleton *et al.* 2002, transfected COS-7 cells with ANKH mutants -11C-T, M48T and E490del and measured a decrease in iPPi (Pendleton et al. 2002). Zhang et al. (2005) in addition to the above mentioned ePPi and ANKH expression changes, also described ENPP1 and TNAP in relation to ANKH mutants (-4G-A, P5L, M48T and E490del) but found no significant changes in expression for any mutant (Zhang et al. 2005). In contrast, Zaka et al. (2006) has shown increased ePPi in stably transduced mouse chondrocyte ATDC5 cells for the P5L mutant and the authors postulated to an increase in ENPP1 activity as the cause but not TNAP activity. The authors also found no change in ePPi or activity of ENPP1 and TNAP for either the P5T or M48T transduced ATDC5 cells (Zaka et al. 2006). Gurley et al. (2006) measured similar rates of PPi transport in frog oocytes and noted that the M48T mutant was able to rescue the destructive phenotype seen in *ank* mice. The authors preferred the gain-of-function phenotype for CPPDD associated mutants but were not able to experimentally demonstrate ePPi increase for CPPDD mutants compared to wild type (Gurley et al. 2006). Wang et al. (2008) reported decreased activity but not expression of TNAP in ATDC5 cells transfected with the E490del mutant compared to wild type suggesting ANKH modulates TNAP activity at its C-terminal region (Wang et al. 2008). A year after, Wang et al. (2009) conducted a complex study on ATDC5 cells to study effects of the M48T mutant on ENPP1, TNAP and PIT-1 and showed altered expression (increased *ENPP1* and *TNAP*, decreased *PIT-1*)

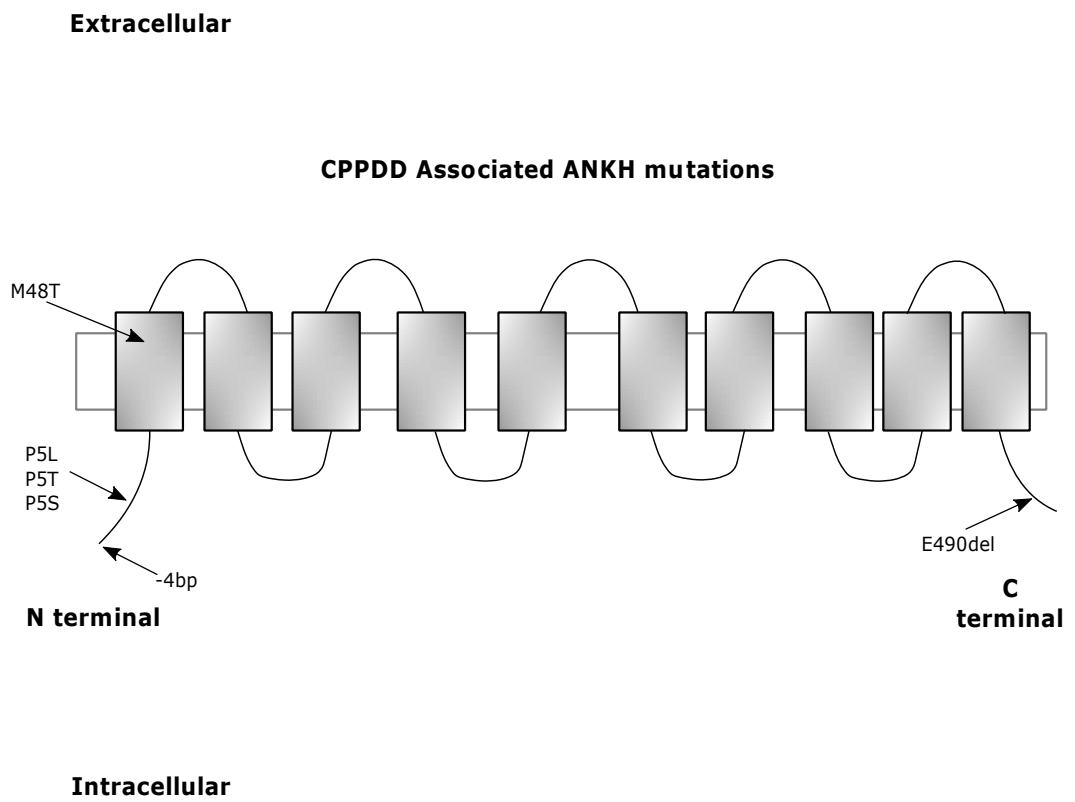


Figure 6 - Schematic representation of CPPDD associated mutant locations on ANKH.

and activity (increased TNAP). However, they concluded by stating that because in wild type control ATDC5 cells expression of *ANKH* and *PIT-1* was increased in co-ordination when they treated with Pi, the observation that *PIT-1* expression was not increased in M48T transfected ATDC5 cells in co-ordination with *ANKH* under the same conditions was due to altered interactions between M48T mutant ANKH and PIT-1 (Wang et al. 2009).

1.3.3 – Other disease associated with ANKH

There are three other diseases associated with ANKH, however none have been studied in much depth, unlike CMD and CPPD. They are – ankylosing spondylitis, cuff tear arthropathy and mental retardation, deafness and ankylosis (MRDA).

1.3.3.1 – Ankylosing spondylitis

Briefly, ankylosing spondylitis was first associated to ANKH in 2003 by Tsui et al. (2003) via genetic linkage analyses of genomes from patients at Toronto Western Spondylitis Clinic and NASC (Tsui et al. 2003). Discovery of 2 regions in the 5' UTR termed ANKH-OR (5' non-coding region) and ANKH-TR (promoter region) were in complete linkage disequilibrium and gave a lambda of 1.9. No mechanistic or clinical data was presented. A more recent study by Liu et al. (2013) also tested TNAP association to ankylosing spondylitis along with ANKH, however they found no susceptibility loci in TNAP but described a single significant SNP (rs26307) for ANKH within an intronic region (Liu et al. 2013).

1.3.3.2 – Cuff tear arthropathy

Briefly, cuff tear arthropathy describes, “chronic massive rotator cuff tear, proximal humeral migration, recurrent joint effusions, and destruction of the joint with collapse of the humeral head” due to osteopenia (low bone density) and altered cartilage structures. Peach et al. (2007) recruited patients from Nuffield Orthopedic Centre in Oxford and directly sequenced *ANKH* and *TNAP* for SNPs associated with cuff tear arthropathy. SNP rs3045 was located within the 3' UTR of *ANKH*, 31 bases after the stop codon, and was more common in patients with cuff tear arthropathy (45%) compared to the control population (20%). Expression of this SNP in CH-8 cell revealed decreased iPPi. SNP rs4654760 was located within intron 10 of *TNAP* and was more common in cuff tear arthropathy patients (32%) compared to control (9%) (Peach et al. 2007).

1.3.3.3 - MRDA

Although only a single study has reported a case of MRDA, the phenotype was determined to be significantly different from CMD and CPPD. The study reported association of mental retardation, deafness and ankylosis with abnormal bone metabolism in a large consanguineous family to the presence of an autosomal recessive L244S *ANKH* mutation. Briefly, the mutation does not affect ePPi in the serum of affected family members and no mechanistic evidence is available for this mutant yet. However, the clinical description also reported a decrease in cartilage calcification and osteopenia both phenotypes somewhat resembling cartilage and bone in *ank/ank* mice (Morava et al. 2011).

1.3.4 – Further research into ANKH molecular function in the pathogenesis of CPPDD and CMD

There is ample evidence to support ANKH function as a homeostatic regulator of PPi concentration in the ECM. However, the mechanisms in which mutants associated with CMD, CPPDD and other related diseases facilitate pathogenesis is still unclear. There is progress in understanding regulation of ANKH and its effects on ossification related mechanisms, however studies have mostly commented on endogenous ANKH function. ANKH function as a PPi transporter has been alluded to in several studies (Gurley et al. 2006, Wang et al. 2008, Wang et al. 2009). However, due to the lack of a crystal structure no positive identification of ANKH function in physical transport of PPi or pore function can be elucidated. The importance of ANKH is directly related to its mutations in patients with CMD, CPPDD and CMD, and hence further mechanistic work describing mutant ANKH processing, translocation and protein-protein interactions, especially ENPP1, TNAP and PIT-1 is needed. How does ANKH facilitate ENPP1 functional activity? Is there a PPi regulatory domain in the cytoplasm that involves steric interactions of ANKH with ENPP1, TNAP and PIT-1? Perhaps mutations in ANKH assert their phenotypes through abnormal compensation in this domain? Several regulatory pathways in ANKH are shared with autophagy and autophagic regulation of mis-folded or non-functional proteins is well established, therefore are there roles for autophagy in the pathogenesis of ANKH associated diseases?

Chapter 2

Aims and Methods

2 – AIMS AND METHODS

2.1 – Aims of this study

2.1.1 – Benefits of understanding mutant ANKH molecular mechanisms in CMD and CPPDD

The fundamental effects of altered PPi in the pathogenesis of CMD and CPPDD has been well established and there is clear evidence for a role of ANKH protein in the homeostasis of PPi in the ECM. However, the majority of evidence of ANKH function is through the understanding of its ultimate pathological manifestation – altered bone formation (CMD) and increased deposition of CPPD crystal (CPPDD) that is caused by the distorted PPi concentrations in the ECM. There is still no clear evidence of a PPi transport function, however it would be reasonable to assume that ANKH function at the cell membrane to increase ePPi is mediated through some sort of passive or active channel property. Furthermore, how ANKH function is mechanistically altered in mutations associated with CPPDD or CMD remains largely unclear. The lack of a crystal structure limits the consequential inferences made by predicted structures and ultimately limits our potential to fully understand ANKH mutant function.

In the ECM of bone forming cells there is evidence for an interacting complex of proteins, comprising of ENPP1, TNAP and PIT-1 that together with ANKH exert physiological control over ePPi concentrations. Experimental evidence of mutated ANKH attenuating or increasing activity and function of ENPP1, TNAP or PIT-1 has also provided direction in better understanding the gain and loss of function phenotypes seen in CMD and CPPDD patients (see section 1.1.9). However, the lack of targeted treatments for ANKH related

CMD and CPPDD highlights the need for better molecular and mechanistic understanding of mutated ANKH.

2.1.2 – Experimental strategy for studying *ANKH* molecular function in CPPDD and CMD

Thus, we hypothesise that mutations within *ANKH* alter essential processing, targeting and functional regulatory domains, and generate non-functional or mis-folded ANKH proteins. Hence, causing differential localisation, pathological changes in ePPi concentration and calcium mineralization and influence expression and activity of ENPP1, TNAP and PIT-1.

There is no published data comparing differential protein localisation of mutant ANKH proteins and their effects on ePPi concentrations and mineralization. The first part of this thesis will visualise the effect of CMD and CPPDD associated ANKH mutants on the localisation of ANKH to cell membrane. Next, we will quantify ePPi and mineralization in CH-8 and HEK293 cells transfected with mutant ANKH and establish a novel and direct link between differential localisation dynamics and altered ePPi leading to pathological mineralization. Previous evidence of ANKH influence on ENPP1, TNAP and PIT-1 expression and function fails to compare and contrast mutant effect over time; therefore we will compare these interactions in more detail whilst contrasting gain and loss of function molecular phenotypes over 72 hours. Given that mutations within *ANKH* generate non-functional or mis-folded proteins we will evaluate their co-localisation with autophagic machinery for evidence of their presence and elucidate novel mechanisms as targets for potential future therapies. Therefore, this thesis will answer whether ANKH mutations cause differential localisation of ANKH protein, and how overexpression of mutant ANKH affects ePPi, mineralization and expression and activity of ENPP1, TNAP and PIT-1.

2.2 – Materials and methods

2.2.1 – Generation of fluorescently tagged GFP *ANKH* and mutant constructs

A full description of generation for each construct is listed in Table 1 (page 46) describing the cloning method, template source, primers and conditions used, plasmid inserted into and restriction enzyme specifics. Several cloning methods were used to create cDNA containing enhanced green fluorescent protein (eGFP) expression constructs for ANKH (NM_054027.4). ANKH mutants were generated using site-directed mutagenesis of the ANKH construct. Dr Yun Zhang provided ANKH cDNA in a pcDNA3.1 vector template (Figure 7A) that was sequenced to confirm exact nucleotides. For visualization of ANKH localisation, cloning into an eGFP expression vector was necessary. We used pEGFP-N3 (Figure 7B) and pEGFP-C1 (Figure 7C) vectors to create C-terminal and N-terminal eGFP constructs for ANKH (provided by Dr Yun Zhang). ANKH cDNA was confirmed to align to Refseq sequence: NM_054027.5 (Figure 8).

2.2.1.1 – Primers

Primer pairs were designed using either Primer3 (Untergasser et al 2012) software or online tool NEBuilder¹ to amplify specific fragments of sequenced pcDNA3.1 plasmids containing cDNA of ANKH. Simple guidelines were followed to ensure good primer characteristics: a similar adenine (A) / thymine (T) to guanine (G) / cytosine (C) ratio, with G or C residues at the 5' and 3' ends to keep fragments in-frame whilst restriction digesting and increase extension stability; avoidance of internal homology and homology between primer pairs to prevent binding to self or primer dimerisation; and similar melting temperature (T_m) of each member of the pair. Primer pairs were verified using the

¹ nebuilder.neb.com

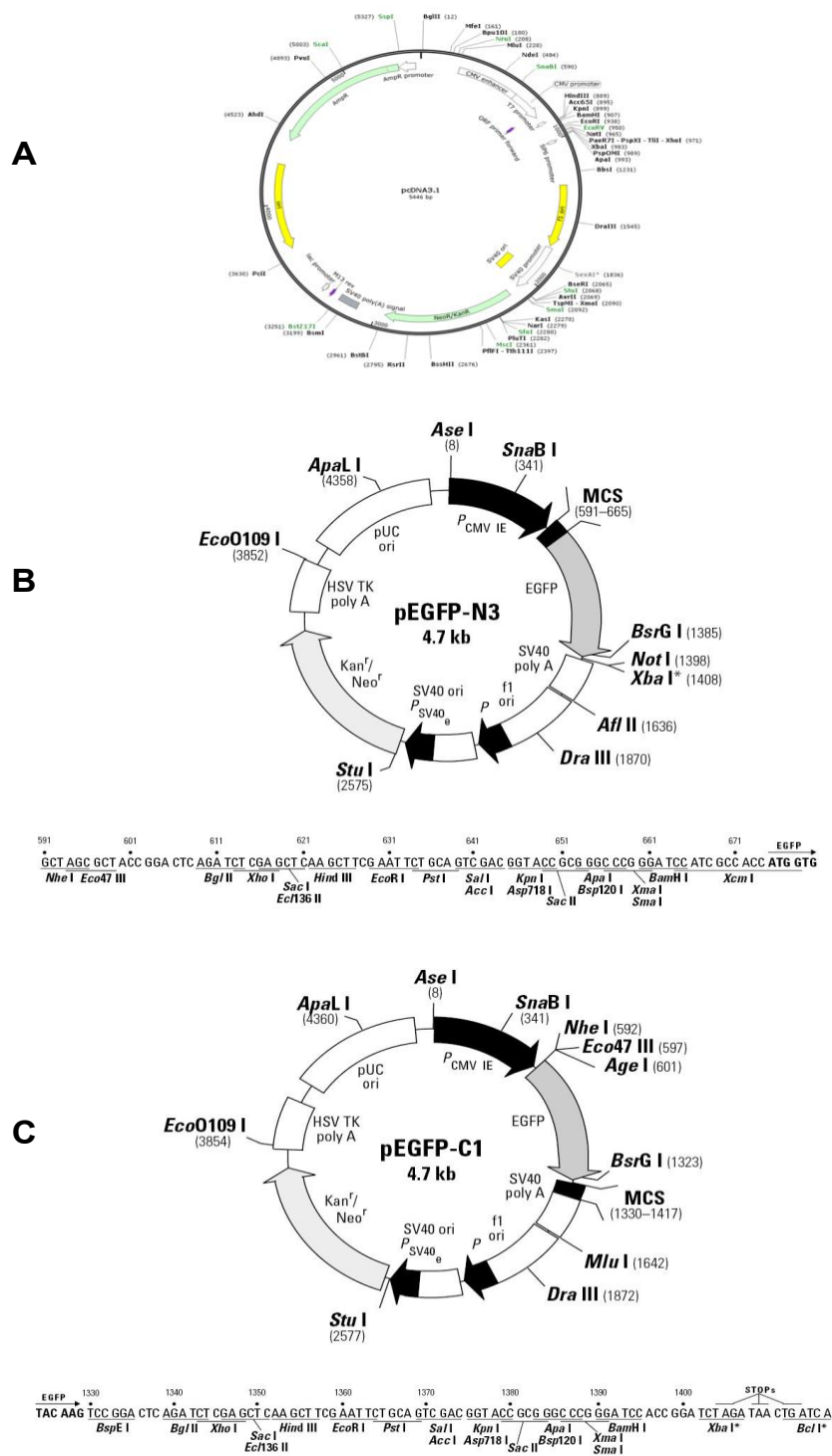


Figure 7 – Plasmid maps of (A) pcDNA3.1 (B) pEGFP-N3 (C) pEGFP-C1.

Multiple cloning sites (MCS) region is enlarged for pEGFP-N3 and pEGFP-C1 to highlight location of restriction sites used in cloning.

pcDNA3.1_ANKH

Homo sapiens ANKH inorganic pyrophosphate transport regulator (ANKH), mRNA

Sequence ID: [NM_054027.5](#) Length: 8231 Number of Matches: 1

Range 1: 339 to 572 [GenBank](#) [Graphics](#)

▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand		
433 bits(234)	5e-119	234/234(100%)	0/234(0%)	Plus/Plus		
Query 140	ATGGTGAAATTC	CGGCGCTCACG	CACTACTGGCC	CTGATCCGGT	CTTGGTGCCCTG	199
Sbjct 339	ATGGTGAAATTC	CGGCGCTCACG	CACTACTGGCC	CTGATCCGGT	CTTGGTGCCCTG	398
Query 200	GGCATCACCAAC	ATAGCCATCGA	CTTCGGGGAGC	AGGCCTTGAAC	CGGGGCATTGCTGCT	259
Sbjct 399	GGCATCACCAAC	ATAGCCATCGA	CTTCGGGGAGC	AGGCCTTGAAC	CGGGGCATTGCTGCT	458
Query 260	GTCAAGGAGGAT	GCGAGTCGAG	ATGCTGGCCAG	CTACGGGCTGG	CGTACTCCCTCATGAAG	319
Sbjct 459	GTCAAGGAGGAT	GCGAGTCGAG	ATGCTGGCCAG	CTACGGGCTGG	CGTACTCCCTCATGAAG	518
Query 320	TTCTTACGGGT	CCCATGAGTG	ACTTCAAAAAT	GTGGGCCTGGT	GTTTGTGAAC	373
Sbjct 519	TTCTTACGGGT	CCCATGAGTG	ACTTCAAAAAT	GTGGGCCTGGT	GTTTGTGAAC	572

Figure 8 – Sequence alignments of pcDNA3.1_ANKH confirmed alignment to NM_054027.5 ANKH mRNA.

in-silico PCR² online tool. Designed primers were custom generated by Life Technologies (currently known as Thermo Fisher) and were received diluted in desalted buffer at 100 μ M, which were diluted into a working concentration of 10 μ M using the supplied dilution buffer and stored at -20°C. Complete list of primers and conditions are stated in Table 1.

2.2.1.2 – Polymerase chain reaction (PCR)

Exponential amplification of cDNA via polymerase chain reaction (PCR) was performed using the Thermo Scientific Extensor Long PCR 2X Master Mix (Thermo Fisher) that includes a high fidelity *Taq* DNA polymerase that has been modified to prevent mis-priming and formation of primer dimers at low temperatures and appropriate concentrations of MgCl₂ and free nucleotides. In a total reaction volume of 25 μ L consisting of 12.5 μ L 2X Extensor Master Mix, 0.5 μ L each forward and reverse primer (10 μ M), 100 ng of template DNA and made to 25 μ L with nuclease free water was thermal cycled along with appropriate negative controls – only forward primer, only reverse primer, no primers, no template (Table 1). All reactions were performed in 0.2 mL microfuge tubes.

The reaction tubes were placed in a Bio-Rad T100 thermal cycler (Bio-Rad) and heated to 94°C for 2 mins allowing initial activation of the DNA polymerase and complete denaturation of plasmid DNA. The three-step process of denaturation, annealing and extension involved in PCR was then started. The reaction mixture was heated for 10 secs at 94°C in order to denature the DNA template strands. The temperature was then lowered to a specific optimum annealing temperature (Table 1) for 30 secs to allow primer binding to their complementary DNA sequences on the single target strands (annealing). Finally, the temperature was raised to 68°C for 90 secs to allow the DNA polymerase enzyme to

² genome.ucsc.edu/cgi-bin/hgPcr

Table 1 – Construct generation

EcoR1-HF (NEB –R3101). BamH1-HF (NEB –R3136)

Plasmid Name	Method	Template	Forward Primer	Reverse Primer	Primer Ta	Restriction Enzymes	Restriction Enzyme Buffer	Ligation Ratio
pEGFP_N3-ANKH	Restriction Cloning	pcDNA3.1-ANKH	ATAAGAATTCATGG TGAAATTCCTGGCG	GCTCGGGATCCTTCA TTCTCCTCTCTCAT	68°C	EcoR1-HF BamH1-HF	Cutsmart	5:1
pEGFP_C1-ANKH	Restriction Cloning	pcDNA3.1-ANKH	ATAAGAATTCATGG TGAAATTCCTGGCG	GCTCGGGATCCTTCA TTCTCCTCTCTCAT	68°C	EcoR1-HF BamH1-HF	Cutsmart	7:1
pEGFP_N3-P5L	Q5 mutagenesis	pEGFP_N3-ANKH	GTGAAATTCCTGGCG CTCAGC	CATGGAATTCGAAGC TTGAGC	63°C	NA	NA	NA
pEGFP_N3-S375del	Q5 mutagenesis	pEGFP_N3-ANKH	TTCTTCCCAGTTCCA GTC	GAAGATCCGCAAAG GAAC	66°C	NA	NA	NA
pEGFP_N3-G389R	Q5 mutagenesis	pEGFP_N3-ANKH	GCATCTCACCAGGTG GCTGATG	GCCCTCACTGTGACT GGA	68°C	NA	NA	NA
pEGFP_N3-E490del	Q5 mutagenesis	pEGFP_N3-ANKH	AATGAAAATGGATCC ATCGC	CTCTCTCATTTCCACG ATG	65°C	NA	NA	NA

extend the primers, using the target strand as template (extension), producing a new complementary DNA strand of the original strand. This three-step process was carried out for 40 cycles generating an exponential increase in the number of copies of target sequence. At the end of the reaction, the extension time was lengthened to 7 mins and then samples were cooled to 4°C for storage.

2.2.1.3 – Agarose gel identification and purification of amplified *ANKH*

Agarose gel was used to separate and identify amplified fragments of DNA after PCR. Briefly, agarose gel electrophoresis separates macromolecules such as DNA according to fragment length and is commonly used to concentrate specific DNA fragments for use in further down stream applications such as cloning. Percentage of agarose (a polysaccharide that forms pores when gelatinous) in the gel matrix determines the resolution of separation and is generally between 0.7-2% dissolved in an appropriate electrophoresis buffer. The action of electrophoresis drives electrical current through the buffer, in which the agarose gel is submerged, and pulls negatively charged DNA molecules towards the positive terminal. Staining with an intercalating agent (commonly ethidium bromide) fluoresces DNA trapped within the agarose gel matrix and allows for easy identification of DNA fragments.

Following PCR amplification, 10 µL of each reaction was mixed with 2 µL 6X Gel Loading Dye, Purple (NEB – B7024S). The samples were then loaded into the wells of a 1% agarose gel dissolved in 1X TBE buffer (Bio-Rad – 1610770) containing a final concentration of 0.5 µg/mL ethidium bromide. PCR products were electrophoresed alongside a Quick-Load Purple 1kb DNA Ladder (NEB – N0552S) at 75V for approximately 1 hour in 1X TBE buffer. Gels were viewed using a UV transilluminator (Bio-Rad) and either a printed or digital image was obtained. PCR products required for

subsequent cloning and sequence analysis were excised under UV light using a clean scalpel, and DNA extracted and purified using the QIAquick Gel Extraction Kit (Qiagen – 28704). Extracted DNA was eluted into 30 µl of supplied buffer EB, quantified using NanoDrop One (Thermo Fisher) and stored at -20°C.

2.2.1.4 – Restriction enzyme digestion of purified *ANKH*

Restriction sites (6-8 bases) are abundantly present in genomic DNA and are specifically recognized by unique restriction enzymes that cleave these palindromic sequences of DNA to leave DNA overhangs termed ‘sticky ends’. By primer design PCR amplification of *ANKH* added specific restriction sites to the 5’ and 3’ end. After gel extraction, in a 50 µL total reaction, 1 µg each DNA fragment was incubated with the appropriate concentration of each restriction enzyme (Table 1) in the appropriate reaction buffer (Table 1) for double digestion for approximately 1 hour at 37°C. Enzymes were purchased from NEB and recommended double digest buffers were found using the NEB Double Digest Finder tool³. Control reactions containing single restriction enzymes, no enzymes or no DNA were run concurrently. After digestion was complete samples were purified using the QIAquick PCR Purification Kit (Qiagen – 28104) to remove reaction buffer, enzymes and excess nucleotides and eluted into 30 µL of supplied buffer EB and stored at -20°C. The use of unique 5’ and 3’ restriction sites meant orientation of the DNA fragment (insert) was correct when ligated into the vector.

³ nebcloner.neb.com

2.2.1.5 – Ligation of *ANKH* into pEGFP-C1 and pEGFP-N3 vectors

Determination of insert to vector ratio was essential in obtaining successful ligation. The online tool NEBioCalculator⁴ easily calculated the required quantities of insert to vector and several attempts using different ratios were needed to successfully clone ANKH into pEGFP-N3 and pEGFP-C1 vectors (Table 1). Generally, a 5:1 ratio was the starting point, in that there are five times as many unique inserts to unique vectors. Ligation was performed using T4 DNA Ligase (NEB – M0202S); an enzyme that catalytically forms the phosphodiester bond between complementary ‘sticky ends’ and seals the ‘nicks’ in the formed single strands. The 20 µL total reaction contained 2 µL 10X T4 DNA Ligase Buffer (NEB – M0202S), 1 µL T4 DNA Ligase, 50 ng digested and purified vector (either pEGFP-N3 or pEGFP-C1), calculated volume of insert according to NEBioCalculator (Table 1) and made up to 20 µL with nuclease free water. Control reactions containing only insert, only vector or no T4 DNA Ligase were incubated concurrently. After gently pipetting and centrifuging the mixtures they were incubated at 16°C for 12 hours, heat inactivation of T4 DNA Ligase at 65°C for 10 mins and then stored at -20°C or stored on ice for immediate transformation into NEB 5-alpha Competent *Escherichia coli* (*E. coli*) cells (NEB – C2987H).

2.2.1.6 – Transformation of constructs into NEB 5-alpha Competent *E.coli*

Competent cells, such as NEB 5-alpha Competent *E.coli* (NEB), are special derivatives of *E.coli* that readily accept foreign DNA under certain temperature conditions. The cells are allowed to outgrow and amplify the foreign DNA for use in downstream application such as transfections. Following heat inactivation, ligation mixtures were cooled in ice along side thawing 50 µL pre-aliquot NEB 5-alpha. Importantly, sufficient time was given for all

⁴ nebiocalculator.neb.com

ice to melt in the aliquots of NEB 5-alpha cells and once both the ligation mixture and NEB 5-alpha cells were sufficiently equilibrated (~5 min), 2 μ L of the ligation mixture was added to 50 μ L pre-aliquot of NEB 5-alpha cells and carefully mixed by gently flicking the tube four-five times. The mixture was placed back into ice, stationary for 5 min after which the tube was rapidly placed into an exact 42°C water bath for exactly 40 secs and directly placed back into the ice for another stationary 2 mins. Next, 950 μ L of Super Optimal broth with Catabolite repression (SOC) Outgrowth Media (NEB – B9020S) was added to the tubes and placed into a shaking (150 rpm) incubator at 37°C for 1 hour. The function of SOC Outgrowth Media is to drive expression of bacterial antibiotic resistance gene, encoded within the transformed construct (Hanahan 1983). Normal bacterial growth media (Lysogeny Broth (LB) media) does not facilitate this expression and can result in lower transformation efficiency when plating onto antibiotic selection agar plates. The same procedure was used to transform constructs pmRFP-LC3 (Addgene - 21075), CFP-LC3 (Addgene – 21073), GFP-ULK1 (Kind thanks to the Sharon Tooze group at the Crick Institute, London) and YFP-GalT (Addgene – 11931).

2.2.1.7 – Antibiotic selection and growing transformed NEB 5-alpha Competent *E.coli*

Antibiotic selection is a simple and robust method of selecting positive transformed bacterial colonies. NEB 5-alpha cells that were successfully transformed with a complete circular construct after ligation would be able to express an antibiotic resistance gene, commonly kanamycin or ampicillin. NEB 5-alpha cells not transformed with a complete circular construct would therefore not grow on LB:Agar plates containing these antibiotics. During SOC media outgrowth, LB:Agar plates containing either 50 μ g/mL kanamycin or 100 μ g/mL ampicillin were poured and allowed to cool naturally under a blue flame. Simply, LB:Agar contains nutrients and macromolecules required for optimum bacterial growth whilst stabilising colonies on a semi-solid surface. Once, the SOC media

outgrowth was complete, 100 μ L of the resulting bacterial growth was aseptically pipetted onto the cooled LB:Agar plate containing the correct antibiotic (Table 1), evenly spread across the surface using an aseptic spreader. After drying for five mins under a 'blue flame' to prevent contamination, plates were placed upside down into a 37°C incubator overnight to facilitate colony formation of transformed NEB 5-alpha cells.

2.2.1.8 – Purification of *ANKH* constructs from NEB 5-alpha Competent *E.coli*

Single colonies were picked and transferred into 5 mL LB media containing appropriate antibiotics, either 50 μ g/ml kanamycin or 100 μ g/ml ampicillin. LB media, a constituent of LB:Agar, is commonly used to grow bacteria such as *E.coli* and contains nutrients such as peptides, vitamins and minerals. After incubation overnight at 37°C, a small aliquot was mixed in a 1:1 ratio with glycerol and stored long term at -80°C. The remaining bacterial culture was transferred to a large flask containing 250 mL LB media with appropriate antibiotics and grown overnight again at 37°C. After the bacterial culture was confluent and resembled a more viscous liquid, it was transferred to 50 mL falcon tubes and centrifuged for 30 mins at 4200 x g in a table top temperature controlled centrifuge cooled to 4°C.

Discarding the supernatant, a light yellow pellet of bacteria was observed. To purify the construct from the bacteria, the commonly used Qiagen bacteria-prep anion-exchange resin column protocol was used. There are three varieties of this protocol, and they differ only in starting bacterial culture volume and reagent volume. Only the most common used, "Midi" is described (Qiagen – 12143). Firstly, 4 mL of buffer P1 was added to the bacterial pellet. Buffer P1 (resuspension buffer) contains RNase A, LyseBlue, Tris-HCL and EDTA. Simply, this buffer allows resuspension of the bacterial pellet in an environment suitable for DNA by disabling nuclease activity and degrading RNA for better purity of DNA.

LyseBlue (thymolphthalein) is a common pH indicator and is used to determine the efficiency of the next alkaline lysis step. After a homogenous suspension was obtained with buffer P1, 4 mL of buffer P2 (lysis buffer) is added and the falcon tube was inverted four-five times and incubated at room temperature for 15 mins. LyseBlue turned the suspension a homogenous blue colour and indicated complete alkaline lysis, via sodium dodecyl sulfate (SDS) and sodium hydroxide (NaOH) degradation of bacteria cellular membrane. Next, 4 mL pre-chilled buffer P3 (neutralizing buffer) was added to the suspension, inverted four-five times and incubated on ice for 15 mins. The suspension turned homogeneously colourless indicating complete neutralization and efficient protein precipitation. Buffer P3 contains 3 M potassium acetate that precipitates SDS bound proteins. Centrifugation of the suspension at 15000 x g for 1 hour at 4°C pelleted the bacteria cellular debris and precipitated proteins and a clear supernatant was observed. Whilst centrifugation, the propriety anion-exchange resin column was equilibrated with buffer QBT. This MOPS, NaCl, Triton X-100 and isopropanol-containing buffer simply primes the resin for the incoming DNA by equilibrating pH and solubilizing the resin. After centrifugation, the clear supernatant was added to the resin column and allowed to enter via gravity flow. Positive charged diethylaminoethanol (DEAE) groups on the surface of the resin bound and fixed the negatively charged phosphate backbone of DNA. The next step removed unwanted bound RNA, proteins and other debris from the resin via a low-salt concentration wash with 20 mL buffer QC. DNA was then eluted from the column with a high-salt concentration wash with 5 mL buffer QF into a clean 15 mL falcon tube. DNA was precipitated by adding 3.5 mL isopropanol, mixing and then centrifuging at 15000 x g for 1 hour at 4°C. Carefully removing the supernatant revealed a small glossy pellet that was washed with 2 mL ethanol and centrifuged again at 15000 x g for 30 mins at 4°C. Finally, the supernatant was again carefully removed leaving behind a small white fluffy pellet that was left to air dry for 15 mins and then resuspended in 500 µL Tris-Cl EDTA (TE) buffer. Eluted DNA was quantified for purity and yield using

NanoDrop One and sent for sequencing (Eurofins Genomics). Sequencing confirmed correct insertion of ANKH into pEGFP-N3 and pEGFP-C1 vectors.

2.2.1.9 – Mutagenesis of pEGFP-N3-ANKH to generate P5L, S375del, G389R and E490del mutants

After sequencing, wild type ANKH constructs (Table 1) were mutated using Q5 Site-Directed Mutagenesis Kit (NEB – E0554S) with primers designed using the online tool NEBaseChanger⁵ (Table 1). A 25 μ L final reaction volume contained 12.5 μ L Q5 Hot Start High Fidelity 2X Master Mix, 1.25 μ L forward primer (10 μ M), 1.25 μ L reverse primer (10 μ M), 1 μ L ANKH construct (25 ng/ μ L) and was made up to 25 μ L with nuclease free water. Reactions were thermal cycled once at 98°C for 30 secs and then 25 cycles of 98°C for 10 secs, 60-66°C for 30 secs (see Table 1) and 72°C for 2 mins. A final single extension at 72°C for 2 mins ensured complete amplification of the construct. Next, 1 μ L of the PCR mixture was mixed with supplied 5 μ L 2X KLD Reaction Buffer, 1 μ L 10X KLD Enzyme Mix and 3 μ L nuclease free water and was incubated at room temperature for 10 mins. The KLD (Kinase-Ligase-DpnI) reaction circularizes the new mutated construct and removes the older template DNA, leaving the new mutant construct ready for transformation in NEB 5-alpha cells and mutant construct generation as described above. After purification and quantification mutant ANKH constructs were sequenced (Eurofins Genomics) for confirmation of correct mutations.

2.2.2 – Cell culture

Cell culture procedures were performed in class II vertical laminar flow hoods. Culture media, media supplements and general consumables were obtained sterile and treated with

⁵ nebasechanger.neb.com

extreme caution to prevent growth and spread of bacteria and other contaminations. Culture media and washing reagents were warmed to exactly 37°C in a dry beads bath and returned into the dry beads bath at regular intervals to avoid thermal stressing of cells. Before entering the laminar flow hood, all containers and gloves were thoroughly sprayed with 70% industrial methylated spirit (IMS). Cells were incubated at 37°C, with a humidified atmosphere of 5% CO₂ in air. Cells were passaged weekly and media replaced as necessary. Gibco supplied all cell culture reagents, unless otherwise stated.

2.2.2.1 – Cell lines

Immortalized cell lines are populations of cells that have been mutated to indefinitely proliferate. Several methods have been used but commonly infection with viral genetics allows partial deregulation of their cellular cycle and infinite reproduction. These cells are advantages in studying *in vitro* as they offer reproducible test conditions that can be altered simply via their cellular growth environment (growth media). Furthermore, immortalized cell lines are more homogenous in their representation compared to primary cultures that can often be invaded by other more rapidly proliferating cells.

2.2.2.2 – Human embryonic kidney cells

Human embryonic kidney (HEK293) cell-line were transformed in 1977 from primary cultures by sheared human adenovirus type 5 DNA (Russell et al 1977) and retained features of tubular epithelial cells (Simmons 1990). HEK293 cells were maintained in high glucose Dulbecco's modified eagle medium (DMEM), supplemented with L-glutamine (580 mg/mL), non-essential amino acids (10% v/v), fetal bovine serum (10%) and penicillin/streptomycin (1%). Passages five or six were used for experiments.

2.2.2.3 – Articular chondrocytes cells

Articular chondrocytes (CH-8) cells were established in 2001 by introduction of recombinant SV40 adenovirus vector containing SV40 early gene into clonal cells from a patient with osteoarthritis. Cytochemical and immunohistological staining showed that immortalized CH-8 cell were positive for TNAP and collagen type 2 indicating they retained their primary characteristics (Yoshimatsu et al 2001). CH-8 cells were maintained in high glucose Dulbecco's modified eagle medium (DMEM), supplemented with L-glutamine (580 mg/mL), non-essential amino acids (10% v/v), fetal bovine serum (10%) and penicillin/streptomycin (1%). Passages 8-16 were used for experiments.

2.2.2.4 – Sarcoma osteogenic cells

Sarcoma osteogenic (Saos2) cells were derived from the primary osteosarcoma of an 11-year-old girl in 1973 during an extensive series of human tumor cell-lines generation (Fogh et al 1977). Saos2 cells also possess several characteristics of osteoblasts (Rodan et al 1987) and are able to maintain an ECM suitable for mineralization (Hausser and Brenner 2005). Saos2 cells were maintained in high glucose Dulbecco's modified eagle medium (DMEM), supplemented with L-glutamine (580 mg/mL), non-essential amino acids (10% v/v), fetal bovine serum (10%) and penicillin/streptomycin (1%). Passages 5-10 were used for experiments.

2.2.2.5 – Serial passaging

Cells were sub-cultured when confluent as determined by light microscopy. Growth media was aspirated and cells were thoroughly washed three times with cell culture grade

phosphate buffered saline solution (PBS) and incubated with minimal amounts of 0.25% trypsin-EDTA for 2-3 mins (trypsinisation). A gentle knock on the side of the culture flask would encourage cell dissociation and reduce time required for trypsinisation. For weekly passaging cells were resuspended in an appropriate volume of growth media to stop trypsinisation and 10 μ L was pipetted onto a hemocytometer to determine cell concentration. For monthly passaging cells were resuspended in an appropriate volume of growth media to stop trypsinisation, decanted into a 50 mL falcon tube and centrifuged at 3000 rpm for 2 mins to remove excess debris and trypsin-EDTA before resuspension in fresh growth media and determination of cell concentration. For a 75cm² flask: HEK293 were seeded 2.5×10^5 , CH-8 were seeded 7.5×10^5 and Saos2 were seeded 2.5×10^5 , and 10 mL of growth media was added before placing into the incubator.

2.2.3 – Chemical transfection

The action of introducing foreign nucleic acids into eukaryotic cells is termed transfection. There are several commonly used methods such as viral transduction that uses adeno- or lentiviral vectors to achieve high transfection rates. We used chemical transfection is a relatively inexpensive and time efficient method to transfect mammalian cells with ANKH constructs for their study. Initial optimisations were conducted using the liposomal-based Lipofectamine LTX (Thermo Fisher – 15338100), however after several attempts a sub-acceptable (<10%) transfection rate was observed. Lipofectamine LTX is also restrictive in the time it can be in contact with cell and required frequent washes and culture media changes. Therefore, FugeneHD (Promega – E2311) was chosen as a low toxicity and high efficiency method of transfection that functioned well in long transfection periods without the need for washing or culture media changes. Transfections were performed in culture media without penicillin/streptomycin (transfection media).

2.2.3.1 – FugeneHD

The actual mechanism of FugeneHD transfection is proprietary information not disclosed by Promega. However, it is known to be a non-liposomal mechanism and contains a “formulation of blended lipids”⁶. Optimisation of FugeneHD:DNA ratio was performed on HEK293 cells cultured on 6 well plates that were 40% confluent 24 hours after seeding. When transfecting 6 well plates, per well (9 cm²) containing 2 mL transfection media and 40% confluent cells, 4.5 µL FugeneHD, 2 µg each construct DNA and 95 µL Opti-MEM Reduced Serum Media (Gibco - 11058021) was incubated together for 10 mins and then added to the well for transfection for a minimum of 24 hours. These ratios were scaled depending on the culture area and culture media volume (Table 2).

2.2.3.2 – Transient transfection vs. Stable transfected cell lines

With increasing availability of CRISPR technology, creation of stable cell-lines has never been more affordable and easy. However, during early stages of this thesis the routine method of creating stable cell lines was through antibiotic selection of clones that had incorporated the gene of interest into the genome. Termed positive selection, eukaryotic cells that had incorporated the gene of interest would also express resistance to antibiotics such G418 via co-incorporation of a resistance gene. However, this method is tedious and unreliable, with several attempts being made in the lab ending without a successful stable clone. Therefore, as transient transfection is a reliable and efficient method of incorporating fluorescently tagged genes into eukaryotic cells for visualization of their protein localisation we opted to use this technique. Transient transfection also does not remove endogenous expression of the gene of interest and the construct overexpresses the

⁶ promega.co.uk/resources/product-guides-and-selectors/protocols-and-applications-guide/transfection

Table 2 – Transfection protocol for T75/T25 flasks, 6 well plates and 35mm glass bottom dishes

Culture Container	Volume of culture media	Volume of Opti-MEM	Volume of FugeneHD	Volume of DNA (per construct)
T75 Flask	10 mL	475 µL	22.5 µL	25 µg
T25 Flask	5 mL	240 µL	11.5 µL	12.5 µg
6 well plate (per well)	2 mL	95 µL	4.5 µl	5 µg
35mm Glass bottom dishes	2 mL	95 µL	4.5 µl	5 µg

fluorescently tagged gene of interest via unregulated upstream promoters such as CMV, perhaps causing biases that cannot be easily addressed.

2.2.4 – Confocal microscopy

2.2.4.1 – Overview

Confocal microscopy offers several advantages over widefield optical microscopy, especially the ability eliminate background information from the focal plane and collect thin optical sections (0.5-1.5 μm) from thick specimens. In fluorescent microscopy the entire specimen is evenly illuminated with light that excites all fluorescent material within the light path at the same time. The resulting emission is detected, along with large unfocused background emission by a detector or camera. The resulting image is degraded due to the lack contrast and defined edges. In contrast, confocal microscope uses point illumination and a pinhole in an optically conjugated plane in front of the detector to eliminate out-of-focus emission – thus the term confocal (Nwaneshiudu et al 2012). The advent of GFP has established a method of confocal imaging for the tracking and localisation of proteins in live organisms and recent improvements in confocal microscope designs have added temperature and CO₂ controlled stages, capable of maintaining cultured cells for long periods of time.

2.2.4.2 – Live cell imaging

HEK293, CH-8 and Saos2 cells were grown on specialized glass bottom dishes (Ibidi) and transfected with appropriate fluorescent constructs of ANKH for 24, 48 and 72 hours. Before imaging, transfection media was aspirated; cells were washed once with PBS and 2 mL growth media was added. For imaging requiring the addition of chemical stains (see

section 2.2.4.5), they were added in their correct concentration to the growth media before addition to the cells. Although some initial testing was done on an older confocal microscope (Zeiss LSM480), the images presented in this thesis are all obtained on the newer LSM880 Airyscan with temperature and CO₂ controlled stage (Zeiss). Confocal dishes were placed inside the incubated stage, clipped down and allowed 5-10 mins to equilibrate to the environment. During this time, the laser power to the microscope was turned on and allowed to warm up. The specific laser wavelength required depended on the transfections and staining present and is listed in Table 3.

Firstly, cells were located using the brightfield setting that allowed viewing through the eyepiece and quick determination of the bottom of the z-axis. Once the cells were in focus, switching to the laser scanning microscope (LSM) mode allowed excitation of the transfected and stained cells and capture of the resulting emission. For large overview images showing multiple cells a 40X oil immersion lens was used, for individual cells and specific intracellular regions the 63X and 100X oil immersion lenses were used. Laser power, detector gain, pinhole size and image resolution was adjusted continuously to optimize the imaging for post editing and maintain optimal Airyscan detector saturation, however it was noted that increasing laser power also diminished the emission of fluorescent signal over time. Once an appropriate region was located, the highest resolution (depending on digital zoom) and lowest sufficient laser power, pinhole and detector gain was used to capture the final image. The single section was approximately 1 μm ; therefore to capture the complete Z-axis of the cell and create a 3D rendering several (~20) overlapping sections were captured in a process called Z stacking. After determination of ideal resolution, laser power, pinhole and detector gain single sections were captured along the Z-axis of the entire cell, with a minimum of 50% overlap to facilitate ideal 3D rendering. Imaging a section every 5-10 secs over several minutes and rendering a movie with a minimum of 20 frames per second captured time-lapse studies.

Table 3 – Confocal microscopy laser settings

Fluorescent Signal	Excitation Laser	Emission
GFP	488 nm	510 nm
RFP	561 nm	584 nm
Hoechst 33342	405 nm	421 nm

2.2.4.3 – Co-localisation analysis

HEK293 cells were grown in glass bottom dishes and co-transfected with ANKH_GFP or mutants and pmRFP_LC3 constructs as described above. 24 hours after transfection cells were visualized for ANKH co-localisation with LC3 on the LSM 880 airyscan as described in section 2.2.4. The culture media was aspirated, cells washed once with PBS and autophagic media was added. After incubation for three hours with autophagic media cells were once again visualised for ANKH co-localisation with LC3. Cells were rescued by aspirating the autophagic media, washing three times with PBS and incubating with growth media for 24 hours and then visualised for the final time for ANKH co-localisation with LC3. For identification and confirmation of co-localisation of fluorescent signal a simple intensity over distance plot was generated. Images of cells with dual emission signals were analyzed on Zen2.1 (Zeiss) for pixel intensity over a straight line. Raw arbitrary grey scale values were obtained and plotted to show co-localisation of signal over the straight line. However, co-localisation of emission signal does not correlate to physical interaction of associated proteins (Moser et al 2017).

2.2.4.4 – Cell membrane expression quantification

Cell membrane expression for ANKH was quantified by imaging 8X8 sections (1.68 mm^2) across the X and Y-axis of the confocal dish and counting transfected cells ($n > 100$) within this area that had only cell membrane expression, only cytoplasmic expression or cells with both cytoplasmic and cell membrane expression. The results are reported as a percentage of the cells with only cell membrane expression using the following equation.

$$\begin{aligned} & \% \text{ of cells with only cell membrane expression} \\ & = \left(\frac{\text{Number of cells with only cell membrane expression}}{\text{Total number of transfected cells counted}} \right) \times 100 \end{aligned}$$

2.2.4.5 – dsDNA staining for confocal imaging

Chemical stains were used to identify nuclear regions. Stain was added to replacement growth media before imaging and allowed sufficient time for their binding and localisation. *Hoechst 33342* (5 µg/mL): Binds double stranded DNA in both live and dead cells and was obtained from Thermo Fisher.

2.2.4.6 – Benefits of imaging live cells compared to fixed samples

During initial microscopy optimisation several attempts were made to image paraformaldehyde (PFA) and methanol fixed HEK293 cells. However, it was abundantly apparent that fixing cells reduced experimental scope and restricted imaging to single time points, furthermore PFA fixation caused emission of fluorescent proteins to diminish compared to live cells actively expressing the fluorescent protein. The attenuation of emission may be attributed to the steric conformational change experienced by proteins when treated with PFA. Formation of covalent cross-links between proteins may have consequences to protein secondary or tertiary structure, and hence fluorescent proteins that emit light due to their steric confirmation would definitely loss function once ‘glued’ into place by PFA. Methanol fixation dehydrates and denatures proteins, hence was immediately dropped as a potential fixing method (Howat and Wilson 2014). Another benefit of live cell imaging was the ability to capture momentary interactions that may

have been missed otherwise. Several interactions with the autophagy marker LC3 for ANKH were reminiscent of the 'kiss and go' mechanism and only lasted 5-10 secs.

However, consideration regarding cell health and response to external stimuli during experiments must be considered. The action of laser confocal microscopy actively damages cellular membrane as shown by the decrease in emission over time and the phenomena of cellular shrinkage after long period of laser stimulation. Transport of confocal dishes from the laboratory to the confocal microscope often meant cells were in an open unregulated environment for several minutes and could respond negatively. This was somewhat countered by allowing cells to equilibrate to their new environment for 5-10 mins inside the confocal microscope incubator stage before imaging. Nonetheless, live cell imaging provides much better scope for understanding protein dynamics in mutated proteins predicted to alter processing and allows long time lapse analyses of co-localised proteins.

2.2.5 – Measuring concentrations of inorganic pyrophosphate

2.2.5.1 – Overview

Inorganic pyrophosphate is a major regulator and contributor of calcium mineralization within the ECM. ANKH function is directly related to ePPi and in order to determine mutant effect on ePPi we measured PPi concentration in the media of HEK293 and CH-8 chondrocytes transfected with ANKH and mutant overexpression constructs. Initial attempts employed a terpyridine-Zinc(II) complex that had been shown to fluorescently detect nanomolar concentrations of pyrophosphate in water (Bhowmik 2014). The terpyridine-Zinc(II) detector acts as a chemosensor specific of PPi rather than other nucleotides tri-phosphates (NPPs) that have similar molecular structures. However, as the

method of generation of the terpyridine complex required several nuclear magnetic resonance (NMR) confirmations it was deemed un-economical for full-scale study. Nonetheless, the synthesis of the terpyridine-Zinc(II) complex was somewhat successful, as we observed some fluorescent staining in HEK293 cells but we were unable to quantify the PPi concentration via spectrophotometry (Figure 1 Appendix).

2.2.5.2 – PPiLight inorganic pyrophosphate assay

A more reliable method of PPi determination was the PPiLight Inorganic Pyrophosphate Assay (Lonza – LT07-610) that catalyzes PPi and AMP to generate ATP. The generation of ATP drives the activity of luciferase to produce light equivalent to the concentration of PPi present in the sample. Initial testing showed good linearity of detection of PPi between 0.1 and 10 μM , however the assay is rated to detect PPi at even lower concentrations (0.02 μM) but we were not able to replicate detection at such low concentrations (Figure 2 Appendix). The assay occurs in two steps, first the PPi in the culture media is converted to ATP, and second the concentration of ATP is detected via light emission on a luminometer. The assay contained two reagents (PPiLight converting reagent and PPiLight detection reagent) that were supplied lyophilized and reconstituted with 10 mL of supplied reconstitution buffer and stored at 4°C for use within the next 24 hours.

Either HEK293 or CH-8 cells were grown and transfected with appropriate constructs in 6-well plates as described above and 100 μL culture media was collected every 12 hours over 72 hours after transfection. For PPi concentration determination, in a luminescence compatible 96-well plate (Perkin Elmer - 6005503) 40 μL of collected culture media was mixed with 20 μL of PPiLight converting reagent and incubated for 30 mins at room temperature. 20 μL of PPiLight detection reagent was added and incubated for a further 30 mins. Luminescence was read on a luminometer with an integrated read time of 0.1 second

and raw arbitrary values were recorded. A standard curve was generated with known concentrations of pyrophosphoric acid (Sigma) and negative controls of fresh culture media, pEGFP-N3 vector only transfected HEK293 and CH-8 cells culture media and water were determined concurrently with experimental samples to minimize technical and plate errors.

2.2.6 – Measuring concentrations of calcium and calcium containing minerals

2.2.6.1 – Overview

Calcium deposition and mineralization in bone formation is the fundamental in the process of ossification. Calcium and Pi form mineral structures called hydroxyapatite (HA), which comprises 70% of bone-dry weight. It has also been suggested that PPi and calcium are antagonistically regulated within the ECM of bone forming cells (Nürnberg et al 2001). It also important to mention that reduced serum calcium levels is a key biomarker in patients with CMD (Hu et al. 2013, Minashima et al. 2010, Kornak et al. 2010, Sheppard et al. 2003, Taylor and Sprague 1989). As HEK293 cells cannot mineralize under normal conditions the concentration of calcium in their culture media would represent a proxy measure of mineralization potential.

2.2.6.2 – Arsenazo III colorimetric calcium assay

Arsenazo III is a metallochromic dye that specifically binds calcium forming a coloured complex that can be measured at 650 nm. HEK293 cells were grown and transfected with appropriate constructs in 6-well plates as described above and 100 μ L culture media was collected every 12 hours over 72 hours after transfection. The RX Monza analyzer workflow (Monza) was used to process Arsenazo III binding to calcium. This workflow

allowed for precise calibration and quality control as system specific calcium standards and human sera were used to eliminate the next day bias. Briefly, as the complete determination of over 200 unique samples would take 3 days to process, at the beginning of each run (3 per day) the RX Monza analyzer was first calibrated using a provided calcium standard (CA2390 - 10.2 mg/dL), then samples processed and finally the run was quality controlled using two human sera samples (QC Level 2 – HN1530_996UN and QC Level 3 – HE1532_772UE). After all samples were processed each unique run was checked for variation in human sera calcium concentration and appropriately quality controlled. For sample calcium determination, 12.5 μ L of collected culture media was mixed with 500 μ L Arsenazo III reagent in a plastic spectrophotometer cuvette and incubated in the RX Monza analyzer at 37°C for 5 mins and then absorption measured at 650 nm. Concentration of calcium was recorded as mg/dL and negative controls of fresh culture media, pEGFP-N3 vector only transfected HEK293 cells culture media and water were determined concurrently with experimental samples to minimize technical errors.

2.2.6.3 – Alizarin red S assay

As CH-8 cells actively mineralize calcium *in vitro* we determined ANKH effect on mineralization via Alizarin red S (ARS) staining. ARS is an anthraquinone dye that chelates to form a reddish salt with calcium containing minerals that can be viewed under light microscopes and easily removed for quantification. To determine ANKH effect on mineralisation CH-8 cells were cultured to 60% confluence and were transfected with appropriate constructs (see section 2.2.3.1). The media was replaced with mineralization media consisting of high glucose DMEM supplemented with 10% FBS, 50 μ g/mL Ascorbic acid (Sigma), 10 mM *B*-glycerol phosphate (Sigma) and 100 nM dexamethasone (Sigma) 24 hours after transfection and the cells were incubated for another 48 hours. Cells were washed twice with PBS for 5 mins, fixed with 3.7% PFA for 15 mins and then

stained with 1 mL pH 4.2 ARS solution (Sigma) for 30 mins at room temperature, then further five times washes with PBS for 5 min until a bright red colour was observed. Of note, it was recommended to wash cells with deionised water (diH₂O), however during initial optimisation washing with PBS developed ARS staining faster and more vibrant. Cells were then imaged using a Zeiss AxioPlan light microscope. To quantify the staining, 500 μ L 10% acetic acid was added to the cells and incubated at room temperature under constant agitation. Cells were scraped, collected, vortexed for 30 secs and heated at exactly 85°C for 10 mins. Lysates were allowed to cool in ice for 5 mins and then centrifuged at 17000 x g for 20 mins. 500 μ L of the supernatant was collected into a new tube and 150 μ L of 10% ammonium hydroxide pH 4.3 was added. 150 μ L was aliquot into 96-well plates and absorbance was read at 405 nM. Results were normalised to ARS standard curve and a negative control of pEGFP-N3 vector only transfected CH-8 cells was evaluated for mineralization concurrently.

2.2.7 – Quantitative PCR

2.2.7.1 – Overview

Expression change of *ENPP1*, *TNAP* and *PIT-1* in response to the presence of mutant ANKH was determined using quantitative PCR (qPCR). The simple and robust method of qPCR to determine gene expression is widely used in molecular biology. Briefly, gene expression is directly correlated to quantity of mRNA present for that gene within a cell and therefore any mechanistic effect that alters gene expression can be validated via quantification of mRNA. Simply, mRNA is extracted from the cell and then reverse transcribed into complimentary DNA (cDNA), which is then exponentially amplified via PCR to determine original quantity of mRNA. Concurrent reverse transcription and PCR

of a housekeeping gene mRNA allows for robust quality control and establishes a dataset comparable between samples.

2.2.7.2 – Primer design

Established primer design guidelines (see section 2.2.1.1) were used to create optimal PCR primers for amplification of *ENPPI*, *TNAP*, *PIT-1* and *β -Actin (ACTB)* (Table 4). However, unlike PCR primers used for amplification of DNA fragments destined for cloning, primers designed for qPCR were also ‘intron skipping’. The term defines a primer that begins in a unique exon and ends in another unique exon, usually the next, hence skipping the intermediate intron. However, longer primers that ‘skip’ several exons have also been used for determination of genomic damage. Nonetheless, designing primers to intron skip increases the likelihood of obtaining ‘clean’ and reliable data. Clean qPCR data was determined to be PCR amplifications that had only one visible peak during amplicon size determination. This is due to the fact that genomic DNA can contaminate mRNA extraction, either through improper extraction techniques or through the use of contaminated equipment. Although every precaution was taken to minimize contamination, the use of exon skipping primers allows for some genomic DNA to be present but not exponentially amplify during qPCR and produce false negative data.

2.2.7.3 – RNA extraction

RNA was extracted using the Qiagen RNeasy kit that used centrifugation and proprietary buffers to extract, bind to silica-based membrane and purify RNA from other cellular materials. HEK293 and CH-8 cells were grown and transfected with appropriate constructs as described above in 6-well plates for 24, 48 and 72 hours. Culture media was aspirated and cells washed once with PBS. Cells were then scraped with minimal amount of PBS

Table 4 – qPCR primers

Target Gene	Forward Primer	Reverse Primer	Primer Ta
<i>ENPPI</i>	GGTGGACTTCT TCCTGTTATTA	GGTGACAATGC TGTAGTGA	60°C
<i>TNAP</i>	GCTTGACCTCC TCGGAAGAC	GGGGCCAGACC AAAGATAGA	60°C
<i>PIT-1</i>	GACTGGCGTCT CTTTCGTA	ATGATGGCAGC ACTGATAAC	60°C
<i>ACTB</i>	TGACCCAGATC ATGTTTGAGA	TACGGCCAGAG GCGTACAGC	60°C

still left, pipetted into an eppendorf and centrifuged at 3000 x g for 5 mins. Excess PBS was removed and 350 μ L buffer RLT, containing guanidine-thiocyanate, added and immediately vortexed to thoroughly resuspended and completely lyse the cells. Buffer RLT also inactivate RNases to ensure purification of intact RNA. 350 μ L 70% ethanol was added and mixed by gently pipetting. Ethanol ensures correct binding conditions of RNA to the silica-based membrane. The mixture is added to the membrane containing spin-column and centrifuged at 8000 x g to bind the RNA. 700 μ L of buffer RW1 is added and centrifuged at 8000 x g for 15 secs to remove unwanted bound biomolecules and RNA fragments shorter than 200 bases. 500 μ L of ethanol containing buffer RPE is added and centrifuged at 8000 x g for 15 secs to removed excess salts from previous buffers. This wash step is repeated but centrifuged for 2 mins and the empty spin column is centrifuged again for an additional 1 min to remove trace amounts of buffer. Total RNA is eluted by adding 30 μ L RNase free water and centrifuging at 8000 x g for 1 min. To further ensure eluted RNA was free of genomic DNA contamination, samples were treated with DNase 1 (Sigma – AMPD1-1KT), that specifically degrades DNA. Simply, 3.5 μ L of supplied DNase 1 reaction buffer and 3.5 μ L DNase 1 were added to the eluted RNA, incubated for 15 mins at room temperature. DNase was inactivated by adding 4 μ L of supplied stop solution and incubating at 70°C for 5 mins. DNase 1 treated RNA was quantified for concentration and purity using Nanodrop One and stored on ice for immediate use.

2.2.7.4 – cDNA synthesis

Reverse transcriptase is an enzyme that generates cDNA from a RNA template. We used the iScript cDNA synthesis kit (BioRad) that contains a supermix with RNase H⁺ moloney murine leukemia virus reverse transcriptase (H⁺M-MLV-RT). This efficient recombinant form of M-MLV-RT degrades RNA during cDNA synthesis to preserve the 1:1 ratio of cDNA from RNA that is important for qPCR. The supermix also contains both oligo dTs

and random hexamer primers to ensure even, unbiased coverage of the mRNA template. Oligo dTs primers are a single stranded sequence (18 bases) of deoxythymine (dT) that bind to the poly(A) tails of RNA. Random hexamers are short (6 bases) sequences of oligodeoxyribonucleotides that randomly bind complementary RNA sequences and prime them for reverse transcription. The reaction buffer contains RNaseA inhibitors to protect RNA during initial setup and avoid skewed data due to RNA degradation. To create a cDNA copy of the extracted RNA we mixed 600 ng of DNase 1 treated RNA with 1 μ L of iScript supermix, 4 μ L iScript reaction buffer and made up to 20 μ L with nuclease free water. The mixture was thermal cycled for 25°C for 5 mins to separate RNA strands, then 42°C for 30 mins for primer annealing and cDNA synthesis and finally 85°C for 5 mins to inactivate H⁺M-MLV-RT enzyme. Reactions were stored at 4°C before use in the PCR reaction. Negative control samples without enzyme (RT control) and without RNA (no template control) were run concurrently.

2.2.7.5 – Gene expression quantification

There are several methods available for the quantification of DNA during qPCR. The most common are – TaqMan and SYBR. Both methods quantify fluorescence changes as specific sequences of cDNA are exponentially amplified during PCR. As we wanted to quantify expression of four genes (*ENPPI*, *TNAP*, *PIT-1* and *β -Actin*) in over 200 unique samples and as SYBR is a simpler method that relies on excellent inputs of well-designed primers and uncontaminated cDNA created from pure RNA we opted to use the SYBR method. We used the iTaq Universal SYBR Green Supermix (Bio-Rad – 1725121) that contains free dNTPs and iTaq DNA polymerase mixed with appropriate dyes and enhancers. Simply, the 2X supermix would exponentially amplify fragments of cDNA as specified by our primers and increase the fluorescent output depending on the amount of DNA synthesized. Genes with low expression would take longer to reach an arbitrary

threshold (C_q) of fluorescence compared to genes with high expression that would reach this threshold much quicker. Definition of the C_q is discussed in section 2.2.7.6. Using the Bio-Rad CFX real-time PCR detection system that displays live fluorescent reads during the PCR cycle, we mixed 60 ng of cDNA with 10 µL SYBR green supermix and 1 µL each forward and reverse primer (10 µM) and made up to 20 µL with nuclease free water in white opaque 96-well PCR plates (see Table 4 for primers). The plate was heated to 95°C for 30 secs for the initial cDNA denaturation and then thermal cycled 40 times at 95°C for 5 secs for template denaturation and 60°C for 30 secs for primer annealing and extension. After each extension cycle the Bio-Rad CFX would record the fluorescence. A melt curve was generated at the end of the cycles, by heating the sample to 65°C for 2 secs and then gradually increasing the temperature in 0.5°C increments up to 95°C and measuring fluorescence at each increment. Data was recorded as arbitrary fluorescence at each thermal cycle and quantitative C_q value was generated using Bio-Rad CFX system defaults.

2.2.7.6 – Thresholding and quality control

During thermal cycling when enough copies of the gene have been amplified there is a linear relationship between cycle number and arbitrary fluorescence. Genes with low expression would reach linearity later than genes with higher expression. The C_q value states the cycle number at which the increase in fluorescence was first linear as an average of every sample on the 96-well plate. Samples that never achieve linearity (cycles above 35) were quality controlled and removed from the data set; this was due to either extremely low gene expression or primer dimerisation in a negative control sample. We achieved 99% sample linearity and all negative control samples showed no amplification indicating the reverse transcription reaction had worked well and primer designs were gene specific and sufficient in amplifying and detecting low gene expression (Figure 3 Appendix).

Melt curve analysis indicated single peaks at 75c for *ENPPI* primer set, 81.5c for *TNAP* primer set, 77c for *PIT-1* primer set and 80c of β -*Actin* primer set. We observed no samples with multiple peaks that would indicate non-specific primer binding and amplification due to contamination or primer dimerisation (Figure 3 Appendix).

2.2.7.7 – qPCR statistical delta-delta analysis

Expression change was determined using the delta-delta method. Changes of *ENPPI*, *TNAP* and *PIT-1* expression in HEK293 and CH-8 cells transfected with wild type ANKH were compared to cells transfected with empty vector. Whilst changes in expression for cells transfected with mutant ANKH were compared to cells transfected with wild type ANKH. Firstly, Cq values for *ENPPI*, *TNAP* and *PIT-1* were normalized to their corresponding β -*Actin* Cq value (first delta). Next experimental changes (wild type ANKH vs. null vector/ mutant ANKH vs. wild type ANKH) in first delta normalized *ENPPI*, *TNAP* and *PIT-1* were compared (second delta). Finally, gene expression change was calculated as $2^{-\text{second delta}}$. A simple two tailed student *t* test was used to determine significance. Statistical significance was set at 0.05.

2.2.8 – ANKH and mutant co-localisation with LC3

HEK293 cells were grown in glass bottom dishes and co-transfected with ANKH_GFP or mutants and pmRFP_LC3 constructs as described above. 24 hours after transfection cells were visualized for ANKH co-localisation with LC3 on the LSM 880 airyscan as described in section 2.2.4. The culture media was aspirated, cells washed once with PBS and autophagic media was added. After incubation for three hours with autophagic media cells were once again visualised for ANKH co-localisation with LC3. Cells were rescued by aspirating the autophagic media, washing three times with PBS and incubating with growth

media for 24 hours and then visualised for the final time for ANKH co-localisation with LC3. Co-localisation was determined as described in section 2.2.4.3.

2.2.9 – Co-immunoprecipitation

2.2.9.1 – Overview

The process of co-immunoprecipitation is used to determine whether two proteins are physically complexed. The traditional method implements magnetic beads covalently coupled with an antibody to specifically bind and separate the proteins of interest from cell protein lysates. We visualised co-localisation of ANKH with LC3 under confocal microscopy. To determine whether they were physically interacting we used magnetic beads conjugated to a GFP antibody to separate ANKH_GFP complexes and checked for LC3 co-immunoprecipitation. We used two kits – Dynabeads (Thermo Fisher) and GFP Trap MA (Chromotek).

2.2.9.2 – Dynabeads

2.2.9.2.1 – Antibody coupling

Dynabeads kit contains 2.8 μm superparamagnetic epoxy beads that optimally bind 5 μg antibody per 1.5 mg beads. For coupling, 1.5 mg beads were first weighed in an eppendorf and washed with 1 mL included buffer C1 and placed into a magnetic holder for separation. Buffer C1 was aspirated and beads were washed again with fresh 1 mL buffer C1 by gentle vortexing. The beads were magnetically separated, buffer C1 aspirated and 250 μL of buffer C1 containing 5 μg anti-GFP antibody (Abcam- ab290) added and vortexed. 250 μL of included buffer C2 was added and gently vortexed again before

incubation on a rotator overnight at room temperature. Next day, the beads were magnetically separated, supernatant containing antibody aspirated, 800 μ L of included buffer HB added and gently vortexed. Buffer HB was aspirated after magnetic separation and 800 μ L of included buffer LB added and gently vortexed. Next, beads were washed three times with 800 μ L buffer SB to completely remove traces of free antibody and salts from previous buffers and finally resuspended in 150 μ L of included buffer SB and stored at 4°C until use.

2.2.9.2.2 – Lysate preparation

The optimal extraction buffer was determined to contain 100 mM NaCl, 2 mM MgCl₂, 1 mM DTT and 1% protease inhibitor. HEK293 cells were grown and transfected with appropriate constructs as described in section 2.2.3.1 into 6-well plates. Subset of cells were collected via scraping 24 hours after transfection, 3 hours after incubation with autophagic media and 24 hours after rescue in growth media. Cells were washed once in PBS before transfer into an eppendorf and centrifugation at 3000 x g for 5 mins and excess PBS was removed. Cells were weighed and extraction buffer added in a 1:9 ratio and incubated on ice for 15 mins with gentle pipetting every 5 mins to assist cell lysis. The cells were centrifuged at 13000 x g for 10 mins at 4°C and supernatant containing protein lysate collected into a fresh eppendorf and stored in ice for immediate use.

2.2.9.2.3 – Co-immunoprecipitation

Eppendorf containing beads were resuspended in 150 μ L buffer SB was magnetically separated, buffer SB was aspirated, 900 μ L extraction buffer added and gently washed by pipetting. Beads were magnetically separated, extraction buffer aspirated, protein lysate added (volume depended on starting cell weight) and incubated overnight at 4°C on a

rotator. Beads were washed three times with 200 μ L extraction buffer by gently pipetting. Beads were then incubated with supplied LWB (containing 0.02% tween20) on a rotator for 5 mins at room temperature and finally bound protein complexes were dissociated by incubating with 30 μ L supplied buffer EB for 5 mins on a rotator, magnetically separating the beads and transferring the supernatant to a fresh tube. Western blot method of analysing co-immunoprecipitated proteins is described in section 2.2.10.

2.2.9.3 – GFP Trap MA

Western blot analysis of Dynabeads co-immunoprecipitated proteins showed ambiguous results and often presented with large dense condensation of magnetic beads within the membrane (Figure 5 Appendix). The process of antibody coupling and use of specific extraction buffers requiring stringent optimisation further complicated the tedious method of Dynabeads. Therefore, an alternative method that implemented pre-coupled beads was used for the majority of the experiments. GFP-Trap MA is a unique system that couples a recombinant camelid antibody raised against GFP to magnetic agarose beads. This system proved to be much simpler and offered repeatable results without the tedious coupling process.

2.2.9.3.1 – Buffer compositions

The following buffers were made for use with the GFP Trap MA kit – *Lysis Buffer*: 10 mM Tris-Cl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.5% NP-40, 1% HALT protease inhibitor cocktail 100X (Thermo Fisher – 78430). *Dilution/Wash buffer*: 10 mM Tris-Cl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 1% HALT protease inhibitor cocktail 100X. *2X SDS sample buffer*: 120 mM Tris-Cl pH 6.8, 20% glycerol, 4% SDS, 0.04% bromophenol blue, 10% β -mercaptoethanol.

2.2.9.3.2 – Lysate preparation

For co-immunoprecipitation HEK293 cells were grown and transfected as described in section 2.2.3.1 into 6-well plates. To harvest cells, culture media was aspirated, 1 mL cold PBS added, cells scraped and transferred to a cooled eppendorf. Centrifugation at 500 x g for 3 mins at 4°C pelleted the cells and supernatant was removed. Cells were washed twice with cold PBS by gently pipetting and centrifuged at 500 x g for 3 mins at 4°C and supernatant discarded. Cells were resuspended in 200 µL cold lysis buffer by pipetting and placed on ice for 30 mins with extensive pipetting every 10 mins. Cell lysate was centrifuged at 13000 x g for 15 mins at 4°C and supernatant transferred to a fresh precooled eppendorf and 300 µL of dilution buffer added, lysate was stored on ice until further use.

2.2.9.3.3 – Co-immunoprecipitation

GFP Trap MA beads were vortexed and per co-immunoprecipitation 25 µL of the beads were transferred into a 500 µL cold dilution buffer in an eppendorf. Beads were magnetically separated and washed twice with cold dilution buffer. After the last wash, beads were magnetically separated until the supernatant was completely clear and then removed. Diluted protein lysate was added to the beads and rotated overnight at 4°C. Next day, beads were magnetically separated, supernatant removed and washed three times with 500 µL dilution buffer and beads magnetically separated. Before western blot analyses beads were resuspended in 30 µL 2X SDS sample buffer and heated to 95°C for 10 mins to disassociate protein complexes. The beads were magnetically separated and the protein containing 2X SDS sample buffer was loaded directly onto western blot gels for analysis.

2.2.10 – Western Blotting

Western blotting is a technique for separating proteins based on their molecular weight and identifying them via detection with antibodies. Antibodies can be raised in several animals to recognise specific antigens, such as GFP. The principal of western blotting is the subsequent detection of these antibodies by a secondary antibody that is raised against the heavy chain of the first animal. These secondary antibodies are often coupled to a chemical sensor such as horseradish peroxidase (HRP) that allows easy chemical detection of their localisation on a membrane.

2.2.10.1 – Antibody selection

Antibodies were purchased from several sources and optimised before their use. A complete list is provided in Table 5 indicating their supplier, animal source, antigen, optimised working concentration, membrane blocking buffer and secondary antibody used.

2.2.10.2 – Lysate preparation

Depending on upstream experimental procedures different types of lysates were used for western blotting. For co-immunoprecipitation studies lysates were either diluted in buffer EB (Dynabeads) and then mixed with appropriate volumes of NuPAGE 4X Sample Buffer (Thermo Fisher – NP0007), heated for 10 mins at 95°C before loading onto western gels, protein samples processed using the GFP Trap MA kit were already mixed with 2X SDS sample buffer (see section 2.2.9.3.1 and section 2.2.9.3.3) and were directly loaded on the

Table 5 – Antibody list and optimised working conditions

Antibody Name	Supplier	Animal Source	Target Antigen	Expected Molecular Weight (Da)	Working dilution	Dilution Buffer	Membrane Blocking Buffer	Secondary Antibody	Secondary Antibody Dilution Buffer
AB290 mAb	Abcam	Rabbit	GFP	27000	1:5000	BSA-TBST	BSA-TBST	AB97051	BSA-TBST
NB600-308 pAb	Novus Biological	Rabbit	GFP	27000	1:2500	BSA-TBST/MILK-TBST	BSA-TBST/MILK-TBST	AB97051	BSA-TBST/MILK-TBST
NB100-2220 pAb	Novus Biological	Rabbit	LC3	LC3II - 17000 LC3I – 19000	1 µg/mL	BSA-TBST/MILK-TBST	BSA-TBST/MILK-TBST	AB97051	BSA-TBST/MILK-TBST
AB128915 mAb	Abcam	Rabbit	GAPDH	37000	1:5000	BSA-TBST	BSA-TBST	AB97051	BSA-TBST
NBP2-38945 pAb	Novus Biological	Rabbit	ENPP1	100000	1:200	BSA-TBST/MILK-TBST	BSA-TBST/MILK-TBST	AB97051	BSA-TBST/MILK-TBST
NBP1-32948 pAb	Novus Biological	Rabbit	TNAP	Expected 57000 Observed ~80000	1:5000	BSA-TBST	BSA-TBST	AB97051	BSA-TBST
27570002-0.1mg pAb	Novus Biological	Rabbit	PIT-1	85000	1:1000	BSA-TBST	BSA-TBST	AB97051	BSA-TBST
AB97051 mAb	Abcam	Goat	Rabbit IgG Secondary	NA	1:5000	BSA-TBST	BSA-TBST	NA	BSA-TBST

western gel. For studies on ANKH mutant effect on protein expression of ENPP1, TNAP and PIT-1, CH-8 cells were grown and transfected with appropriate constructs as described in section 2.2.3.1 for 24, 48 and 72 hours in 25cm² flasks and collected as pellets in an eppendorf after trypsinisation. Whole cell protein lysates were extracted by incubating with 100 µL lysis buffer (see section 2.2.9.3.1) on ice for 30 mins with extensive pipetting every 10 mins, centrifuged at 13000 x g for 15 mins at 4°C and supernatant containing protein transferred to a fresh eppendorf and stored at -20°C until use.

2.2.10.3 – Lysate quantification

For experiments requiring comparison of protein expression lysates were first quantified by BCA assay before loading on western blot gels. Briefly, the bicinchoninic acid assay (BCA) measures the reduction of copper (II) into copper (I) by protein residues (biuret reaction) and subsequent chelation of bicinchoninic acid with copper (I) forming a coloured product measurable at 562 nm. For protein quantification 10 µL of protein lysate or protein standard was added to 200 µL working solution of BCA containing 50:1 ratio of BCA reagent A and BCA reagent B (Thermo Fisher - 23227) in a clear bottom 96-well plate and incubated for 30 mins at 37°C (purple colour was observed). The plate was allowed to cool before reading absorbance at 562 nm on a SpectraMax i3X plate reader (Molecular Devices). For protein standards, 2 mg/mL stock solution of bovine serum albumin (BSA) was diluted in deionised water to produce concentrations of 1.5 mg/mL, 1 mg/mL, 0.75 mg/mL, 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL and determined alongside a blank sample containing water. A standard curve was generated from arbitrary absorbance values and protein concentration of experimental samples determined.

2.2.10.4 – Polyacrylamide gel electrophoresis

Polyacrylamide gels (PAGs) varying in gel percentage and loading volume were used depending on the proteins of interest molecular weight and abundance of protein after lysate preparation and quantification. Polyacrylamide gel electrophoresis (PAGE) is a simple method of separating proteins according to molecular weight. The Mini-PROTEAN system from Bio-Rad was used for all PAGE. PAGs were purchased pre-cast at appropriate percentages and loading well volumes. Before loading, protein samples were denatured by heating at 95°C for 10 mins in SDS containing sample buffer (either 4X NuPAGE or 2X SDS sample buffer – see section 2.2.10.2). Precast PAGs were placed into the electrophoresis chamber, clamped and 1X Tris-Glycine SDS buffer (TGS) was poured to sufficiently cover the electrodes. Care was taken to ensure no air bubbles were trapped in the loading wells. Protein samples were allowed to cool before carefully pipetting 90% max volume of loading well containing equal quantity of protein into the gel wells. PageRuler Prestained NIR Protein Ladder (Thermo Fisher – 26635) was used to determine protein molecular weights ranging from 11,000 Da to 250,000 Da (5 µL per PAG). Protein samples were first ‘stacked’ by gently electrophoresing at 50V for 10 mins. Stacking aligns protein samples by distributing them evenly within the first 1/10th of the PAG and prevents uneven running due to un-matched protein:sample buffer ratios between wells. The PAG were then electrophoresed completely for approximately 1 hour at 75V to ensure variations of 1,000 Da could be detected. Variation in time taken could be attributed to uncontrollable factors such as ambient temperature, PAG made date and true voltage passing through electrodes (effected by rust build up on the terminal connectors). It was determined that placing the Mini-PROTEAN system on ice whilst electrophoresis both improved running times and avoided un-even samples (smiling). Proteins would separate into tighter and evenly sized bands possibly due to better TGS buffer ion recovery and thermal effects on protein diffusion.

2.2.10.5 – Protein transfer

Proteins separated with PAGE were transferred onto a polyvinylidene difluoride (PVDF) membrane using the TransBlot Turbo Transfer System (Bio-Rad). The PAG containing separated proteins and PVDF membrane were sandwiched between several layers of filter paper pre-soaked with an optimized proprietary buffer and proteins are transferred from the PAG into the PDVF via the application of an electrical current (25V, 2.5A) for 7 mins. After transfer, PVDF membranes were transferred into 50 mL falcon tubes and washed three times with 10 mL 1x tris-buffered saline containing 1% tween20 (TBST) by placing on a rotator.

2.2.10.6 – PVDF membrane blocking

Blocking reduces the un-specific background when PVDF membranes are chemically visualised. Depending on primary antibody quality, PVDF membranes in 50 mL falcon tubes were blocked for 1 hour at room temperature on a rotator with 5 mL either 5% Marvel milk in TBST or 5% BSA in TBST (Table 5). PVDF membranes to be used with monoclonal antibodies were always blocked with 5% BSA in TBST. Whilst PVDF membranes to used with polyclonal antibodies were first tested by blocking with 5% BSA in TBST, but if excess background or un-specific binding was visualised 5% Marvel milk in TBST was used for experiments. Of note, antibodies supplied by Developmental Studies Hybridoma Bank at the University of Iowa (DSHB) are suspended in sera and PVDF membranes to be used with these antibodies were always blocked with 5% Marvel milk in TBST.

2.2.10.7 – Immunodetection

After blocking, PVDF membranes in 50 mL falcon tubes were washed three times with 10 mL TBST on a rotator. For immunolabeling of separated proteins primary antibodies were diluted in 5 mL of their appropriate blocking buffer (Table 5) and incubated with the PVDF membrane overnight on a rotator at 4°C. Next day, PVDF membranes were washed three times with 10 mL TBST. Secondary antibody was purchased from Abcam and raised in Goat for recognition of rabbit (ab97051) immunoglobulin G (IgG) heavy or light chains and conjugated to horseradish peroxidase (HRP). Depending on primary used, secondary antibodies were diluted 1:5000 in 5 mL appropriate blocking buffer (Table 5) and incubated with PVDF membrane on a rotator for 1 hour at room temperature. For detection for bound antibodies, the PVDF membranes were washed three times with 10 mL TBST on a rotator and placed carefully, to avoid trapped air bubbles, onto the viewing plate of the ChemiDoc XRS+ (Bio-Rad). Bound secondary antibodies conjugated with HRP were detected using Clarity ECL Western Substrate (Bio-Rad – 1705060S). Clarity ECL contains luminol that is oxidised to 3-aminophthalate by HRP and emits light. Minimal amounts of Clarity ECL, enough to cover the PVDF membrane was pipetted onto the membrane and using the *western* protocol for *chemiluminescence* on the ChemiDoc XRS+ localisation of HRP conjugated secondary antibody was visualised. Protein ladder was visualised using the *western* protocol for *colorimetric* detection. Bio-Rad proprietary software (Image Lab) was used to *merge* the *chemiluminescence* and *colorimetric* images and determine molecular weight of protein bound to antibodies. For experiments comparing expression of protein, arbitrary intensity of *chemiluminescence* of samples was normalised to *chemiluminescence* of their GAPDH (housekeeping protein). Primary antibodies were incubated together with anti-GAPDH primary, as expected protein weights were sufficiently different (Table 5).

2.2.11 – ENPP1 activity assay

CH-8 cells were grown and transfected with ANKH and mutant constructs as described in section 2.2.3.1 for 24, 48 and 72 hours. Protein lysates were collected as described in section 2.2.9.3.2. Equal amounts of protein lysate were incubated with 200 μ L 2.15 mM *p*-nitrophenyl thymidine 5'-monophosphate (Sigma) for 1 hour and room temperature. 20 μ L 1 M NaOH was added to stop the reaction and absorbance was measured at 405 nm. In the presence of ENPP1, *p*-nitrophenyl thymidine 5'-monophosphate is catalyzed to form *p*-nitrophenyl thymidine and increases absorbance at 405 nm.

2.2.12 – TNAP activity assay

The simple NBT/BCIP (Thermo Fisher - 34042) assay was used to determine TNAP activity in CH-8 cells grown and transfected with ANKH and mutant constructs in 6-well plates for 24, 48 and 72 hours as described in section 2.2.3.1. Commonly used in immunohistochemistry for the staining of bound antibodies conjugated with alkaline phosphatases, in the presence of TNAP the combination of nitro-blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3'-indolylphosphate *p*-toluidine salt (BCIP) produces an insoluble deep purple precipitate that can be quantified by light microscopy. After transfection, cells were washed three times with PBS and fixed using 1 mL 3.7% PFA for 15 mins at room temperature. After which, PFA was aspirated, cells washed three times with PBS and left to air dry at room temperature for approximately 20 mins. Cells were then incubated at 37°C for approximately 1 hour with 500 μ L NBT/BCIP solution to allow development of purple precipitate.

Unfortunately, after 1 hour incubation no purple precipitation was observed and cells were left overnight. Ultimately, a precipitate was only observed after 4 days of incubation and

deemed unreasonable for quantification. Due to time constraints we abandoned this method. However, it was very likely that during the initial testing, reagent concentration was used as optimised for immunohistochemistry staining of abundant antibodies rather than detection of endogenous TNAP expressed at relative low concentration of PFA fixed cells. Given more time, optimisation of NBT/BCIP concentration, incubation time and fixation methods would offer good results.

2.2.13 – Statistics

Assuming equal variance, 2-tailed *t*-test was used to evaluate significance for normally distributed data, whilst a Mann-Whitney *u*-test was performed on non-normally distributed data. An *F*-test was performed to check equal and unequal variance. Statistical significance was set at $p < 0.05$. Unless stated otherwise, in all figures, scale bars = 20µm; *** indicates p value < 0.01 ; * indicates p value < 0.05 . # indicates p value < 0.1 ; neg.= Negligible/Raw value below threshold.

Chapter 3

Results (I): Mutations in ANKH alter intracellular localisation dynamics

3.1 – Results (I): Mutations in ANKH alter intracellular localisation dynamics

3.1.1 – Introduction

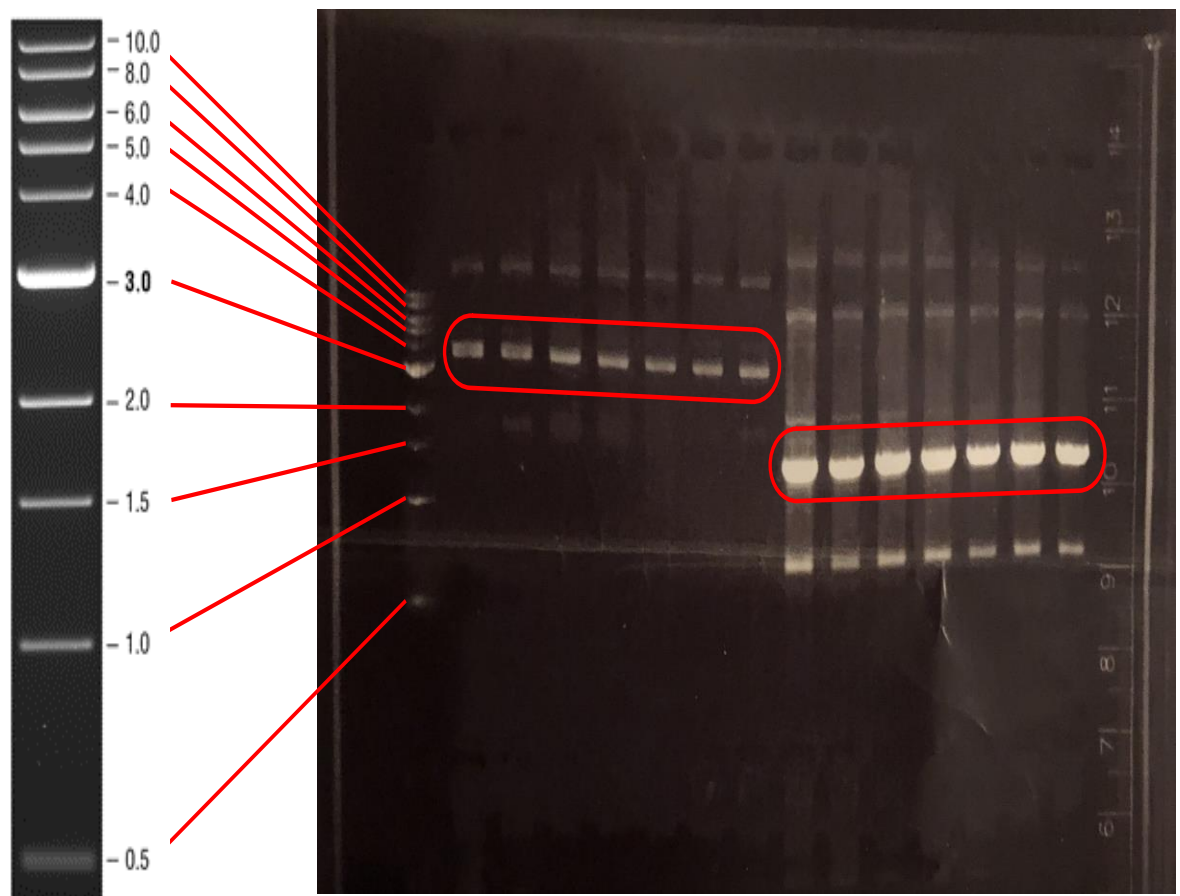
HEK293, CH-8 and Saos2 cells transfected with wild type ANKH-GFP (wt. ANKH) or mutant ANKH-GFP (P5L, S375del, G389R and E490del) were imaged for localisation of GFP signal using confocal microscopy (section 2.2.4). Previous work has clearly demonstrated wt. ANKH localises specifically to the cell membrane (Abhishek and Doherty 2011, Carr et al. 2009, Gurley et al. 2006, Pendleton et al. 2002, Seifert et al. 2016, Zaka et al. 2006). Localisation of ANKH to other intracellular regions has also been observed (Seifert et al. 2016).

3.1.2 – Generation and confirmation of ANKH pEGFP-C1 and pEGFP-N3 vectors

We first confirmed amplification of wt. ANKH by size determination gel electrophoresis (Figure 9). Next we confirmed the correct sequence was present by direct sequencing of the pEGFP-N3 ANKH construct (Figure 10). Lastly, we confirmed mutagenesis had worked correctly by sequencing the pEGFP-N3 P5L, S375del, G389R and E490del constructs (Figure 11)

3.1.3 – Terminal placement of GFP does not alter ANKH localisation

To eliminate any steric bias caused by orientation of the GFP tag, we created ANKH expression constructs with either a C-terminal GFP tag (pEGFP-N3) or a N-terminal GFP tag (pEGFP-C1). Imaging wt. ANKH and mutant ANKH localisation in HEK293 cells revealed no visible differences for ANKH constructs with C-terminal or N-terminal GFP tags (Figure 12).



ULK4-732 2.1kb

ULK4-580 1.7kb

Figure 9 – Agarose gel images of wt. ANKH.

Right red circle highlights correct sized bands excised for downstream cloning of wt.

ANKH. Red lines highlight corresponding 1KB DNA ladder bands.

pEGFP_N3-ANKH

Homo sapiens ANKH inorganic pyrophosphate transport regulator (ANKH), mRNA
Sequence ID: [NM_054027.5](#) Length: 8231 Number of Matches: 1

Range 1: 339 to 1814 [GenBank](#) [Graphics](#)

▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
2726 bits(1476)	0.0	1476/1476(100%)	0/1476(0%)	Plus/Plus
Query 1	ATGGTGAAATCCCGGCGCTCACGCACTACTGGCCCCGATCCGGTTCTTGGTGCCCTG	60		
Sbjct 339	ATGGTGAAATCCCGGCGCTCACGCACTACTGGCCCCGATCCGGTTCTTGGTGCCCTG	398		
Query 61	GGCATCACCAACATAGCCATCGACTTCGGGGAGCAGGCCCTTGAACCGGGGCATTGCTGCT	120		
Sbjct 399	GGCATCACCAACATAGCCATCGACTTCGGGGAGCAGGCCCTTGAACCGGGGCATTGCTGCT	458		
Query 121	GTCAAGGAGGATGCAGTCGAGATGCTGGCCAGCTACGGGCTGGCGTACTCCCTCATGAAG	180		
Sbjct 459	GTCAAGGAGGATGCAGTCGAGATGCTGGCCAGCTACGGGCTGGCGTACTCCCTCATGAAG	518		
Query 181	TTCTTCACGGGTCCCATGAGTGACTTCAAAAATGTGGGCCTGGTGTTTGTGAACAGCAAG	240		
Sbjct 519	TTCTTCACGGGTCCCATGAGTGACTTCAAAAATGTGGGCCTGGTGTTTGTGAACAGCAAG	578		
Query 241	AGAGACAGGACCAAAGCCGTCTGTGTATGGTGGTGGCAGGGGCCATCGCTGCCGTCTTT	300		
Sbjct 579	AGAGACAGGACCAAAGCCGTCTGTGTATGGTGGTGGCAGGGGCCATCGCTGCCGTCTTT	638		

ANKH N3-ANKH

Figure 10 – Sequence alignments of wt. ANKH within the pEGFP-N3 vector.

Only the first four rows of aligned sequence are shown but we attained 100% alignment of pEGFP-N3_ANKH to complete NM_054027.5 ANKH mRNA.

pEGFP_N3-ANKH-P5L

Homo sapiens ANKH inorganic pyrophosphate transport regulator (ANKH), mRNA
Sequence ID: [NM_054027.5](#) Length: 8231 Number of Matches: 1

Range 1: 339 to 1233		GenBank	Graphics	Next Match		Previous Match	
Score	Expect	Identities		Gaps	Strand		Plus/Minus
1648 bits(892)	0.0	894/895(99%)		0/895(0%)	Plus/Plus		
Query 28	ATGGTGAATTCCTGGGCTT	CAGCGACTACTGGCCCTGATCCGGTCTTGGTGGCCCTG		87			
Sbjct 339	ATGGTGAATTCCTGGGCTT	CAGCGACTACTGGCCCTGATCCGGTCTTGGTGGCCCTG		398			
Query 88	GGCATCACCACATATG	CGACTTCGGGGAGCAGGCTTGAACCGGGGCAATGCTGCT		147			
Sbjct 399	GGCATCACCACATATG	CGACTTCGGGGAGCAGGCTTGAACCGGGGCAATGCTGCT		458			
Query 148	GTCAAGGAGATGCACTCGAGATGCTGGCCAGCTACGGCTGGCGTACTCCCTCATGAAG			207			
Sbjct 459	GTCAAGGAGATGCACTCGAGATGCTGGCCAGCTACGGCTGGCGTACTCCCTCATGAAG			518			
Query 208	TTCTTCACGGGTCCCATGAGTGACTTCAAAAATGTGGGCTTGTTGTTGTGAACAGCAAG			267			
Sbjct 519	TTCTTCACGGGTCCCATGAGTGACTTCAAAAATGTGGGCTTGTTGTTGTGAACAGCAAG			578			
Query 268	AGAGACAGGACCAAGCCGCTCTGTATGGTGGCAGGGGCAATCGCTGCCGCTCTT			327			
Sbjct 579	AGAGACAGGACCAAGCCGCTCTGTATGGTGGCAGGGGCAATCGCTGCCGCTCTT			638			

A

pEGFP_N3-ANKH-S375del

Homo sapiens ANKH inorganic pyrophosphate transport regulator (ANKH), mRNA
Sequence ID: [NM_054027.5](#) Length: 8231 Number of Matches: 1

Range 1: 1328 to 1814		GenBank	Graphics	Next Match		Previous Match	
Score	Expect	Identities		Gaps	Strand		Plus/Minus
880 bits(476)	0.0	484/487(99%)		3/487(0%)	Plus/Plus		
Query 1	CTGCATGGCTCTGTCTCACTCAGCTCTGTTCGTGATGTTTGGACACCCCAAGGTGCTGA			60			
Sbjct 1328	CTGCATGGCTCTGTCTCACTCAGCTCTGTTCGTGATGTTTGGACACCCCAAGGTGCTGA			1387			
Query 61	GAAATCTTGATGACATCATCGAGTGGACTTTGGCTTTGCAAGAACTCTGTGTTGTTCC			120			
Sbjct 1388	GAAATCTTGATGACATCATCGAGTGGACTTTGGCTTTGCAAGAACTCTGTGTTGTTCC			1447			
Query 121	TTTGGCGATTTTCTTCTCCAGTTCCAGTCACAGTGAAGGCGCATCTCACCAGGCTG			177			
Sbjct 1448	TTTGGCGATTTTCTTCTCCAGTTCCAGTCACAGTGAAGGCGCATCTCACCAGGCTG			1507			
Query 178	GCTGATGACATCGAGAAACCTTCTGCTTGGCCCAAGCTCTGTGCTGGGATCATGCT			237			
Sbjct 1508	GCTGATGACATCGAGAAACCTTCTGCTTGGCCCAAGCTCTGTGCTGGGATCATGCT			1567			
Query 238	CCTCATGCGCAGCTCGTGGTCTACCTTACCTGGGGGTGACAGTGGGCTGGGCTG			297			
Sbjct 1568	CCTCATGCGCAGCTCGTGGTCTACCTTACCTGGGGGTGACAGTGGGCTGGGCTG			1627			

B

pEGFP_N3-ANKH-G389R

Homo sapiens ANKH inorganic pyrophosphate transport regulator (ANKH), mRNA
Sequence ID: [NM_054027.5](#) Length: 8231 Number of Matches: 1

Range 1: 938 to 1814		GenBank	Graphics	Next Match		Previous Match	
Score	Expect	Identities		Gaps	Strand		Plus/Minus
1615 bits(874)	0.0	876/877(99%)		0/877(0%)	Plus/Minus		
Query 66	TTTATTCTCTCTCTCATTTCCAGATGTCTGTCACTCTCTGTGCGAGGCAATGTCTGT			125			
Sbjct 1814	TTTATTCTCTCTCTCATTTCCAGATGTCTGTCACTCTCTGTGCGAGGCAATGTCTGT			1755			
Query 126	CATGGCAGAGTCTTCCCCCTCGGTGGCGACTCATTTCCATCTTCTTTTCTGCTTCCG			185			
Sbjct 1754	CATGGCAGAGTCTTCCCCCTCGGTGGCGACTCATTTCCATCTTCTTTTCTGCTTCCG			1695			
Query 186	GTAGACATAGCAGCAGCGATGGCGACATGGTGGATTCTCCCAAGAGCCCGCAGGAG			245			
Sbjct 1694	GTAGACATAGCAGCAGCGATGGCGACATGGTGGATTCTCCCAAGAGCCCGCAGGAG			1635			
Query 246	GGAGCCCAAGCCCAAGGTCGACCGTGCACCCCAAGTGGGTAGGACACAGGCTGGC			305			
Sbjct 1634	GGAGCCCAAGCCCAAGGTCGACCGTGCACCCCAAGTGGGTAGGACACAGGCTGGC			1575			
Query 306	GATGAGGACGATGATCGCAGCAGAGCTGGGGGCAAGGACGAGGTTTCTTCACTGT			365			
Sbjct 1574	GATGAGGACGATGATCGCAGCAGAGCTGGGGGCAAGGACGAGGTTTCTTCACTGT			1515			
Query 366	CATCAGCACCCTGGTGGATGGCCCTCACTGTGACTGGAACTGGGAAAGAGGAAAT			425			
Sbjct 1514	CATCAGCACCCTGGTGGATGGCCCTCACTGTGACTGGAACTGGGAAAGAGGAAAT			1455			

C

pEGFP_N3-ANKH-E490del

Homo sapiens ANKH inorganic pyrophosphate transport regulator (ANKH), mRNA
Sequence ID: [NM_054027.5](#) Length: 8231 Number of Matches: 2

Range 1: 1442 to 1814		GenBank	Graphics	Next Match		Previous Match	
Score	Expect	Identities		Gaps	Strand		Plus/Minus
669 bits(362)	0.0	370/373(99%)		3/373(0%)	Plus/Minus		
Query 66	TTTATTCTCTCTCTCATTTCCAGATGTCTGTCACTCTCTGTGCGAGGCAATGTCTGT			125			
Sbjct 1814	TTTATTCTCTCTCTCATTTCCAGATGTCTGTCACTCTCTGTGCGAGGCAATGTCTGT			1755			
Query 126	CATGGCAGAGTCTTCCCCCTCGGTGGCGACTCATTTCCATCTTCTTTTCTGCTTCCG			185			
Sbjct 1754	CATGGCAGAGTCTTCCCCCTCGGTGGCGACTCATTTCCATCTTCTTTTCTGCTTCCG			1695			
Query 186	GTAGACATAGCAGCAGCGATGGCGACATGGTGGATTCTCCCAAGAGCCCGCAGGAG			245			
Sbjct 1694	GTAGACATAGCAGCAGCGATGGCGACATGGTGGATTCTCCCAAGAGCCCGCAGGAG			1635			
Query 246	GGAGCCCAAGCCCAAGGTCGACCGTGCACCCCAAGTGGGTAGGACACAGGCTGGC			305			
Sbjct 1634	GGAGCCCAAGCCCAAGGTCGACCGTGCACCCCAAGTGGGTAGGACACAGGCTGGC			1575			
Query 306	GATGAGGACGATGATCGCAGCAGAGCTGGGGGCAAGGACGAGGTTTCTTCACTGT			365			
Sbjct 1574	GATGAGGACGATGATCGCAGCAGAGCTGGGGGCAAGGACGAGGTTTCTTCACTGT			1515			
Query 366	CATCAGCACCCTGGTGGATGGCCCTCACTGTGACTGGAACTGGGAAAGAGGAAAT			422			
Sbjct 1514	CATCAGCACCCTGGTGGATGGCCCTCACTGTGACTGGAACTGGGAAAGAGGAAAT			1455			

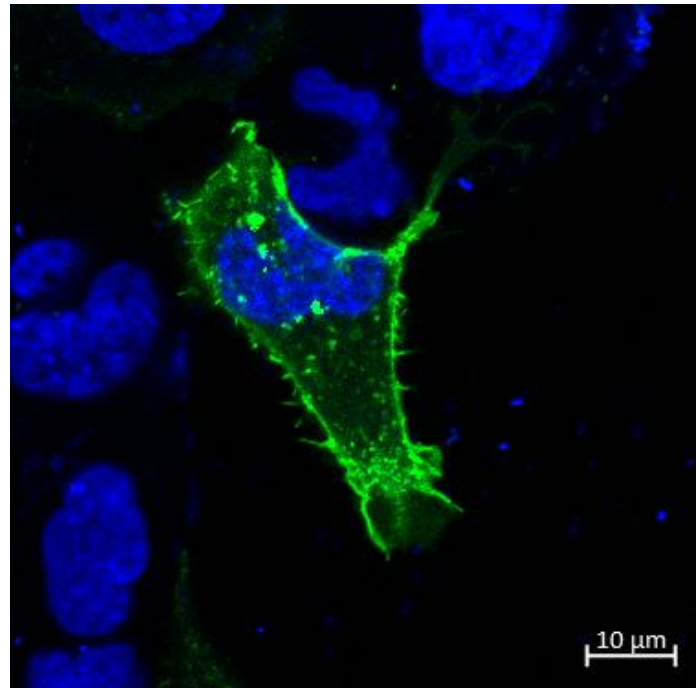
D

Figure 11 – Sequence alignments of ANKH mutant P5L, S375del, G389R and E490del.

Only the first four rows of aligned sequence are shown. We attained correct mutagenesis (red circles) of (A) Substitution of C>T of pEGFP-N3_ANKH-P5L at base position 14 (B) Deletion of bases TCC at base position 1123 of pEGFP-N3_ANKH-S375del (C) Substitution of C>T of pEGFP-N3_ANKH-G389R at base position 1165 (D) Deletion of bases GAG at base position 1468 of pEGFP-N3_ANKH-E490del.

pEGFP_C1-ANKH

Hoechst 33342



pEGFP_N3-ANKH

Hoechst 33342

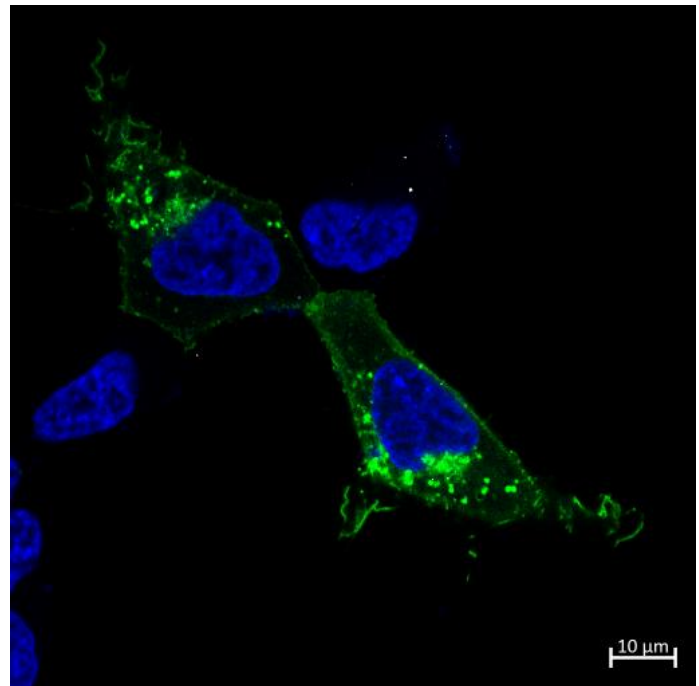


Figure 12 – Confocal microscopy of pEGFP-C1_ANKH and pEGFP-N3_ANKH.

Confocal microscopy of HEK293 cells transfected with either pEGFP-C1_ANKH or pEGFP-N3_ANKH revealed no observable differences in localisation of GFP signal. *Note* – Brightness differences are attributed to incorrect control of gain and exposure settings during early confocal testing.

3.1.4 – CH-8 and Saos2 cells exhibit auto fluorescent punctate bodies like structures

During initial optimisation of CH-8 and Saos2 cells for confocal imaging, we observed abundant punctate bodies-like structures within cytoplasmic regions of un-transfected CH-8 and Saos2 cells. Imaging by exciting at 405nm, 458nm, 488nm, 514nm, 561nm or 633nm and capturing long-pass emission revealed these punctate bodies-like structures auto-fluoresced at all wavelengths (Figure 13A). We were unable to experimentally evaluate these punctate bodies-like structures due to time limitations, however it may be reasonable to assume that the composition of these structures is related to bone cell function – such as calcium based minerals but no previous publications have described similar findings. Furthermore, when transfecting CH-8 and Saos2 cells with ANKH-GFP constructs we were unable to clearly visualise ANKH localisation due to over saturation of the detector from nearby punctate bodies-like structures (Figure 13B). Nonetheless, as ANKH localisation to cell membrane has been reported in other non-bone cells (Gurley et al. 2006, Seifert et al. 2016, Zaka et al. 2006), we elucidated mutant effect on ANKH localisation dynamics solely in HEK293 cells.

3.1.5 – Aims of this section

The main aim of this section was to observe localisation dynamics of mutant ANKH proteins associated with CPPDD (P5L and E490del) and CMD (S375del and G389R). Whether ANKH localisation is altered in a time-dependent manner was also observed. Specific localisation of wt. ANKH and mutant ANKH-GFP was imaged using confocal microscopy of transfected HEK293 cells with varying imaging techniques, such as 3D Z-stacking and time-lapse used to describe any observed changes over-time.

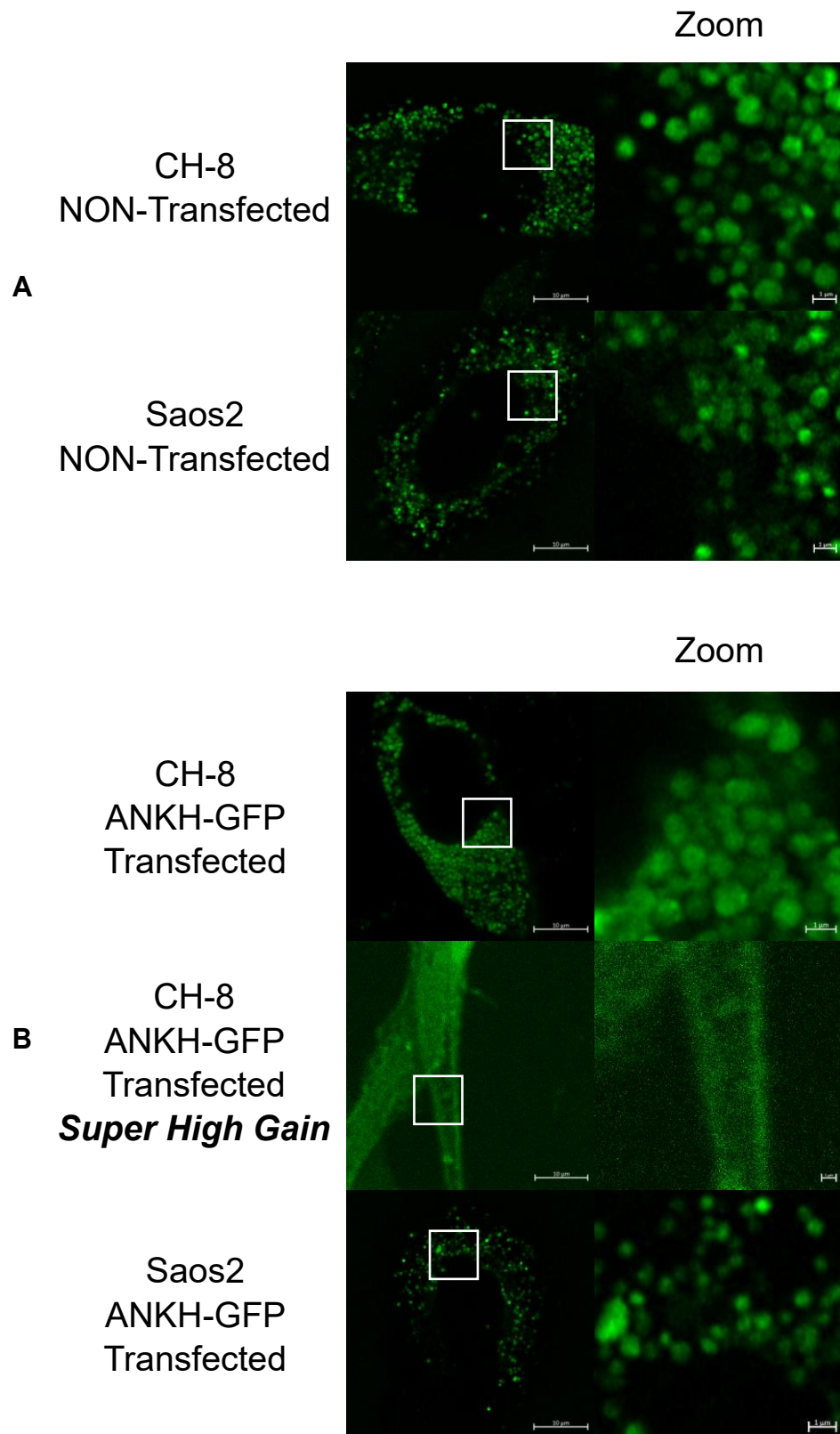


Figure 13 - CH-8 and Saos2 cells exhibit auto fluorescent punctate bodies like structures.

Confocal microscopy of CH-8 cells showed small structures (zoom) present within the cytoplasm of the cells that auto fluoresced at all wavelengths (excitation: 405nm, 458nm, 488nm (shown), 514nm, 561nm and 633nm; emission: LP 405nm, LP 458nm, LP 488nm (shown), LP 514nm, LP 561nm and LP 633nm) in (A) non-transfected and (B) ANKH-GFP transfected cells. *Super high gain* demonstrates that ANKH-GFP was expressed but due to over saturation of signal from auto fluorescent structures it was not possible to sufficiently image whole cells.

3.1.6 – Wild type ANKH localises specifically to the cell membrane but is non-uniform

Wt. ANKH showed clear localisation to the cell membrane and perinuclear area in HEK293 cells (Figure 14A) as reported in other cell types such as mouse osteoblastic cells MC3T3-E1, human adult fibroblasts (HAF), adenocarcinomic human alveolar basal epithelial cells (A549), human cervical cancer cells HeLa and monkey COS7 cells (Carr et al. 2009, Seifert et al. 2016). Further clarification using 3D confocal Z-stack rendering also showed the localisation was non-uniform but general to the cell membrane (Movie 1). To quantify the percentage of transfected cells that had specific cell membrane expression, we imaged an 8x8 area (1.68mm^2) and counted transfected cells ($n>100$). We recorded the numbers of cells with wt. ANKH expression only at the membrane, cells with wt. ANKH expression within cytoplasmic regions and cells with wt. ANKH expression in both the cell membrane and cytoplasmic regions. At 24 hours after transfection 57% of HEK293 cells expressed wt. ANKH specifically at the cell membrane. At 48 hours after transfection we observed that 66% of HEK293 cells expressed wt. ANKH specifically at cell membrane. This remained the same at 72 hours after transfection, as we again observed 66% of HEK293 cells expressed wt. ANKH specifically at the cell membrane (Figure 14B).

3.1.7 – P5L mutations delays ANKH recruitment to the cell membrane

The proline to leucine change at residue 5 of ANKH delayed the recruitment of ANKH to cell membrane. At the 24 hours after transfection we observed only 34% of transfected HEK293 cells expressed P5L specifically at the cell membrane. However, at 48 and 72 hours, transfected cells displaying specific cell membrane localisation reached 64% and 73% and resembled the cell membrane specific – non-uniform localisation dynamics observed in wt. ANKH (Figure 15A and Figure 15B).

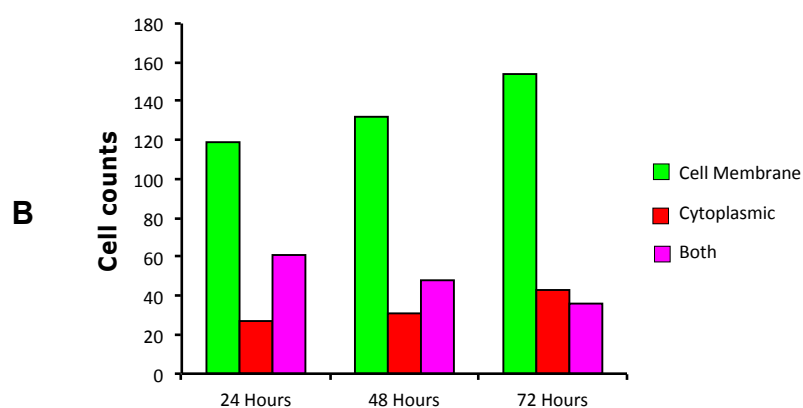
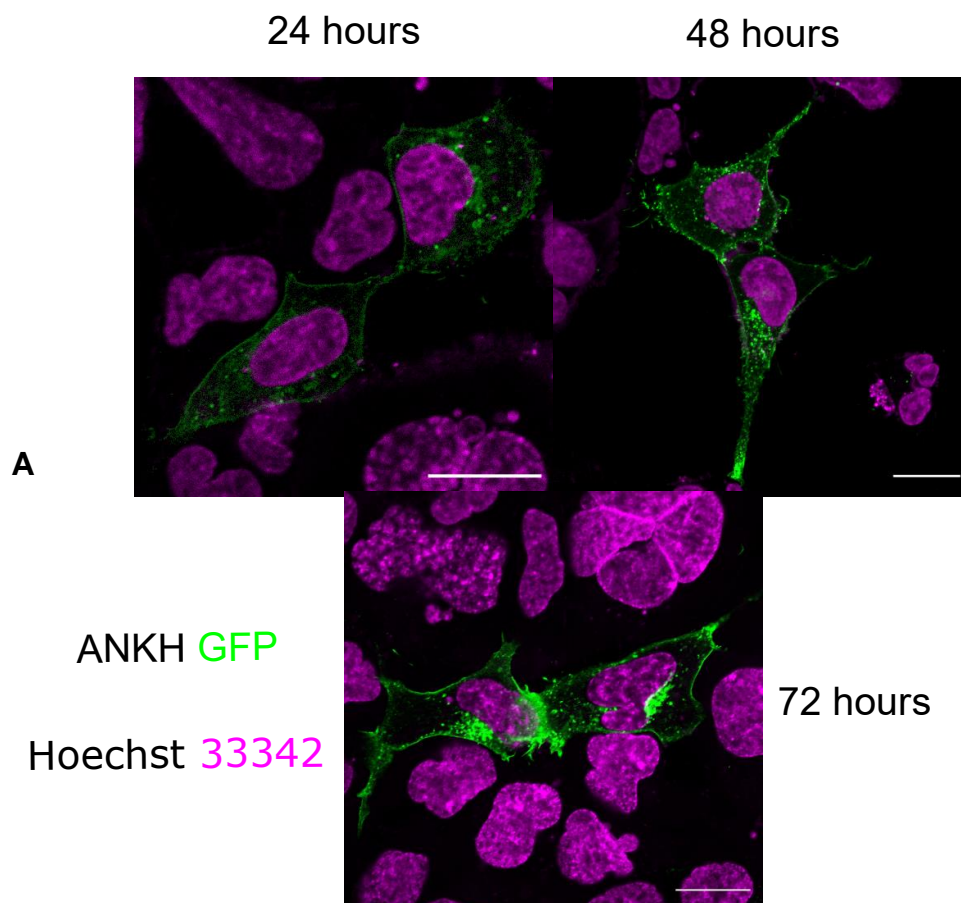


Figure 14 – Wild type ANKH localises primarily to cell membrane.

Confocal microscopy of HEK293 cells transfected with pEGFP-N3_ANKH revealed (A) GFP localisation primarily to the cell membrane at 24, 48 and 72 hours after transfection. (B) Tiling and 8x8 area (1.68mm²) we counted 57% of transfected HEK293 cells had specific cell membrane expression at 24 hours, increasing to 66% at 48 hours and remained at 66% at 72 hours after transfection. Absolute cell count of one biological replicate.

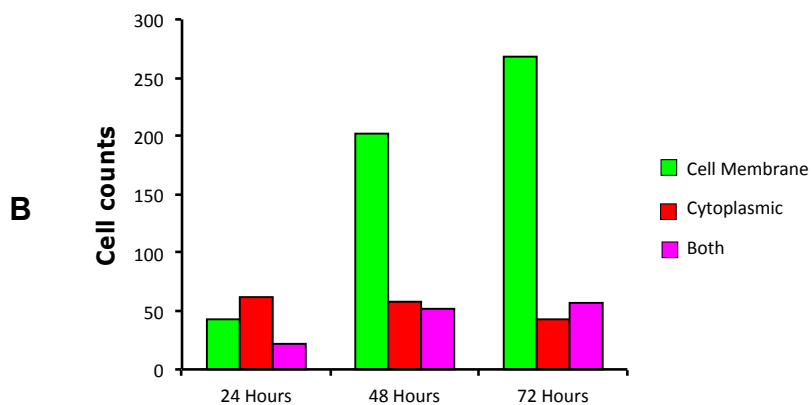
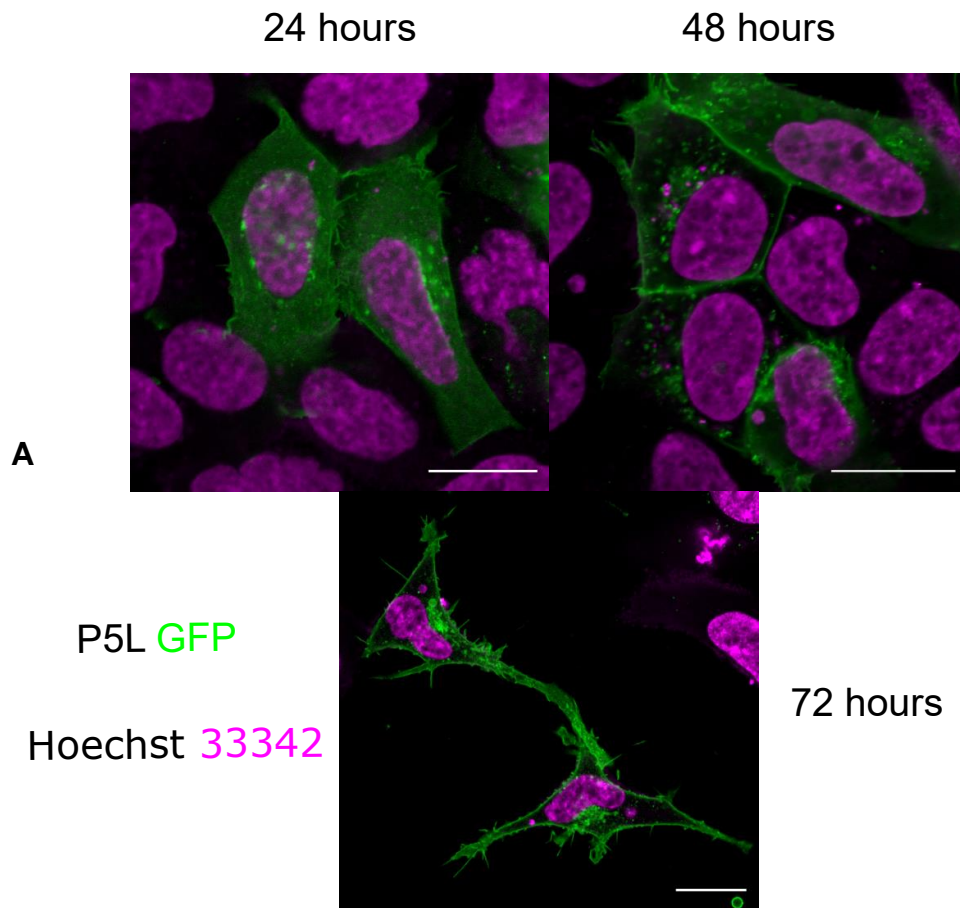


Figure 15 – P5L mutation delays cell membrane localisation of ANKH.

Confocal microscopy of HEK293 cells transfected with pEGFP-N3_ANKH-P5L revealed (A) GFP localisation primarily to the cytoplasmic region at 24 hours after transfection but specifically to cell membrane at 48 and 72 hours after transfection. (B) Tiling and 8x8 area (1.68mm²) we counted 34% of transfected HEK293 cells had specific cell membrane expression at 24 hours, increasing to 64% at 48 hours and 73% at 72 hours after transfection. Absolute cell count of one biological replicate.

3.1.8 – S375del mutation completely abolishes ANKH cell membrane localisation

The deletion of serine at residue 375 rendered ANKH completely cytoplasmic at 24, 48 and 72 hours after transfection. We measured 0% specific cell membrane localisation in transfected HEK293 cells for all time points. We could not identify any specific cytoplasmic regions that may have increased localisation, however we observed an almost-cytoskeletal like localisation at 24 hours after transfection and a diffuse generalised cytoplasmic localisation at 48 and 72 hours after transfection (Figure 16A and Figure 16B). Comparing to null vector (pEGFP-N3) transfected cells that expressed GFP only, we concluded that the observed localisation for S375del transfected cells was true. As GFP only transfected HEK293 cells displayed evenly diffused signal of GFP within the nucleus and cytoplasm, a trademark of GFP only expression (Figure 16C).

3.1.9 – G389R mutation delays cell membrane recruitment of ANKH and forms punctate bodies

Similar to the P5L mutation, the glycine to arginine change at residue 389 of ANKH delayed cell membrane localisation of ANKH. G389R expressed mostly within the cytoplasm with just 28% of transfected HEK293 cells displaying specific cell membrane localisation at 24 hours after transfection. At 48 and 72 hours the mutant protein was recruited into the cell membrane and also formed vesicle-like puncta with 66% and 72% of transfected cells, respectively, displaying specific cell membrane localisation (Figure 17A and Figure 17B).

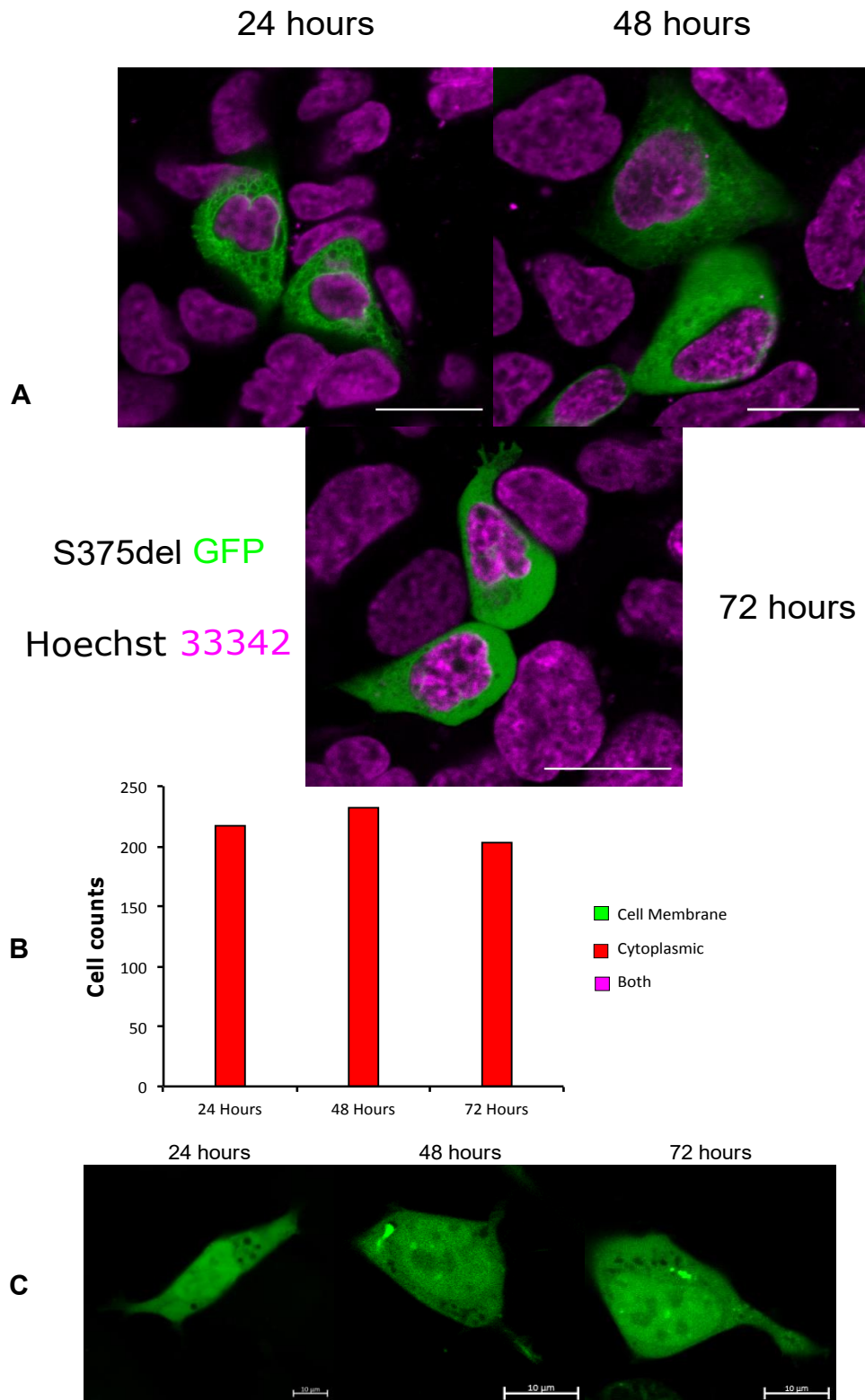


Figure 16 – S375del mutation abolishes ANKH cell membrane localisation

Confocal microscopy of HEK293 cells transfected with pEGFP-N3_ANKH-S375del revealed (A) GFP localisation to the cytoplasmic region at 24, 48 and 72 hours after transfection. (B) Tiling and 8x8 area (1.68mm²) we counted 100% of transfected HEK293 cells had only cytoplasmic expression at 24, 48 and 72 hours after transfection. Absolute cell counts from one biological replicate. (C) HEK293 cells transfected with pEGFP-N3 null vector show ubiquitous GFP signal in the nuclear and cytoplasmic region, whilst pEGFP-N3_ANKH-S375del transfected HEK293 cells show only cytoplasmic expression.

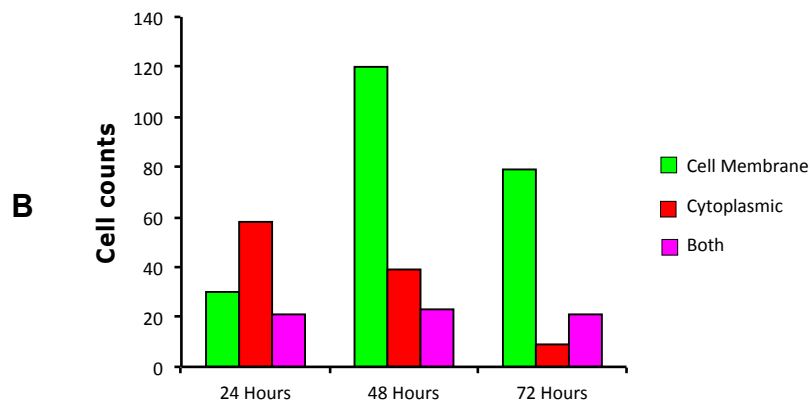
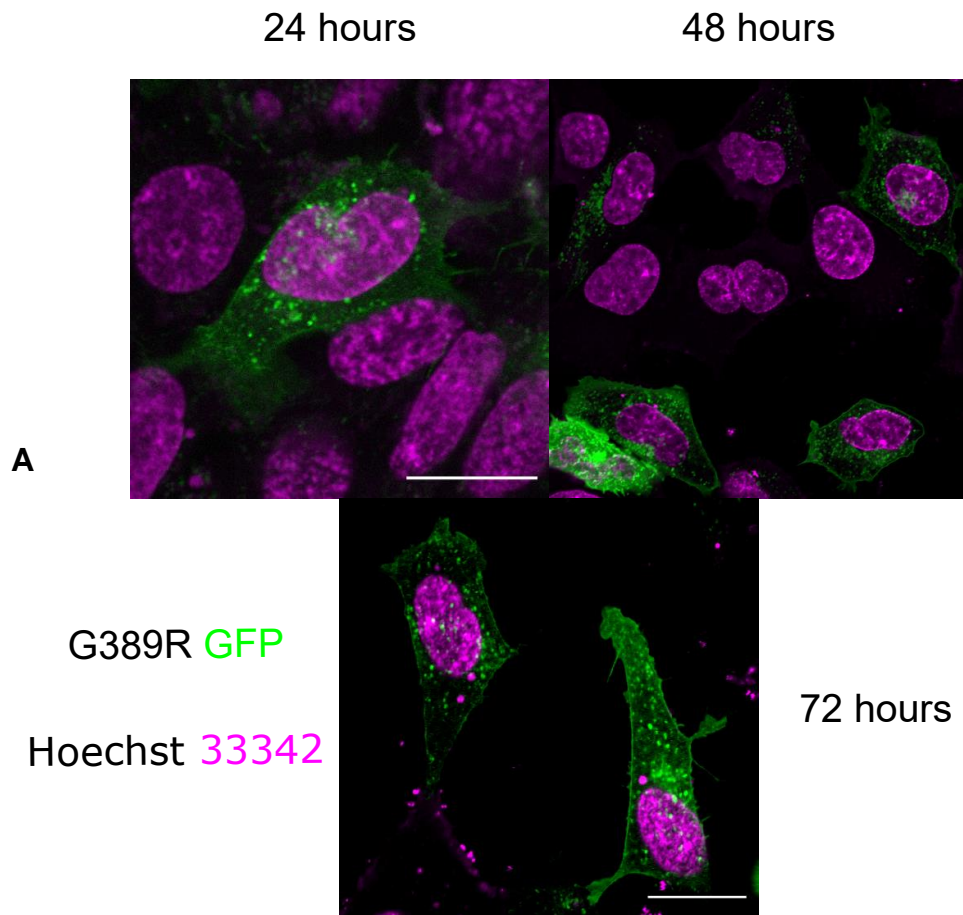


Figure 17 – G389R mutation delays cell membrane localisation of ANKH and forms punctate bodies.

Confocal microscopy of HEK293 cells transfected with pEGFP-N3_ANKH-G389R revealed (A) GFP localisation primarily to the cytoplasmic region at 24 hours after transfection forming punctate bodies like structures but specifically to cell membrane at 48 and 72 hours after transfection still forming punctate bodies. (B) Tiling and 8x8 area (1.68mm²) we counted 28% of transfected HEK293 cells had specific cell membrane expression at 24 hours, increasing to 66% at 48 hours and 72% at 72 hours after transfection. Absolute cell counts from one biological replicate.

3.1.10 – E490del mutation causes ANKH to localise primarily to the cytoplasm and forms punctate bodies

Deletion of a glutamate at residue 490 renders ANKH ineffective at inserting into the membrane compared to wt. ANKH. At 24 hours after transfection, the E490del protein was variably expressed, with only 35% of transfected HEK293 cells showing specific cell membrane expression. We observed the formation of vesicle-like punctate bodies at 48 and 72 hours after transfection and quantified only 7% and 29%, respectively, of transfected HEK293 cells with specific cell membrane expression (Figure 18A and Figure 18B).

3.1.11 - Mutants G389R and E490del co-localise with LC3 but do not physically interact or influence autophagic flux

Autophagic recycling of mis-folded or defective proteins has been well established; therefore, we described co-localisation of mutant ANKH proteins with the autophagy marker LC3 as indication of the presence of defective proteins. LC3 is used to define the autophagosome, the structure that engulfs proteins destined for recycling. The presence of mutant ANKH protein within the cytoplasm, especially when forming vesicle-like punctate bodies structures for G389R and E490del, was indication towards the presence of defective proteins. Co-transfection of mutant ANKH constructs with pmRFP-LC3 revealed co-localisation of G389R and E490del with LC3 when imaged with confocal microscopy (Figure 19A, Movie 2 and Movie 3). Drawing a straight line across co-localised signals and plotting intensity against distance, we measured co-localisation of autophagy marker LC3 with E490del under normal, autophagy induction and rescue conditions, and with G389R under autophagy induction and rescue conditions (Figure 19A). We observed no co-localisation for wt. ANKH, P5L or S375del with LC3 under any conditions (Movie 4, Movie 5 and Movie 6). Furthermore, immunoprecipitation of G389R and E490del revealed

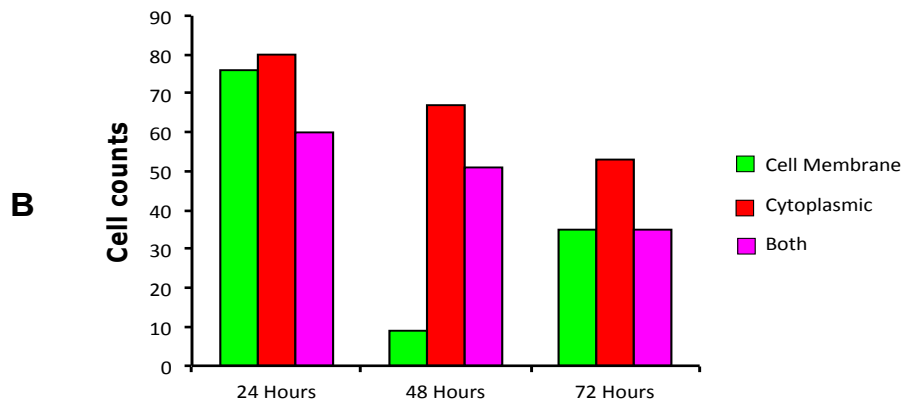
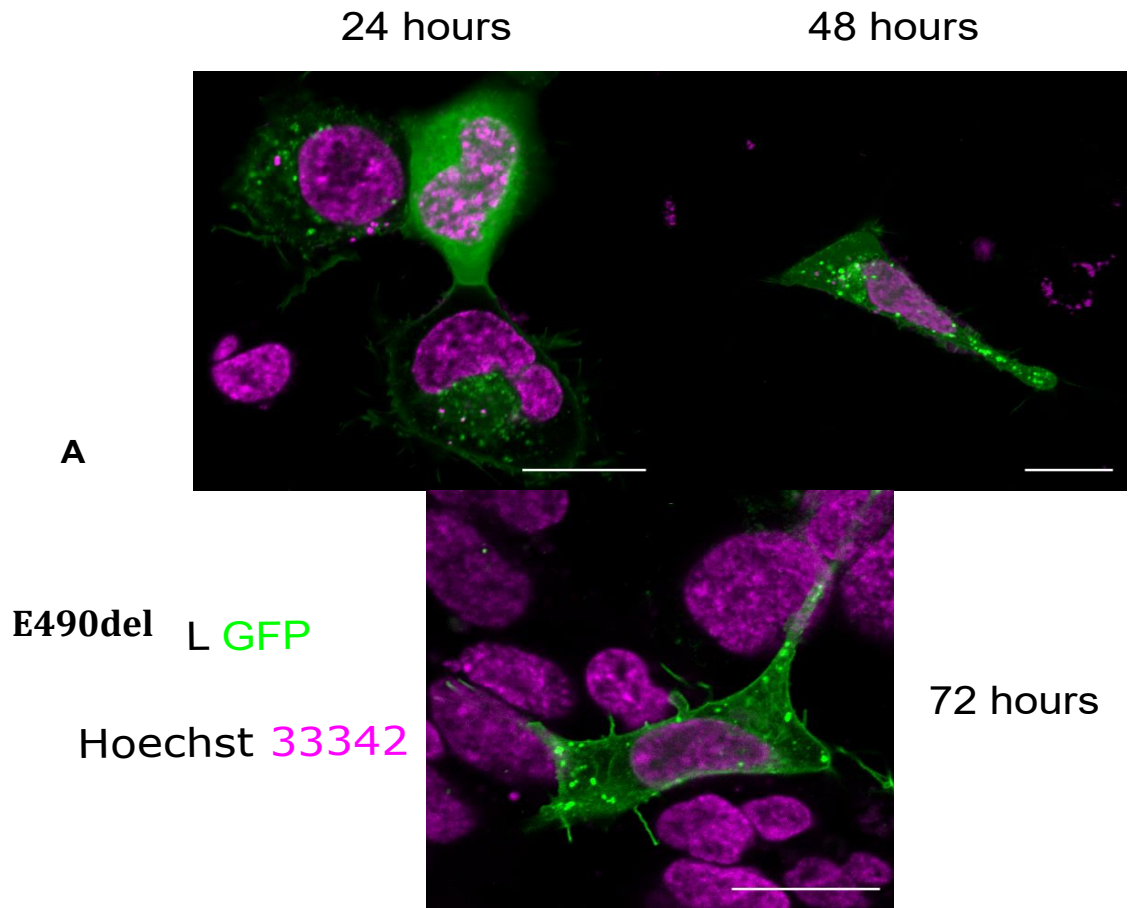


Figure 18 – E490del mutation causes ANKH to remain cytoplasmic and form punctate bodies.

Confocal microscopy of HEK293 cells transfected with pEGFP-N3_ANKH-E490del revealed (A) GFP localisation primarily to the cytoplasmic region at 24, 48 and 72 hours after transfection and forms punctate bodies. (B) Tiling and 8x8 area (1.68mm²) we counted 35% of transfected HEK293 cells had specific cell membrane expression at 24 hours, decreasing to 7% at 48 hours and recovering to 29% at 72 hours after transfection. Absolute cell counts from one biological replicate.

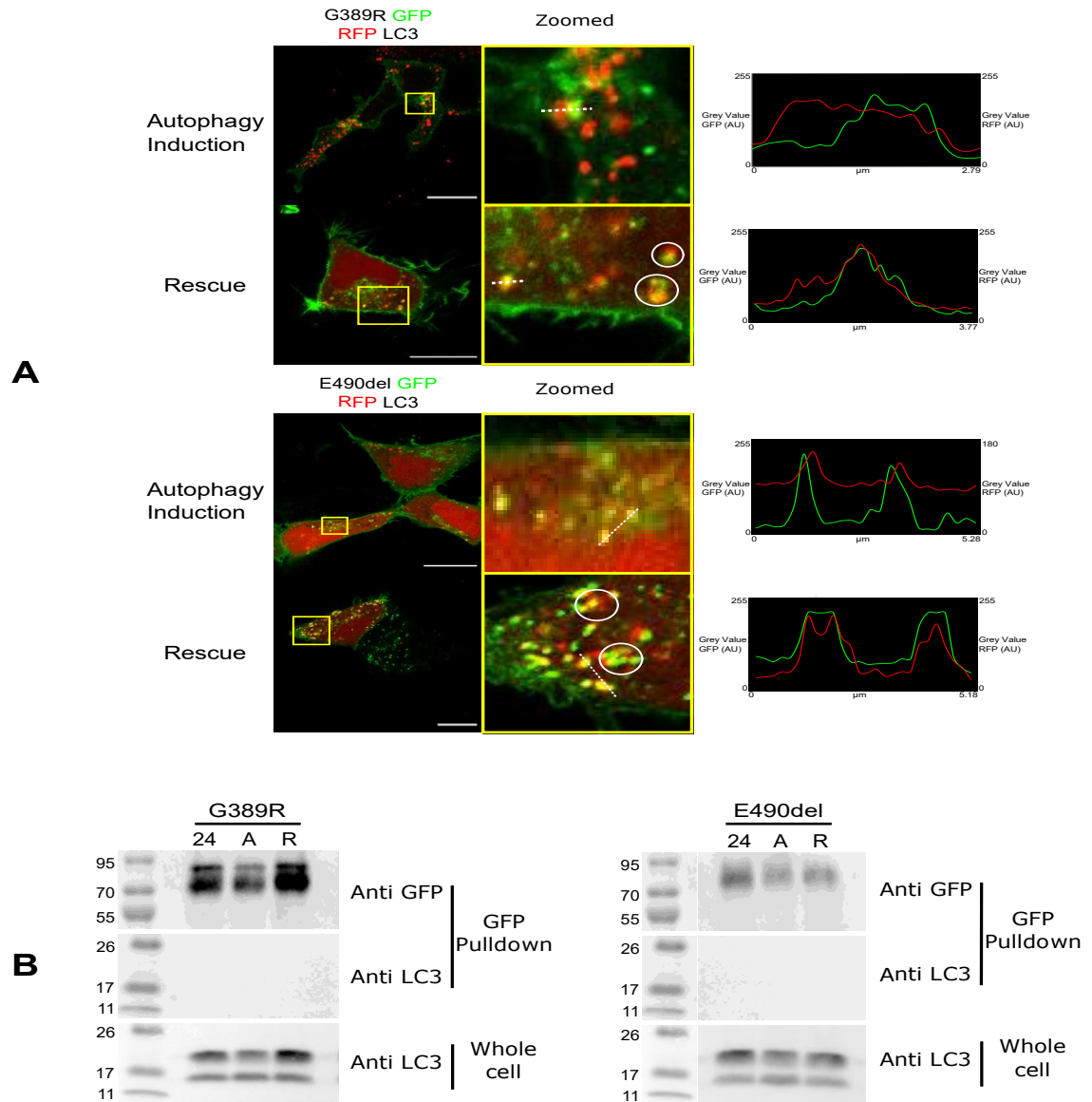


Figure 19 – Mutants G389R and E490del co-localise of LC3 but do not physically interact.

(A) Confocal imaging of HEK293 cells overexpressing G389R or E490del ANKH-GFP (green) and RFP-LC3 (red): left showed co-localisation of G389R or E490del and LC3 during autophagy induction and rescue (circled in zoom), right – intensity over distance (dotted line in zoom) plot confirming co-localisation of G389R or E490del and LC3 (B) Lysates of HEK293 cells overexpressing G389R or E490del ANKH-GFP and RFP-LC3 (I): one of the three subsets was collected at 24 hours (24), media was replaced in the remaining subsets with autophagic media for 3 hours and the second subset of lysates were collected (A), the remaining subset was rescued by replacing media with normal growth media for further 24 hours and lysates were collected (R); top: anti GFP WB of GFP pull down showing specific IP of G389R or E490del ANKH-GFP (~75,000 Da) with GFP Trap MA beads, middle: anti-LC3 WB of GFP pull down – highlighting G389R or E490del did not physically interact with LC3, bottom: anti-LC3 WB of whole cell lysate before GFP pull down showing presence of LC3 (~17,000 Da).

no co-immunoprecipitation of LC3 after western blot analysis of purified lysates under any condition (Figure 19B).

3.1.12 – Discussion

Firstly, to rationalise our choice of mutant ANKH constructs P5L, S357del, G389R and E490del. We chose these as being the most established and well-characterised mutants within the literature. The P5L mutation has been extensively studied and provided us with a good calibrator for our model. The S357del is thought to be the most severe clinically and has several case studies. The G389R is clinically associated with both CPPDD and CMD in the literature and provided us with an interesting data set to compare our molecular data to. Lastly, the E490del is most extensively studied mutation within the C terminal and would have better referencing to compare our data.

ANKH function at the cell membrane to transport PPi into the ECM is well established, however, contrasting mechanistic evidence of mutated ANKH altered intracellular trafficking and localisation has not been previously reported. We initially discovered ANKH localisation to the cell membrane to be non-homogenous and a striking distinction between the localisation dynamics of each ANKH mutants. P5L and G389R both localised more slowly but specifically to the cell membrane, S375del did not localise specifically to the cell membrane at any time and while E490del showed some cell membrane localisation it was mostly cytoplasmic. These fragmented localisation dynamics; even between mutants causing similar clinical features, highlights the complex and divisive function of ANKH in the pathogenesis of CPPDD and CMD. Clustering of wt. ANKH at the cell membrane indicates the possibility of localised domains containing ANKH (Garcia-Parajo et al. 2014). Perhaps the complex interplay of ANKH with ENPP1, TNAP and PIT-1 requires

these proteins to be within close proximity, as indicated by ANKH effect on modulating ENPP1 and PIT-1 activity (Wang et al. 2008, Wang et al. 2009, Zaka et al. 2006).

The delay in cell membrane recruitment for CPPDD associated mutant P5L may indicate a processing defect caused by the change in amino acid from proline to leucine. Recently, Molnar et al. (2016) described that even though leucine to proline is a non-polar to non-polar substitution, the presence of proline residues within close proximity of α -helices in transmembrane proteins can expose hydrogen bridge acceptors that provide a ‘partial extra charge’ (Molnár et al. 2016). Therefore, loss of this partial charge would invariably affect protein anchoring within the ER, especially if the defect is at the N-terminal. Glucose transporter 1 (GLUT1) is a well-studied multipass transmembrane protein that is anchored into the ER membrane via a signal anchor sequence within the first α -helix. Disruption of the first α -helix has been shown to delay GLUT1 protein translocation to the cell membrane, inverted orientation into the ER membrane and loss of protein function (Blodgett et al. 2008, Pascual et al. 2008). There is also evidence that unlike proteins destined for cytosolic regions, multipass transmembrane proteins are targeted for cell membrane translocation by interacting with ER machinery at several locations along the peptide chain (Molnár et al. 2016). Perhaps, this allows for a more robust anchoring of the peptide into the ER during translocation and ‘flow’ of membrane material from ER to the Golgi apparatus and finally to the cell membrane. This would also explain the delay rather than complete halt of mutant P5L ANKH protein translocation from ER to cell membrane. The serine deletion at residue 375 occurs within a predicted cytosolic loop of ANKH and our data clearly demonstrates the importance of this residue in physiological processing and cell membrane targeting of ANKH. The complete ablation of cell membrane or specific cytoplasmic regional localisation indicates the S375del mutation to be severely detrimental, a novel mechanistic observation that relates to the severe pathology of CMD patients with this mutation compared to others. Although some cytoskeletal localisation

was observed at 24 hours after transfection in some transfected cells, mostly S375del ANKH protein was observed diffusely expressed within the cytoplasm of HEK293 cells similar to what is expected of soluble cytosolic proteins. Most proteins bound for insertion into a membrane are first anchored into the ER, via recognition of their signal anchor sequence by signal recognition particle (SRP) that binds to SRP receptors on the surface of the ER and forces translocation of the newly translated protein into the ER membrane through the SEC61 translocon complex, a pore through the ER membrane (Berndt et al. 2009). On the other hand, soluble cytosolic proteins contain hydrophilic residues towards the outside of their tertiary structure and are translated by ribosomes in the cytoplasm (Stephens and Nicchitta 2008). Serine is a polar hydrophilic residue, and hence it would seem contradictory to assume that a decrease in hydrophilic potential would cause the pathological reduction in cell membrane insertion. In theory, as mentioned above, multipass membranes may interact with and are recognised by ER machinery at several locations along the peptide chain (Feige and Hendershot 2013) and simply serine at residue 375 is essential for proper protein insertion and anchoring. Interestingly, the bilateral transport capability of the SEC61 translocon complex may better explain the observed molecular phenotype. Although the standard mechanism of the SEC61 complex is unilateral translocation of newly synthesised peptide into the ER lumen or membrane, damaged or mis-folded proteins can be retrograde translocated from the ER into the cytoplasm for degradation via the ER-associated degradation (ERAD) pathway (Ruggiano et al 2014). Furthermore, we did not observe co-localisation of S375del with LC3, indicating that degradation of mutant S375del ANKH protein is not specific for macroautophagy but rather another protein degradation mechanism, of which the ERAD pathway is the most compatible for insoluble defective proteins (Ruggiano et al. 2014).

Classically, the G389R mutation is associated with CMD, however recently it was described in a familial study co-segregating with CPPDD (Baynam et al. 2009). Our

confocal microscopy data has somewhat replicated this association, as we observed a delayed cell membrane recruitment of mutant G389R similar to mutant P5L, which is clinically only reported in cases with CPPDD. Structurally, the change from glycine to arginine at residue 389 seems to hinder steric processing of the mutant protein. Glycine is a simple robust non-polar residue, whilst arginine is a polar acidic residue more than double in molecular weight (75,000 Da vs. 174,000 Da). Simply, this added 'bulk' might delay varied stages of protein synthesis and translocation to cell membrane. Moreover, glycine residues are often found in parts of the protein forbidden to all other amino acids (*e.g.* tight turns in structure) (Betts and Russell 2003) and G389R was observed to co-localise with LC3 in autophagy induced and rescue conditions. This highlights the possibility that a disastrous conformational change could be present in mutant G389R ANKH protein, however, this change does not eliminate translocation of G389R protein to the cell membrane and only under increased autophagic flux was the mutant protein targeted by autophagic machinery. Furthermore, glycine residues are known to bind ATP in several protein kinases (Betts and Russell 2003, Hemmer et al. 1997) and are often extremely conserved, appearing in their classic X-Gly-X-X-Gly motif. Although no such motif is present in ANKH, substituting to an arginine residue that contains a complex guanidinium group severely increases the potential to bind negatively charged phosphate groups as arginine can form multiple hydrogen bonds compared to the none of glycine (Betts and Russell 2003).

The E490del mutation is associated with a gain-of-function phenotype in CPPDD patients, yet our confocal data measured very low percentages of HEK293 cells with specific cell membrane expression over 72 hours (35%, 7% and 29%). This interesting observation firstly highlights the requirement of other effectors in ANKH related diseases, as even though E490del ANKH protein was not present in large quantities at the cell membrane there is association to a phenotype that directly requires increased ANKH quantity or

function. Secondly, the co-localisation of E490del with LC3 in all autophagy conditions further highlights the need for other effectors, as the protein was constantly targeted by autophagic machinery, even under basal conditions and hence, is probably non-functional or mis-folded. The glutamate at residue 490 is proposed to be in the C-terminal cytosolic region of ANKH; in theory, this residue should not interfere with known mechanism of protein targeting. Multipass transmembrane proteins are anchored into the ER at their transmembrane domains (Feige and Hendershot 2013). There is evidence that C-terminal domain such as PDZ, 80-90 amino acids, can anchor cell surface receptors to the cell membrane (Lee and Zheng 2010). However, we found no evidence of any known domain for amino acids 453-492 (C-terminal cytoplasmic region) of ANKH using bio-informatics software^{7,8}. Nevertheless, proteins in the cell membrane are often recycled via endocytosis as a natural mechanism of homeostasis. Short stretches of peptides contain a cargo internalisation signal (CIS) that can be recognised by several clathrin-associated sorting proteins (CLASPs) for clathrin-dependant endocytosis (CDE), or others in clathrin-independent endocytosis (CIE). The mechanisms of these pathways are expansive, complex and beyond the scope of this thesis and are reviewed extensively (Betts and Russell 2003, Hemmer et al. 1997). Nonetheless, if the CIS for ANKH lies within the C-terminal domain then it would be reasonable to assume increased recycling of the defective mutant protein. Furthermore, proteins destined for recycling after CIE/CDE are often degraded via the lysosomal system (Mayor et al. 2014), a novel observation confirmed by our data regarding E490del co-localisation with LC3. Lastly, the rationale for determining whether LC3 physically interacted with ANKH was to highlight any potential role of ANKH in LC3 mediated autophagy. As the contributing membrane of the autophagosome membrane is highly debated with several possibilities, we thought ANKH, a cell membrane localised protein could potentially influence autophagy. Our data does not support a physical interaction between ANKH and LC3 and as such a role for ANKH in

⁷ ebi.ac.uk/interpro

⁸ modpepint.informatik.uni-freiburg.de

LC3 mediated autophagic flux is unlikely. Perhaps, ANKH may contribute to maintaining the PPi concentrations within the autophagosome, however no data has suggested this.

3.1.13 – Scientific implications of our data

These fragmented localisation dynamics, even between mutants causing similar clinical features, underlines the complex and divisive function of ANKH in the pathogenesis of CPPDD and CMD. Our novel contrasting data highlights delayed processing, complete ablation of cell membrane targeting and increased interaction with autophagic machinery as key facilitators of mutant mechanistic effect in CPPDD and CMD. Most interesting, is the requirement of other effectors in ANKH mediated pathological effect, as observed for mutant E490del that is associated with a gain-of-function phenotype but we detected decreased ANKH localisation to cell membrane. As ANKH function to increase ePPi is at the cell membrane, there must be other contributing factors that produce the gain-of-function phenotype for the E490del mutant rather than simply implying increased ANKH transport function. The P5L mutation delayed ANKH localisation to cell membrane, however this mutation is associated with a gain-of-function in literature, and whether the delayed processing is the cause of pathology seems unlikely and rather is a mechanistic side effect. The equivocal association of ANKH mutation G389R with both CPPDD and CMD was replicated in our confocal data as we observed localisation dynamics similar to the gain-of-function P5L. However, mechanistically as mutant G389R was able to localise sufficiently to the cell membrane, the pathological effect seen in CMD patients with the G389R mutation would highlight the transport function of ANKH to be impaired. Conversely, the S375del is a simple loss-of-function due to complete loss of ANKH protein at the cell membrane. To clarify these fragmented localisation dynamics we next measured mutant effect on ePPi concentrations and mineralization potential.

Chapter 3

Results (II): ANKH mutant effect on extracellular inorganic pyrophosphate and mineralization

3.2 – Results (II): ANKH mutant effect on extracellular inorganic pyrophosphate and mineralization

3.2.1 – Introduction

HEK293 and CH-8 cells were transfected with wt. ANKH or mutant constructs and their effect on ePPi, eCa and mineralization was determined. PPi concentration was measured in the culture media of HEK293 and CH-8 cells 24, 48 and 72 hours after transfection using the PPLight Inorganic Pyrophosphate assay (see section 2.2.5). Calcium concentration was measured in culture media of HEK293 cells 24, 48 and 72 hours after transfection using the Monza RX workflow (see section 2.2.6.2). Mineralization potential was determined in transfected CH-8 cells after mineralization was induced for 72 hours using the Alizarin Red S method (see section 2.2.6.3).

3.2.2 – ANKH regulates extracellular inorganic pyrophosphate

Homeostatic regulation of phosphate concentration within the ECM derives the appropriate ratio of ePi and ePPi essential for HA deposition, disruption of which can lead to pathological deposition of CPPD, an alternative calcium-based mineral. Cheng and Pritzker (1983) reported that in aqueous solution $[Pi]/[PPi]$ greater than 100 forms HA, whilst $[Pi]/[PPi]$ less than 3 forms CPPD and Thouverey et al. (2009) also demonstrated similar results when treating chicken EMVs with varying $[Pi]/[PPi]$ (Cheng and Pritzker 1983, Thouverey et al. 2009). ANKH function at the cell membrane to transport PPi from intracellular stores into the ECM is well established, however, studies on mutant mechanistic effect on the concentration of ePPi has been limited. As deregulation of PPi is the major contributor to the pathogenesis of CPPDD and CMD, and we observed

localisation dynamics of mutant proteins counter-intuitive to their assigned association, we first confirmed mutant effect on PPi using the PPiLight Inorganic Assay.

We calibrated the working range of PPiLight Inorganic Pyrophosphate Assay using known concentrations of pyrophosphoric acid and determined a linear relationship between 0.1 and 10 μM (Figure 2 Appendix). Initial testing of culture media collected from CH-8 cells transfected with wt. ANKH, mutant constructs or null-vector revealed PPi concentrations were below threshold ($<0.1 \mu\text{M}$) at 24, 48 and 72 hours after transfection (Figure 6A Appendix). In culture media of HEK293 cells transfected with wt. ANKH or mutant constructs, PPi concentration was again below threshold at 24 hours after transfection but within the linear range at 48 and 72 hours for all constructs tested. PPi concentration in culture media for null-vector transfected and non-transfected HEK293 cells were below threshold at 24, 48 and 72 hours after transfection (Figure 6B Appendix).

Culture media of HEK293 cells transfected with wt. ANKH overexpression construct had 2.6 μM PPi at 48 hours and 3.2 μM PPi at 72 hours after transfection (Figure 20A). Compared to wt. ANKH culture media of HEK293 cells transfected with mutant P5L had significantly increased PPi (2.12 fold, $P = 0.032$) at 72 hours (Figure 20B). For mutant S375del we measured a trending decrease in PPi at 72 hours (0.55 fold, $P = 0.08$) (Figure 20C). Mutant G389R non-significantly decreased PPi at 48 (0.51 fold, $P = 0.47$) and 72 hours (0.79 fold, $P = 0.68$) (Figure 20D). Lastly, for mutant E490del we found a non-significant decrease in PPi at 48 hours (0.41 fold, $P = 0.57$) with a return to baseline at 72 hours (Figure 20E). Averages of 6 biological replicates conducted over three experimental repeats.

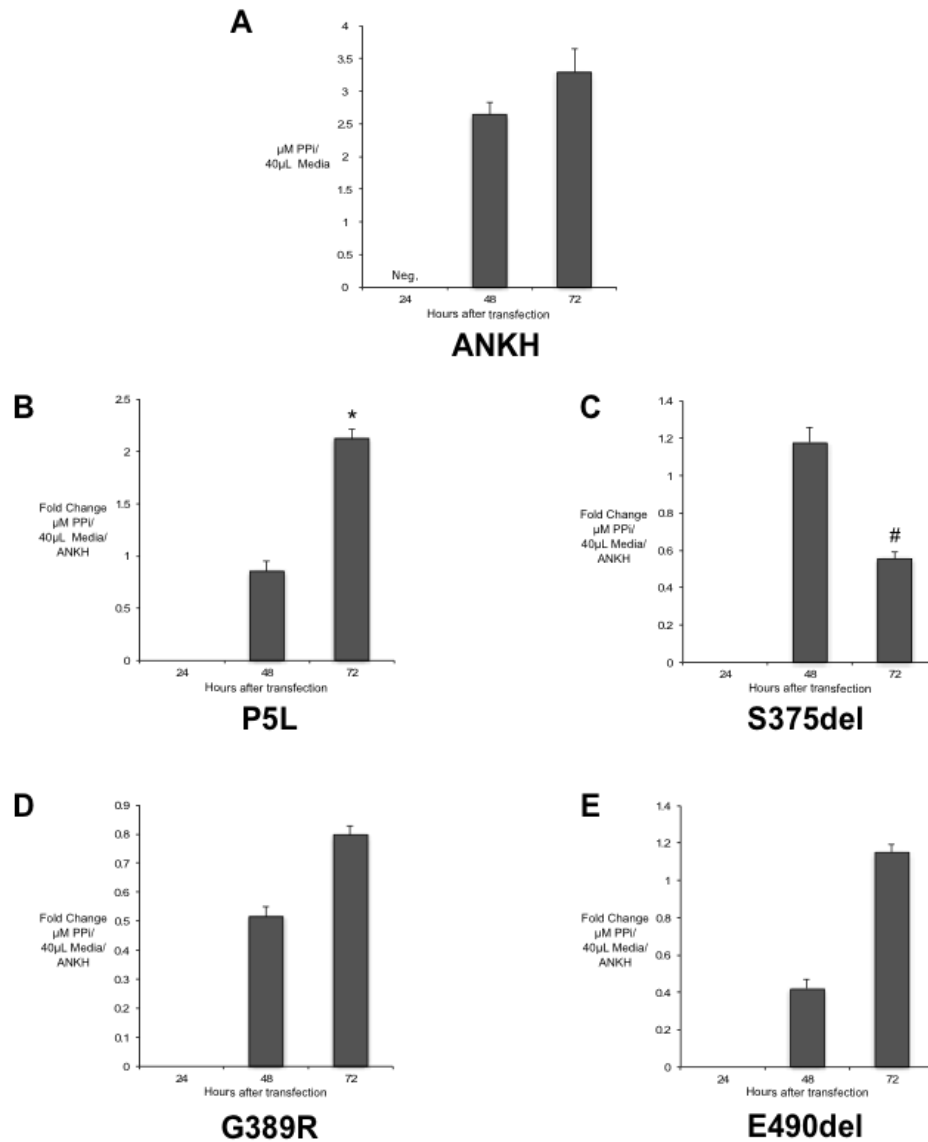


Figure 20 – ANKH mutant effect on PPi concentration in culture media of HEK293 cells. PPiLight Inorganic Pyrophosphate assay measuring concentration of PPi in culture media of HEK293 cells transfected with (A) pEGFP-N3-ANKH measured 2.6 μM PPi at 48 hours and 3.2 μM PPi at 72 hours after transfection per 40 μL culture media. (B) Compared to wt. ANKH culture media of HEK293 cells transfected with mutant P5L had significantly increased PPi (2.12 fold, $P = 0.032$) at 72 hours. (C) For mutant S375del we measured a trending decrease in PPi at 72 hours (0.55 fold, $P = 0.08$). (D) Mutant G389R non-significantly decreased PPi at 48 (0.51 fold, $P = 0.47$) and 72 hours (0.79 fold, $P = 0.68$). (E) Lastly, for mutant E490del we found a non-significant decrease in PPi at 48 hours (0.41 fold, $P = 0.57$) with a return to baseline at 72 hours.

3.2.3 – Overexpression of mutant ANKH increases mineralization compared to wt. ANKH

The downstream effect of altered PPi concentrations in the ECM of bone cells is abnormal deposition of calcium minerals such as HA and CPPD. CH-8 cells have been shown to maintain an ECM in culture that is able to mineralize and deposit HA and CPPD (Yoshimatsu et al. 2001). We measured the effect of wt. ANKH and mutant ANKH overexpression on the mineralization potential of CH-8 cells by culturing them in mineralization media for 72 hours and staining for calcium containing minerals using Alizarin Red S. After visualising the extent of mineralisation with a light microscope, bound Alizarin stain was removed and quantified (see section 2.2.6.3).

Wt. ANKH overexpression in CH-8 cells significantly decreased mineralization (37.15 nM, $P = 5.2 \times 10^{-4}$) compared to null-vector control (44.26 nM) (Figure 21A). P5L significantly increased mineralization (44.64 nM, $P = 0.01$) in CH-8 cells compared to wt. ANKH. S375del drastically increased mineralization (52.68 nM, $P = 5.12 \times 10^{-3}$). For mutant G389R we measured a significant, if small, increase in mineralization (40.62 nM, $P = 0.001$). Lastly, mutant E490del also significantly increased mineralization (42.79 nM, $P = 0.01$) (Figure 21B and Figure 21C). Averages of three biological replicates conducted over three experimental repeats.

3.2.4 – Calcium concentrations are not related to CPPDD and CMD associated mutant overexpression

Extracellular calcium concentrations are also reported to inversely correlate to concentrations of PPi in the ECM of ATDC5 cells (Zaka et al. 2006) and calcium dependant mechanisms such as PKC and ERK1/2 can modulate expression of ANK mRNA (Cailotto et al. 2007, Netter et al. 2004, Oca et al. 2010).

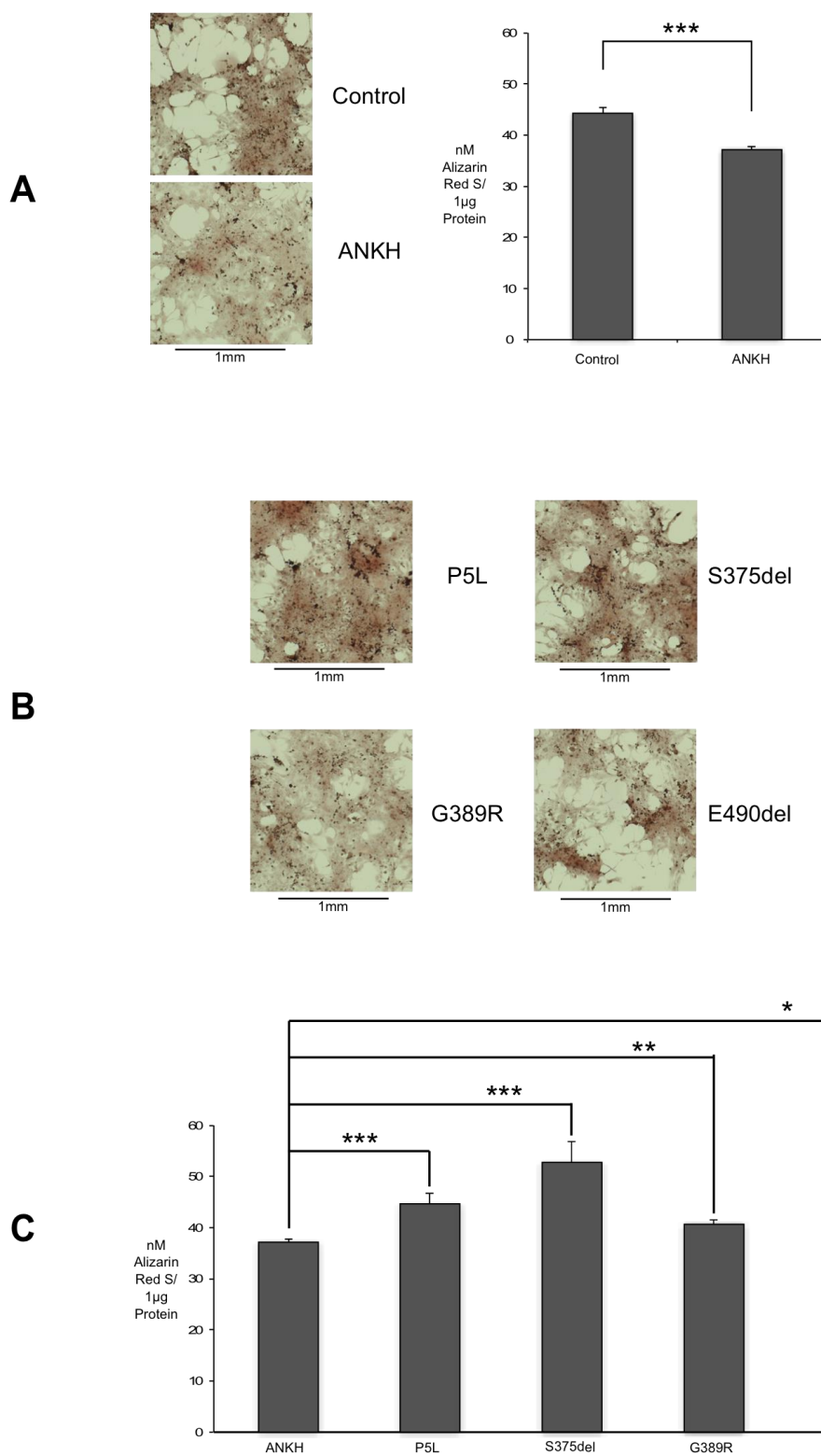


Figure 21 – Mutations in ANKH increase mineralization in CH-8 cells.

Light microscopy of bound Alizarin Red S stain (reddish) on CH-8 cells and quantification of bound stain revealed (A) CH-8 cells transfected with pEGFP-N3_ANKH significantly decreased mineralization (37.15 nM, $P = 5.2 \times 10^{-4}$) compared to null-vector control (44.26 nM) (B) Light microscopy of CH-8 cells transfected with mutant ANKH constructs. (C) Quantification of bound Alizarin Red S in CH-8 cells transfected with pEGFP-N3_ANKH-P5L significantly increased mineralization (44.64 nM, $P = 0.01$) compared to wt. ANKH. Transfection with pEGFP-N3_ANKH-S375del drastically increased mineralization (52.68 nM, $P = 5.12 \times 10^{-3}$). Transfection with pEGFP-N3_ANKH-G389R increased in mineralization (40.62 nM, $P = 0.001$). Transfection with pEGFP-N3_ANKH-E490del also significantly increased mineralization (42.79 nM, $P = 0.01$).

Serum calcium is also used as a biomarker for disease progression in clinical evaluation of CPPDD and CMD patients (Béjia et al. 2004, Dutra et al. 2012, Gruber et al. 2012, Prontera et al. 2011). We therefore measured changes in calcium concentration in the culture media of HEK293 cells transfected with overexpression constructs of wt. ANKH and mutant ANKH at 24, 36, 48, 60 and 72 hours after transfection (see section 2.2.6.2).

We normalised our data to the native non-cellular fluctuations measured in culture media only control sample and attributed this fluctuation to the maintenance of media pH over time at 37°C and 5% CO₂. Non-transfected HEK293 cells maintained calcium concentrations effectively and we measured changes attributed to cellular division and cell growth, but this affect would be enhanced or diminished in cells overexpressing wt. ANKH and representative of ANKH function (Figure 22A). Wt. ANKH overexpression increased eCa concentrations at 36 (18.93%, $P = 0.037$) hours after transfection compared to non-transfected controls (Figure 22B). The P5L mutant further deregulated eCa concentration, decreasing at 36 (19.55%, $P = 0.02$), 48 (4.40%, $P = 0.05$) and 60 (5.10%, $P = 0.01$) but increasing at 72 (17.08%, $P = 0.02$) hours after transfection compared to wt. ANKH. The S375del mutant severely decreased eCa at 72 (29.61%, $P = 0.03$) hours after transfection compared to wt. ANKH. The G389R also slightly decreased eCa at 48 (5.45%, $P = 0.03$) hours after transfection compared to wt. ANKH. The E490del mutant did not significantly alter eCa concentration at any time point compared to ANKH (Figure 22C). Averages of 6 biological replicates conducted over three experimental repeats.

3.2.5 – Discussion

The pathological presentation of CPPDD and CMD are polar molecular phenotypes resulting from either increased or decreased PPi. The generation of HA under normal physiological condition is strictly controlled by the concentration of PPi in the ECM of

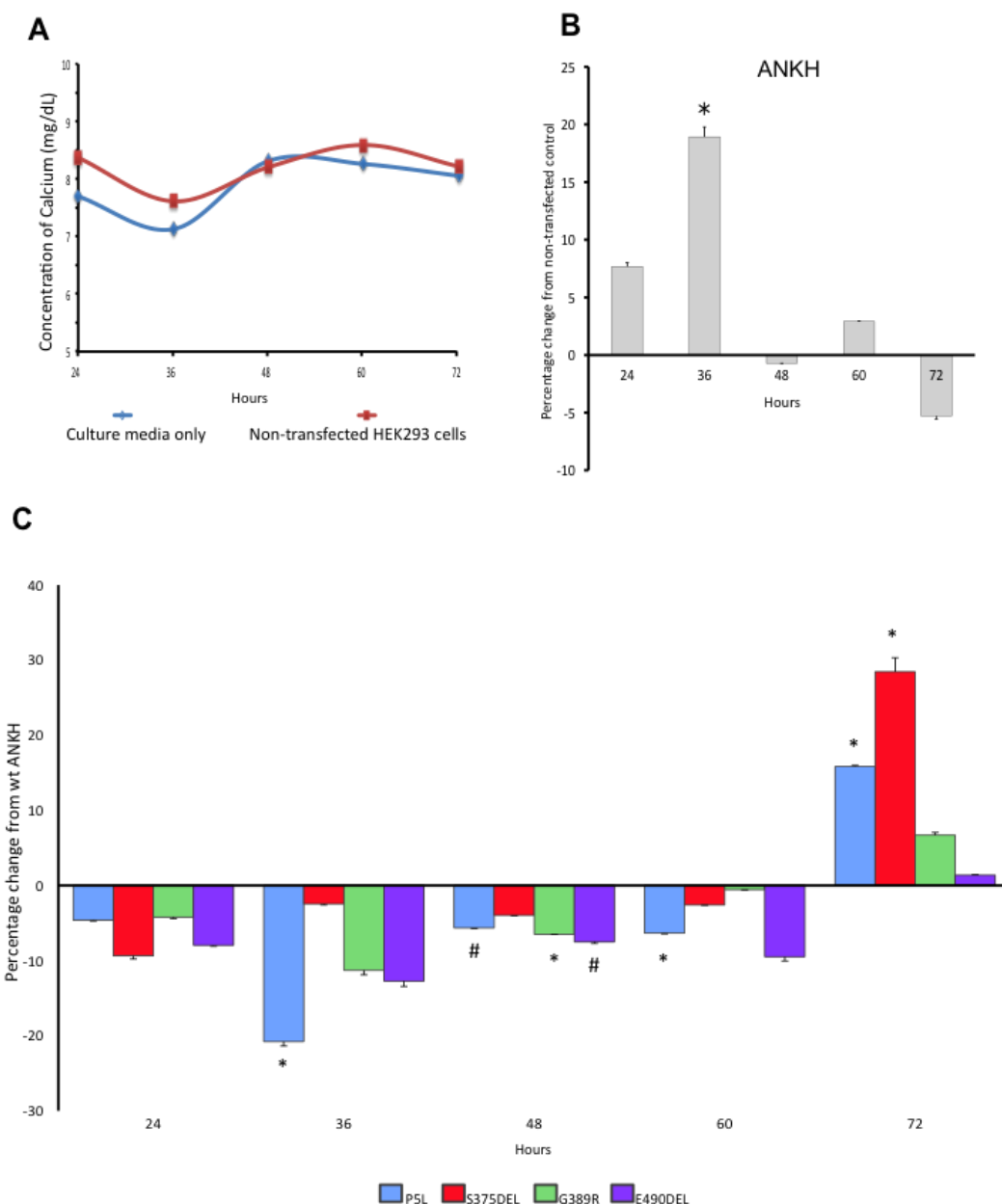


Figure 22 – Calcium concentration in the culture media of ANKH or mutant transfected HEK293 cells.

(A) Calcium concentration measured by Arsenazo III assay of culture media (blue) and non-transfected HEK293 cells (red) between 24 and 72 hours. (B) Percentage change in calcium concentration in culture media of HEK293 cells transfected with pEGFP-N3_ANKH compared to non-transfected HEK293 cells measured a significant increase only at 36 hours (18.93%, $P = 0.037$). (C) HEK293 cells transfected with ANKH mutants P5L (blue), S375del (red), G389R (green) and E490del (purple) measured variable changes in calcium concentration in their culture media compared to wt. ANKH.

bone forming cells. Direct inhibition of HA crystal nucleation due to excess PPi has been demonstrated both clinically and *in vitro* (Cheng and Pritzker 1983, Thouverey et al. 2009), however, excess PPi can also lead to pathological mineralization of CPPD causing the arthritis-like phenotype in CPPDD patients (Williams 2015). Decreasing concentrations of PPi will therefore attenuate the inhibition of HA crystal nucleation, leading to pathological bone deposition as observed in clinical presentation of CMD (Singh et al. 2016). Our experiments also showed an effect of ANKH on eCa concentrations that could be relevant to both CMD and CPPDD. In CMD, clinical evaluation includes measurement of serum calcium as a biomarker for disease progression, and previous studies on ANKH function have also observed an inverse relationship between ePPi and eCa (Abhishek and Doherty 2016, Sheppard et al. 2003, Singh et al. 2016, Zaka et al. 2006). Since HEK293 cells do not naturally exhibit calcium mineralization, eCa concentrations may be a proxy measurement of HA and CPPD crystal formation because both require calcium and deregulation of eCa could provide insights into the biochemical process maintained by ANKH. Overexpression of wt. ANKH buffered eCa similarly to non-transfected controls and only significantly increased eCa at 36 hours, possibly as a compensation for the increase in ePPi. Both the P5L CPPDD gain-of-function and G389R CMD loss-of-function mutants deregulated eCa similarly (decreasing concentration between 36 and 60 hours and increasing at 72 hours, an observation that mimics the grouping of their slower but specific cell membrane localisation dynamics). Thus, the G389R mutant is rather ambiguous in its classification as CMD and CPPDD associated, and the effect of this mutant on eCa concentration may not differentiate between CMD and CPPDD. The inverse relationship between ePPi and eCa was supported by the S375del mutant that significantly increased eCa at 72 hours, although this was later than the corresponding decrease in ePPi possibly indicating a directional relationship between ePPi and eCa concentrations. However, the eCa concentration may also act to buffer the ECM, especially when ePPi concentrations are high, as measured for

the P5L mutant. This would be important in maintaining physiological pH and appropriate membrane ionization. Overall, eCa appears to differentiate poorly between CPPDD and CMD and the major effect of ANKH mutants is through influencing ePPi.

In HEK293 cells overexpressing wt. ANKH we measured an increase in ePPi (48 hours; 2.6 μ M and 72 hours; 3.2 μ M) as expected compared to null-vector control that was below our 0.1 μ M threshold. Yet, we measured decreased mineralization in CH-8 cells overexpressing wt. ANKH (37.15 nM) compared to null-vector controls (44.26 nM). Therefore, modestly increasing ANKH quantity is not sufficient for increased deposition of CPPD in our model, but the corresponding modest increase in ePPi was sufficient for increased inhibition of HA, representing the overall decrease in mineralization. This implies that ANKH function at the cell membrane is not limited to transport of PPi, as also previously indicated by ANKH ability to increase ENPP1 activity (Zaka et al 2006). Perhaps saturation of this ANKH:ENPP1 interaction was the limiting factor responsible for the modest increase in ePPi and hence, reduction of mineralization. It is thought that ENPP1, not ANKH, generates the majority of the PPi in the ECM (Mackenzie et al. 2012). Possibly, ANKH function to transport intracellular stores of PPi into the ECM is a more controllable, less powerful mechanism and utilised for micro-management of ECM PPi concentration. Whereas, ANKH function to regulate and modulate ENPP1 represents a non-redundant role, such that if ENPP1 is already generating the majority of the PPi in the ECM then the modest PPi transport capability, compared to ENPP1 generation of PPi, of ANKH is somewhat irrelevant. Thus, if ENPP1 activity is indeed modulated by ANKH at the cell membrane then there exists a dynamic mechanism that is able to control PPi concentration in the ECM and is not reliant on either extracellular sources (NTPs for ENPP1) or intracellular sources (iPPi for ANKH transport).

Mutations associated with CPPDD have been shown to increase concentrations of ePPi in several cell types (Wang et al. 2008, Wang et al. 2009, Zaka et al. 2006, Zhang et al. 2005, Zhang and Brown 2005). However, the converse reduction in ePPi for CMD associated mutants has not been reported in molecular studies. Firstly, we present data that confirms previous observations of the P5L mutation to be a true gain-of-function (Zaka et al. 2006, Zhang et al. 2005), as we measured a significant increase in ePPi (2.12 fold) at 72 hours after transfection and mineralization (44.64 nM) compared to wt. ANKH (37.15 nM). In relation to the delayed but specific cell membrane localisation observed for mutant P5L ANKH protein (see section 3.1.7) we can hypothesise that functional pathology for the P5L mutation occurs at the cell membrane. Whether this gain-of-function is directly caused by increased ANKH transport capability or other factors, such increased ENPP1 activity remains experimentally uncertain. Taken together the increase in ePPi and mineralization indicates the mineralization to also be specific for CPPD. However, in context of disease progression the increase in ePPi (2.12 fold) does not directly correlate to the required ratio for CPPD deposition ($[Pi]/[PPi]>3$) but this is countered by the late onset characteristics of CPPDD. The mean age at disease onset is 29 years (Williams et al. 2002), unlike CMD that presents early in development (Sheppard et al. 2003). Our data therefore further highlights the need for a slow build up and concentration of PPi in the ECM of affected patients over several decades for disease progression as observed in clinical descriptions of P5L affected CPPDD patients (Williams et al. 2002).

Our novel data showed overexpressing ANKH mutation S375del decreased ePPi (0.55 fold) and increased mineralization (52.68 nM), concordant with our observation that this mutant does not localise to cell membrane. The extreme increase in HA mineralization is also clinically representative, as patients with the S375del mutation display a more severe phenotype, relative to other CMD associated mutations (Chen et al. 2017, Nürnberg et al. 2001). Regardless of the upstream defect causing the S375del protein to remain

cytoplasmic (see section 3.1.8), our molecular data for the S375del mutation further emphasises that normal ANKH function to increase ePPi requires ANKH localisation to the cell membrane. Furthermore, our data emphasises the drastic clinical severity of CMD and CPPDD, whilst CPPDD can be described as clinically relevant but not life threatening, CMD patients have greatly reduced life expectancy and require surgical intervention (Sheppard et al. 2003). Comparing to the P5L mutant that almost doubled ePPi the converse halving of ePPi measured for the S375del mutant produces a much more severe clinical phenotype. This highlights a fundamental biochemical difference between homeostatic regulation of increased or decreased PPi in the ECM, such that an excess of a raw material is better counteracted by relevant proteins/mechanisms such as TNAP and or PIT-1 but missing raw material is harder to generate and hence compensate for. Possibly due to the fact that the lack of raw material was due to defects in its generation pathway in the first place. PPi in the ECM of bone forming cells acts to both limit ossification, via inhibition of HA nucleation and provides the raw material for HA as a precursor for ePi (Williams 2015). Hence, it may be reasonable to assume evolutionary preference for increased ePPi rather than decreased ePPi was beneficial as reflected in clinical presentation of CPPDD vs. CMD.

We measured trending decreases in ePPi at 48 (0.51 fold) and 72 (0.79) hours after transfection and an increase in mineralization (40.62 nM) for the G389R mutation but had observed cell membrane localisation dynamics similar to the P5L gain-of-function mutation (see section 3.1.9). Mineralization was likely to be increased HA deposition due to the decreased ePPi and this equivocal data highlights the association of mutant G389R with both a gain-of-function and loss-of-function clinical phenotype. Nonetheless, our molecular data indicates that although the G389R mutation does not interrupt ANKH localisation to the cell membrane, there must be a loss-of-function in either PPi transport capability or interaction with ENPP1. As no previous molecular data has described the

G389R mutation to interact with ENPP1, we predict a loss in PPi transport capability due to the relatively moderate increase in mineralization and somewhat milder clinical phenotype, especially compared to the S375del mutation. Mechanistically, arginine binds negatively charged phosphates more strongly than glycine and could interrupt normal PPi binding and transport due to abnormally increased affinity at residue 389 (Betts and Russell 2003, Hemmer et al. 1997, Kovalenko et al. 2005).

Interestingly, the E490del mutation did not significantly alter ePPi concentrations compared to wt. ANKH overexpression at any time point but still increased mineralization (42.79 nM). This highlights the requirement of other effectors in the pathogenesis of CPPDD for the E490del mutation, as we had observed decreased cell membrane localisation (see section 3.1.10) but similar ePPi concentrations compared to wt. ANKH. For the E490del mutation to produce a comparable molecular phenotype to wt. ANKH but not be able to effectively localise to cell membrane would require either greatly increased transport capability or initiation of the compensatory network of ENPP1, TNAP or PIT-1. As we observed co-localisation of the E490del mutant ANKH protein with LC3 under basal conditions and postulate an increased recycling mechanism as the cause of decreased cell membrane expression, perhaps initiation of the compensatory network due to the lack of ANKH at membrane may indeed be causal of the disease phenotype.

3.2.6 - Conclusions

Taken together with our localisation data we have clarified that although mutants associated with the same disease can have different localisation dynamics, their effect on PPi and mineralization still represents their association with CPPDD or CMD. We replicated previous observations for the CPPDD associated P5L mutation to increase ePPi and pathologically deposit CPPD but highlight that this causal effect occurs at the cell

membrane. The E490del mutation, also associated with CPPDD, did not increase ePPi compared to wt. ANKH but pathologically increased mineralization. In context, the E490del mutation's ability to decrease cell membrane localisation but maintain ePPi concentrations similar to wt. ANKH clearly highlighted the need for other factors in its pathogenesis. We highlighted the increased recycling of the E490del mutant protein would perhaps cause pathological compensation by the ENPP1, TNAP and PIT-1 complex due to the decreased presence of ANKH at the cell membrane. Simply, the CMD associated S375del mutation is a true loss-of-function and our novel data clearly characterises the complete loss of cell membrane localisation causing decreased ePPi and increased mineralization. Perhaps, in contrast to the E490del mutant that does not completely abolish cell membrane localisation, for the S375del mutant the compensatory network is not capable of rescuing the molecular phenotype. We measured contrasting data for the G389R mutation that further emphasises its clinical association with both CPPDD and CMD. The mutation does not hinder cell membrane localisation but trended to decrease ePPi and pathologically increase mineralization. Our data somewhat inclines towards a loss of transport function as both clinically and now molecularly the observed phenotype is mild. Building on evidence that the majority of PPi in the ECM is generated by ENPP1, we predict that G389R mutation maintains efficient ENPP1 interaction but is unable to transport PPi. Compared to the S375del mutation that does not interact with ENPP1 due to loss of cell membrane localisation of ANKH presenting a much more severe clinical and, according to our data, molecular phenotype. The severity of the mutants tested can be ranked accordingly from their pathological effect on mineralization: S375del being the most severe (52.68 nM), second P5L (44.64 nM), third E490del (42.79 nM) and fourth G389R (40.62 nM).

Our calcium measurement data was rather ambiguous and did not offer clear insight into the molecular biology of these mutants. It is possible that although changes in PPi

concentration can effect calcium concentrations, due to the varied nature of calcium homeostasis especially in context of bone formation other factors are present. Calcium as a proxy measurement of mineralization in HEK293 cells may also represent a fundamental experimental drawback. It is evident that CH-8 cells maintain PPi at lower concentrations than HEK293 cells in their culture media due to HEK293 cells not being able to deposit minerals, reflecting their non-specialised function. This fortunately permitted measurement of PPi in HEK293, as PPi was able to concentrate and reflected ANKH function. However, this same advantage in using HEK293 cells to measure PPi would also contribute to altered calcium concentrations that would not represent ANKH function. Calcium in the ECM is used in a variety of pathways such as calmodulin signalling (CaM), regulation of enzyme activity (PKC), permeability of ion channels (binding annexins) and modulating cellular homeostasis through regulation of gene expression (Bagur and Hajnóczky 2017). Hence, several mechanisms are dependant on proper calcium concentration independent of ANKH function, and represent the primary modulators of calcium homeostasis.

Chapter 3

Results (III): ANKH mutant effect is modulated by ENPP1, TNAP and PIT-1

3.3 – Results (III): ANKH mutant effect is modulated by ENPP1, TNAP and PIT-1

3.3.1 – Introduction

Evidence for a complex containing ANKH, ENPP1, TNAP and PIT-1 in maintaining proper ECM conditions for HA deposition is overwhelming (Couto et al. 2012, Orimo 2010, Rodrigues et al. 2012, Tsui 2012, Wang et al. 2008, 2009, Zaka et al. 2006, Zhang et al. 2005, Zhang et al. 2007). The extent of interaction between these four proteins is an emerging and promising direction of study for the pathogenesis of CPPDD and CMD. Several studies have previously reported ANKH mutant ability to modulate activity or expression of ENPP1, TNAP and PIT-1 (see section 1.1.9). Our confocal and biochemical data has further highlighted the requirement of other effectors to potentiate the phenotypes causal for CPPDD and CMD. For instance, the E490del mutation decreased cell membrane localisation and increased protein recycling but still functioned similar to wt. ANKH, clearly indicates the requirement of compensatory mechanisms. Therefore, to elucidate ANKH mutant effect on ENPP1, TNAP and PIT-1 we measured their expression changes at 24, 48 and 72 hours after wt. ANKH and mutant transfection using qPCR (see section 2.2.7), SDS-PAGE (see section 2.2.10) and ENPP1 (*p*-nitrophenyl thymidine 5'-monophosphate) specific activity assays (see section 2.2.11) in HEK293 and CH-8 cells.

3.3.2 – *ENPP1*, *TNAP* and *PIT-1* expression in CH-8 cells overexpressing ANKH

Specialized cells such as CH-8 cells may also modulate gene expression, in addition to ePPi, of important bone homeostasis genes such as *ENPP1*, *TNAP* and *PIT-1* more strictly compared to HEK293 cells (Pontén et al. 2009), as we were unable to find any significant changes in expression for any of the ANKH mutants. Although, we averaged six biological replicates for each mutant during the initial experiment we found no statistical differences

comparing wt. ANKH to null-vector and mutant ANKH to wt. ANKH (Figure 7 Appendix). Any effect ANKH mutations may have had in CH-8 cells was incredibly small and we concluded an unrealistic sample size would have been required. Drawing on experience of ANKH mutant effect on CH-8 cells' inability to concentrate PPi in culture media we relied on mutant effect in HEK293 cells to determine *ENPP1*, *TNAP* and *PIT-1* expression change.

3.3.3 – ENPP1, TNAP and PIT-1 expression in CH-8 cells overexpressing ANKH

Two attempts were made to describe ENPP1, TNAP and PIT-1 protein expression changes in CH-8 cells transfected with wt. ANKH and mutant constructs. However, we were unable to consecutively replicate our data and abandoned further repeats due to time limitations (Figure 8 Appendix).

3.3.4 – *ENPP1*, *TNAP* and *PIT-1* expression in HEK293 cells overexpressing wt. ANKH

Wt. ANKH in HEK293 cells showed gene expression of *ENPP1* decreased at 24 hours (0.485 fold, $P = 0.059$) but increased at 48 (2.4 fold, $P = 0.032$) and 72 hours (1.6 fold, $P = 0.035$). A similar but non-significant trend was measured for *TNAP* at 24 (0.56 fold, $P = \text{NS}$), 48 (1.2 fold, $P = \text{NS}$) and 72 (0.61 fold, $P = \text{NS}$) hours after transfection. *PIT-1* increased significantly at 72 hours (2.00 fold, $P = 0.018$). ENPP1 activity in CH-8 cells overexpressing wt. ANKH mimicked the drop in *ENPP1* expression at 24 hours (0.70 fold, $P = 2.02 \times 10^{-5}$) and increase at 48 (1.93 fold, $P = 7.9 \times 10^{-5}$) but returned to baseline at 72 hours (Figure 23A).

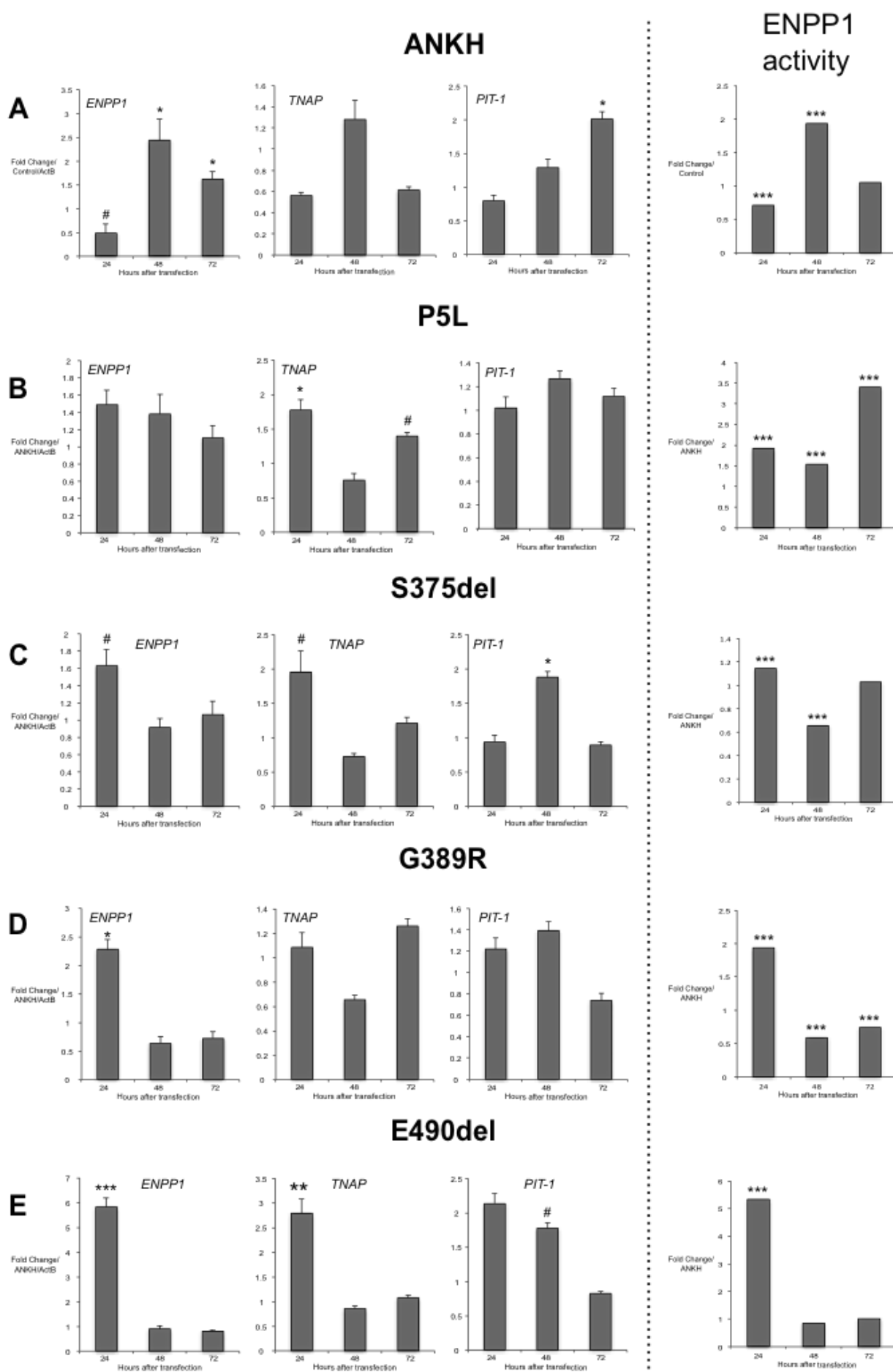


Figure 23 – ANKH mutant effect on *ENPP1*, *TNAP* and *PIT-1* expression and ENPP1 activity.

HEK293 cells transfected with (A) pEGFP-N3_ANKH (B) pEGFP-N3_ANKH-P5L (C) pEGFP-N3_ANKH-S375del (D) pEGFP-N3_ANKH-G389R (E) pEGFP-N3_ANKH-E490del were collected as lysates 24, 48 and 72 hours after transfection and expression of *ENPP1*, *TNAP* and *PIT-1* was measured using qPCR (left of dotted line). Lysates were also tested for ENPP1 activity using *p*-nitrophenyl thymidine 5'-monophosphate substrate (right of dotted line). Wt. ANKH effect is compared to Null vector transfected HEK293 cells and normalised to β -Actin. Mutant effect is compared to wt. ANKH and normalised to β -Actin.

3.3.5 – *ENPP1*, *TNAP* and *PIT-1* expression in HEK293 cells overexpressing mutant ANKH

3.3.5.1 – P5L

P5L significantly increased *TNAP* in HEK293 cells at 24 (1.72 fold, $P = 0.044$) and 72 hours (1.39 fold, $P = 0.055$) but did not change *ENPP1* or *PIT-1*. In CH-8 cells we observed increased ENPP1 activity at 24 (1.92 fold, $P = 7.56 \times 10^{-4}$), 48 (1.54 fold, $P = 5.1 \times 10^{-5}$) and 72 hours (3.39 fold, $P = 2.72 \times 10^{-6}$) (Figure 23B).

3.3.5.2 – S375del

For the S375del mutation we measured non-significant increases in *ENPP1* (1.62 fold, $P = 0.089$) and *TNAP* (1.95 fold, $P = 0.052$) at 24 hours and found a significant increase in *PIT-1* (1.877 fold, $P = 0.04$) at 48 hours in HEK293 cells. ENPP1 activity in CH-8 cells was found to increase at 24 hours (1.14 fold, $P = 9.63 \times 10^{-5}$) but decrease at 48 hours (0.65 fold, $P = 3.25 \times 10^{-5}$) (Figure 23C).

3.3.5.3 – G389R

G389R mutation was found to significantly increase *ENPP1* at 24 hours (2.28 fold, $P = 0.02$) and did not significantly change *TNAP* or *PIT-1* at any time in HEK293 cells. Similar to *ENPP1* expression, we measured an increase in ENPP1 activity in CH-8 cells at 24 hours (1.93 fold, $p = 1.21 \times 10^{-8}$) but decreased activity at 48 (0.59 fold, $p = 1.73 \times 10^{-4}$) and 72 (0.74 fold, $P = 6.99 \times 10^{-4}$) hours (Figure 23D).

3.3.5.4 – E490del

Strikingly in HEK293 cells, E490del increased expression of *ENPP1* (5.82 fold, $P = 7.3 \times 10^{-5}$), *TNAP* (2.79 fold, $P = 0.001$) and *PIT-1* (2.13 fold, $P = 0.045$) at 24 hours and trended to increase *PIT-1* at 48 hours (1.77 fold, $P = 0.058$) compared to wt. ANKH. ENPP1 activity in CH-8 cells overexpressing E490del resembled *ENPP1* expression at 24 hours with a remarkable increase (5.33 fold, $P = 1.3 \times 10^{-5}$) (Figure 23E).

3.3.6 – Discussion

Our molecular data provides a more complete, contrasting and mechanistic description of ANKH mutants associated with CPPDD and CMD. We have confirmed some previous observations for the P5L mutant (Zaka et al. 2006) and added novel interactions for S375del, G389R and E490del mutations with ENPP1, TNAP and PIT-1. However, it is important to recognise the effect of wt. ANKH overexpression compared to endogenous ANKH expression on this complex as we measured changes in *ENPP1*, *TNAP* and *PIT-1*. Firstly, we initially tested both *β-actin* and *GAPDH* to determine if there was a bias in our cell line, however we found similar Cq values when corrected with either *β-actin* or *GAPDH* (data not shown) and hence, chose to perform the full scale experiments using only *β-actin*. In summary, at 24 hours after transfection we measured non-significant decreases in both *ENPP1* (0.485 fold) and *TNAP* (0.56 fold) concordant with the lower levels of ePPi (below threshold) and wt. ANKH cell membrane localisation (57%). At 48 hours after transfection we measured increased *ENPP1* (2.4 fold) and *TNAP* (1.2 fold) expression, once again concordant with increased ePPi (2.6 μM) and wt. ANKH cell membrane localisation (66%). Lastly, at 72 hours after transfection we measured increased expression of *ENPP1* (1.6 fold) and *PIT-1* (2 fold) but decreased *TNAP* (0.61 fold) with increased ePPi (3.2 μM) and stationary percentage of wt. ANKH cell membrane

localisation (66%). Key aspects of ANKH function can be elucidated from the above data. Firstly, it is evident that *ENPP1* expression is related to percentage ANKH cell membrane localisation as we measured an almost linear relationship over 72 hours. This would further emphasise a symbiotic role of ANKH and ENPP1, building on previous observations (Zaka et al. 2006), perhaps either require the presence of the other for their function and the increase in ePPi cannot be attributable to ANKH function alone. Secondly, the increased ANKH localisation and *ENPP1* expression causing increased ePPi invariably signalled increased *TNAP* expression to facilitate the removal of excess PPi and generate ePi. As TNAP is the major clearance mechanism for free PPi in the ECM (Hessle et al. 2002) there must exist a mechanism to increase expression of *TNAP* in response to elevated PPi, with studies suggesting similar requirements for renal failure due to increased TNAP (Lomashvili et al. 2008). Previous studies have conflicted on ANKH mutant effect on TNAP expression and activity, with Zhang et al. (2005) and Zaka et al. (2006) showing no effect but Wang et al. (2008) postulating an interaction between the C-terminal of ANKH and TNAP to modulate activity. Although we were unable to determine TNAP activity in response to increased ANKH expression, our data leans towards a secondary messenger mechanism for a non-direct interaction of ANKH and TNAP; most likely, elevated PPi signalling increased expression rather than increased ANKH expression facilitating increased TNAP activity. Lastly, for *PIT-1*, we measured a delayed increase in expression concordant with previously increased *TNAP*, generating excess ePi and hence signalling increased *PIT-1* for clearance. The requirement for a physical interaction between ENPP1 and ANKH due to their parallel upstream function would seem essential, however as TNAP and PIT-1 have an antagonistic downstream function it would seem likely that their expression and activity does not directly require interaction with ANKH. However, further work is needed to confirm this hypothesis.

Nonetheless, mutations in ANKH can assert their pathology through alteration of ENPP1, TNAP and PIT-1 expression and activity. Confirming previous data, the P5L mutation increased ENPP1 activity (3.39 fold) but not *ENPP1* expression indicating the mutation facilitates increased interaction of ANKH and ENPP1 at cell membrane, but our data does not confirm any physical interaction and this would be an exciting next step in determining the true effect of the P5L mutation. Although the mutation delayed cell membrane localisation, at 72 hours after transfection once majority of the protein had entered the cell membrane (73%) we measured an increase in ePPi (2.12 fold) that was concordant with the increased ENPP1 activity. Mechanistically, the loss of a hydrogen bridge acceptor due to proline to leucine change at the N-terminal of ANKH may further potentiate the interaction and would be causal for the pathogenesis in patients with the P5L mutation. Since TNAP expression increased initially and has a role in reducing excess ePPi, we suggest that there should be a feedback mechanism to upregulate TNAP under conditions of high ePPi but cannot compensate for the mutation effect. As previously discussed in section 3.2.5, the role of ENPP1 to generate the majority of the PPi in the ECM is well established. Our ENPP1 expression and activity data for the P5L mutant further emphasises a somewhat symbiotic relationship between ANKH and ENPP1. The ANKH:ENPP1 interaction is clearly important in maintaining PPi (Zaka et al. 2006), however the extent of ANKH contribution is not experimentally certain. As discussed in section 3.2.5, it may be reasonable to assume that as ANKH and ENPP1 both act to increase ePPi, a non-redundant role for ANKH in which ANKH functions as the primary modulator of ENPP1 activity could be plausible.

For the S375del mutation we provide novel data that highlights the severity of the mutation. As we observed complete ablation of cell membrane targeting, the compensatory increases in *ENPP1* (1.62 fold) and *TNAP* (1.95 fold) at 24 hours and *PIT-1* (1.877 fold) at 48 hours after transfection could not rescue the phenotype. Interestingly, we measured an

initial increase in ENPP1 activity (1.14 fold) at 24 hours after transfection concordant with the increase in expression, however at 48 hours when ENPP1 expression was similar to wt. ANKH we measured a decrease in ENPP1 activity (0.65 fold). This further highlights the role of ANKH to potentiate ENPP1 activity, as when there was no ANKH protein present at cell membrane there was a decrease in ENPP1 activity but not expression. Of note, without visualisation of the S375del mutant's inability to localise to the cell membrane, this interaction may have appeared to be caused by changes in the physical protein-protein interaction and could explain why some conflicting data has been presented by previous studies describing ANKH protein interactions. Nonetheless, our data for the S375del mutant further emphasises the compensatory network between ANKH, ENPP1, TNAP and PIT-1. If considering just function of each component, then it would seem that ANKH and ENPP1 perform a redundant role. However, building on our data for the P5L mutant that highlights the ANKH:ENPP1 interaction as symbiotic and supplemented with our data for the S375del mutant that showed that increased expression of ENPP1 was not sufficient to counteract the lack of ANKH at the cell membrane, we can elucidate that ANKH function to increase ePPi requires ANKH localisation to the cell membrane. Furthermore, the increases in *TNAP* and *PIT-1* are evidently associated with the lack of ANKH at the cell membrane and previous work by Wang et al. (2009) has shown ANKH to physically interact with PIT-1 (Wang et al. 2009). Whether ANKH localisation to the cell membrane is required for TNAP activity remains uncertain, as we were unable to measure TNAP function (see section 2.2.12). However, taken together it is clear that ANKH has effects on ENPP1, TNAP and PIT-1 that rely on ANKH localisation to the cell membrane.

Similarly, the G389R mutation increased *ENPP1* expression (2.28 fold) with a concordant increase in ENPP1 activity (1.93 fold) at 24 hours after transfection but measured decreased ENPP1 activity at 48 (0.59 fold) and 72 hours (0.74 fold) after transfection. As we observed majority cell membrane localisation for the G389R mutation was not altered

(66% at 48 hours and 72% at 72 hours) but measured decreases in ePPi (0.51 fold at 48 hours and 0.79 fold at 72 hours) we can postulate either a loss of transport function or altered interaction with ENPP1 as causal in patients with the G389R mutations. The initial increases of *ENPP1* at 24 hours are concordant with decreased cell membrane expression of the G389R mutant and further highlights ENPP1 compensatory role. Therefore, although both mechanisms of pathogenesis are valid avenues for future studies, the decreased activity but not expression of ENPP1 at 48 and 72 hours after transfection indicates preference for the G389R mutation to interfere with the ANKH:ENPP1 interaction. However, owing to the nature of the substitution (glycine>arginine), a destructive conformational change in the transport channel also leading to increased protein recycling gives preference to a loss of transport function. Perhaps, the association to both CPPDD and CMD is reflected in these two mechanisms, altered interaction with ENPP1, although leading to a CMD molecular phenotype in our data, could somehow lead to increased ePPi if another unknown factor is present (such as mutated ENPP1). The loss of transport function would invariably associate with a decrease in ePPi causing CMD. Combination of these mechanisms is also a possibility, perhaps the destructive conformational change does disrupt transport function but also increases protein recycling leading to decreased permanent interaction with ENPP1, hence reducing its activity.

Uniquely, our molecular data for the E490del mutant indicates that it is the compensatory mechanism of ENPP1, TNAP and PIT-1 itself that is causal in CPPDD patients with the E490del mutation. We observed decreased cell membrane expression (7-35%) due to increased recycling of the mutant protein, leading to similar concentrations of ePPi as wt. ANKH overexpression but increased mineralization. However, disease causality was due to overpowered compensatory increases in *ENPP1* (5.82 fold), *TNAP* (2.79 fold) and *PIT-1* (2.13 fold) with concordant extreme increase in ENPP1 activity (5.33 fold). As discussed in section 4.5, we postulated that for the E490del mutation to produce a molecular

phenotype similar to wt. ANKH but also decrease cell membrane localisation there must be other factors. Deletion of the glutamate residue at 490 is responsible for increased recycling of the mutant protein, however the exact mechanism for this are unclear. We were unable to identify any known cell membrane targeting domains within C-terminal region of ANKH and anchoring of multipass transmembrane proteins is conducted through recognition of their transmembrane domains. However, we postulate increased endocytosis and subsequent degradation of E490del mutant ANKH protein which upregulates *ENPP1*, *TNAP* and *PIT-1* as compensation for the loss of ANKH at the cell membrane. It is noteworthy that the clinical presentation of CPPDD associated with the E490del is less severe than other forms that might be owing to some of the ANKH getting to the membrane but at lower efficiency.

3.3.7 - Conclusions

Previous molecular studies have described single ANKH mutant effect on modulation of the ENPP1, TNAP and PIT-1 complex. We aimed to provide a more robust contrasting data set containing several mutations that would better explain the localisation differences observed. Whilst confirming previous observations for the P5L mutation effect on ENPP1, our novel data for other mutants provides a clearer direction for future study in ANKH related diseases and elevates the field from primarily observational clinical studies with little mechanistic information, especially for CMD associated mutations, and provides better direction for discovery of tractable drug targets.

Chapter 4

CONCLUDING DISCUSSION

4 – CONCLUDING DISCUSSION

4.1 – Existing knowledge and limitations

4.1.1 – Current knowledge of ANKH function in regulating bone formation and mineralization

The published literature on ANKH has concentrated on clinical mineralization abnormalities in bones and joints due to mutations affecting the *ANKH* gene. Fibroblasts from *Ank* mice, routinely used as a model for destructive arthritis, were first used to describe a pathological role for ANKH in decreasing ePPi and increasing iPPi (Ho et al. 2000). This was reversed when overexpressing ANK. Ho et al. also found ANKH to be ubiquitously expressed in several tissues (heart, brain, liver, spleen, lung, muscle and kidney) and highly conserved (9/492 amino acids substituted in human vs. mouse ANK). In addition, when treating COS-7 cells overexpressing ANK at the cell membrane with extracellular probenecid there was no decrease in iPPi, suggesting ANK is a cell membrane localised PPi transporter (Ho et al. 2000). Several recent studies have also shown ANKH localisation to cell membrane (Gurley et al. 2006, Seifert et al. 2016). Structural modeling predicts ANKH to contain 9-12 transmembrane domains that may orient as a six membered parallel assembly (Ho et al. 2000, Nürnberg et al. 2001). Nonetheless, whether ANKH is indeed a true PPi transporter remains unclear. Although Gurley et al. and Carr et al. attempted to demonstrate actual membrane transport of PPi by ANKH, they were unable to measure PPi transport in the oocyte system (Carr et al. 2009, Gurley et al. 2006). They hypothesized that the membrane localisation and predicted structure of ANKH would function either as a PPi transporter itself, or as a regulator of a PPi transporter already present in oocyte membranes.

4.1.2 – Current knowledge of ANKH mutants in the pathogenesis of CPPDD and CMD

CPPDD typically presents with destructive arthritis and may mimic rheumatoid arthritis, gout or osteoarthritis and is the commonest form of inflammatory monoarthritis in the elderly, occurring in up to 40% of those over 65 years of age (Abhishek and Doherty 2016, Zhang and Brown 2005). In contrast, CMD is a rare disorder characterised by hyperostosis/sclerosis of the skull and abnormal modelling of the long bones and individuals with severe forms of CMD can have reduced life expectancy as a result of compression of the foramen magnum (Nürnberg et al. 2001). CMD is caused by decreased ePPi, which allows increased HA deposition and altered bone modelling via chondrogenesis, osteoblastogenesis and osteoclastogenesis (Minashima et al. 2010). Mutations near either end of *ANKH* are mostly associated with CPPDD while mutations in the middle have been reported to cause CMD. P5L CPPDD associated mutations caused increased expression of ANKH and was further reported to increase expression and activity of ENPP1 (Zaka et al. 2006, Zhang et al. 2005). E490del CPPDD associated mutation deregulated TNAP activity but was highlighted to be involved with other unknown factors (Pendleton et al. 2002, Wang et al. 2008). CMD related mutants have largely been restricted to clinical case studies. One case report of S375del showed a decrease in ePPi that was consistent with the predicted loss-of-function but no mechanistic molecular evidence was provided (Wu et al. 2016). G389R, reported in several cases of CMD and recently also in CPPDD, was predicted to be a loss-of-function variant (Baynam et al. 2009, Nürnberg et al. 2001). However, the biological effect and cellular function of these mutant variants remain largely unexplored with sparse mechanistic evidence in their pathogenesis.

4.1.3 – Limitations and benefits of using HEK293 cells to study ANKH related function

A major part of this thesis was the use of HEK293 cells to study mutant ANKH localisation and affect on ENPP1, TNAP and PIT-1. HEK293 cells are a robust non-specialised cell type that allowed for easy growth, transfection and confocal visualisation. Due to auto-fluorescent punctate bodies being present in both CH-8 and Saos2 cell types we were unable to replicate wt. ANKH localisation to cell membrane in these cells (Figure 13B). However, as ANKH is known to express in several tissues we thus confirmed wt. ANKH localisation in HEK293 cells (Figure 14A). Under light microscope visualisation of CH-8 and Saos2 cells, we observed large structures resembling the punctate bodies viewed in confocal imaging (Figure 9 Appendix) and can postulate that these structures may be essential to the bone forming capability of these cells. However, we were unable to find any previous publications that have visualised similar structures in these cells. We were unable to measure ePPi in CH-8 chondrocyte cells but HEK293 effectively concentrated PPi in their culture media due to the lack of their ability to deposit minerals. We were also unable to detect significant changes in *ENPP1*, *TNAP* or *PIT-1* expression in CH-8 cells but measured several changes in response to overexpression of mutant ANKH in HEK293 cells. However, as pathological ANKH function is altering mineralization in CPPDD and CMD and replication of the physiological process of bone ossification is not possible in HEK293 the major drawback is their clinical transference. It would seem unreasonable to impart major clinical significance to data obtained from unaffected cell types but rather use them as primary associations of clinical significance, to be replicated in more clinically relevant tissues and organisms. Our data clearly indicated CH-8 cells regulated their ECM and bone forming genes more strictly than HEK293 cells, and this allowed measurement of ANKH effect that was previously deemed unobtainable by several studies (Williams et al. 2002, Zaka et al. 2006, Zhang et al. 2005). Perhaps, due to their non-specialised nature, ANKH mutant overexpression in HEK293 cells was not strictly compensated and allowed

to pathologically progress, as HEK293 cells would not have the appropriate differential characteristics to combat such a pathological change. However, it was due to their non-specialised nature that allowed determination of ANKH mutant effect in HEK293 cells, not limited by strict homeostatic control exerted by expression of ossification specific genes as in chondrocytes.

4.2 – Conclusions regarding wild type ANKH molecular function

We first confirmed some important previous observations for wt. ANKH to certify our model was effective in reproducing known ANKH functions. The predicted role of ANKH to transport PPi from the subcellular region into the ECM of bone forming cells requires ANKH localisation to cell membrane. Several previous studies have shown wt. ANKH to localise to cell membrane in a variety of cell types, including non-bone cell types such as HeLa, A549 and HAF/human adult fibroblasts (see section 3.1.5). Our data also confirms wt. ANKH primarily localises to cell membrane in HEK293 cells, however we noted that this expression was clustered in a non-uniform manner. We also observed perinuclear wt. ANKH expression highlighting previous observations by Seifert et al. (2016), that ANKH has a role in regulating endosomal membrane trafficking (Figure 14A). Our data does not further any hypothesis relating to ANKH subcellular function as we observed no co-localisation with LC3 and no effect on autophagic flux when overexpressing wt. ANKH (Figure 19C, Figure 19D and Figure 19E). However, the clustering of wt. ANKH at the cell membrane indicated the possible presence of a domain containing ANKH, perhaps with ENPP1, TNAP and PIT-1 (Movie 1).

Next, we confirmed once wt. ANKH was localised to cell membrane it does act to increase ePPi, however simply overexpressing wt. ANKH does not increase mineralization. Although Uzuki et al. (2014) measured increased ANK protein and mRNA corresponding

to increased CPPD deposition in affected tissues from patients suffering with osteoarthritis with CPPDD, they failed to describe whether these patients were carrying a mutant gene. The antibodies used in their study were designed against amino acids 118-130 (Ab1) and 477-492 (Ab3) and these would not differentiate between wt. ANKH or the CPPDD associated mutation P5L. Overexpression of mutant P5L was previously shown to increase expression of endogenous ANKH in CH-8 cells (Zhang et al. 2005). Furthermore, the RNA probes used by Uzuki et al. would not differentiate wt. ANKH from any known mutant variant (Uzuki et al. 2014). In our model, moderate overexpression of ANKH does not sufficiently increase ePPi to deposit CPPD ($[Pi]/[PPi]>3$), however enough to inhibit HA accretion ($[Pi]/[PPi]$ between 3-99) and overall decrease mineralization. Interestingly, wt. ANKH overexpression and ePPi increase was linked to a corresponding increase in *ENPP1* expression. We observed maximum cell membrane localisation at 48 and 72 hours after transfection (both time points = 66%) an increase in ePPi (2.6 μ M at 48 hours and 3.2 μ M at 72 hours) and significantly increased *ENPP1* expression (2.4 fold at 48 hours and 1.6 fold at 72 hours). However, ENPP1 activity was only significantly increased at 48 hours (1.93 fold) but not changed at 72 hours. Firstly, this indicated a synergistic expression profile for ANKH and ENPP1 but more importantly as ENPP1 is known to generate the majority of the PPi in the ECM (Nam et al. 2011) and ANKH is postulated to regulate ENPP1 activity (Zaka et al. 2006), perhaps it was saturation of the ANKH:ENPP1 interaction that limited the increase in ePPi as indicated by the unchanged ENPP1 activity at 72 hours.

The non-significant changes to *TNAP* expression further emphasise that ePPi concentrations were not pathologically increased, however the 2-fold increase in *PIT-1* at 72 hours, when ePPi levels were highest, indicates that there was excess ePi due to increased HA accretion inhibition. Wang et al. (2009) showed ANK and PIT-1 can be co-immunoprecipitated together, however in our study, expression of PIT-1 did not

correspond with ANKH expression but rather the increase in ePPi. Therefore, although ANKH may modulate PIT-1 activity, its expression may be regulated by changes in ePPi and hence ePi. TNAP expression and activity was shown to decrease in the presence of ANKH mutant E490del, whether this was due to a physical interaction was not determined and Wang et al. (2008) measured the presence of some low molecular weight inhibitors causing the decrease. We were unable to measure TNAP activity but *TNAP* expression profile (24 hours: 0.56 fold, 48 hours: 1.2 fold, 72 hours: 0.61 fold) was somewhat similar to *ENPP1* expression profile (24 hours: 0.485 fold, 48 hours: 2.4 fold, 72 hours: 1.6 fold) but did not directly correspond to ANKH cell localisation (24 hours: 56%, 48 hours: 66%, 72 hours: 66%) and ePPi (24 hours: below threshold, 48 hours: 2.6 μ M, 72 hours: 3.2 μ M), perhaps indicating that TNAP is regulated independently of ANKH as postulated by Wang et al. (2008) and maybe a rate limiting step in generating ePi from excess ePPi.

4.2.1 – P5L

Previously, the P5L mutant variant of ANKH was shown to attenuate NF- κ B signalling due to altered interactions with MYBB1a and SPHK1 (Minashima et al. 2014). Overexpression of mutant P5L was shown to increase ANKH and collagen type X expression in CH-8 cells (Zhang et al. 2005) and increased ENPP1 activity in ATDC5 cells (Zaka et al. 2006). Therefore, it was evident that the pathological phenotype observed in CPPDD patients with the P5L mutation may not be limited to a pathological increase in ANKH PPi transport function. Our confocal imaging (Figure 15) highlighted an initial defect in the processing of the P5L mutant protein (24 hours: 34% cell membrane localisation) and we reason that the proline to leucine change interferes with anchoring into the ER membrane due to the loss of a partial charge disrupting the confirmation of the first α -helix (Molnár et al. 2016). However, once inserted into the cell membrane this confirmation change facilitates an increased interaction with ENPP1 and increases its

activity (24 hours: 1.92 fold, 48 hours: 1.54 fold, 72 hours: 3.39 fold). As we measured no significant change in *ENPP1* expression, the increase in ePPi (72 hours: 2.12 fold) and resulting increased deposition of CPPD (see section 3.2.3) was attributed to the increased ENPP1 activity of protein already at the membrane rather than increased expression. As a consequence of the increasing ePPi, we measured increase in *TNAP* expression at 72 hours (1.39 fold), however the initial increase at 24 hours (1.72 fold) maybe due to other ANKH and ePPi independent factors such as the ones described by Wang et al. (2008) for the E490del mutant (Wang et al. 2008). Therefore, the pathologically significant increase in ePPi and concordant increase in mineralization truly identifies the P5L mutation to be a gain-of-function and CPPDD associated but not simply due to an increase in PPi transport capability.

4.2.2 – *S375del*

Associated with an extremely severe form of CMD, the *S375del* mutation caused ANKH to remain completely to the cytoplasm and abolished cell membrane localisation (Figure 16). The resulting decrease in ePPi (0.55 fold) and extreme increase in mineralization (see section 3.2.3) was not rescued by the compensatory increases in *ENPP1* (1.62 fold), *TNAP* (1.95 fold) and *PIT-1* (1.877 fold). The lack of ANKH at the cell membrane decreased ENPP1 activity (0.65 fold) concordant with our data for the P5L mutant's ability to increase ENPP1 activity at the cell membrane. There is no previous mechanistic molecular data for the *S375del* mutant and our novel association of cytoplasmic localisation with decreased ENPP1 activity provides further insight into ANKH function at cell membrane, steering future research direction to not limit studies to ANKH transport function alone. Mechanistically, the serine deletion may cause retrograde translocation of the mis-folded or defective protein from the ER into the cytoplasm, to be degraded by the ERAD but not autophagic pathways as determined by the lack of co-localisation with LC3 (Figure 19C).

4.2.3 – G389R

Unique in the mutants we tested, the G389R mutation is clinically associated with both CMD and CPPDD and our novel molecular data replicates this equivocal association. Gurley et al. (2006) injected frog oocytes with G389R ANKH and measured decreased PPI transport attributed to ANKH function. Our data was non significant for the G389R mutation but we measured trending decreases in ePPI at 48 (0.51 fold) and 72 hours (0.79 fold) and a mild increase in mineralization (see section 3.2.3). However, most interestingly the G389R mutant protein was found co-localised with LC3 after autophagic induction and taken together with the mutant ability to effectively insert into the cell membrane, our mechanistic determination for the G389R mutation inclines towards a loss of transport channel function facilitated by glycine to arginine substitution. As glycine residues are often found in position restricted to other residues, the potential for a drastic conformational change is plausible, however most likely it is the introduction of hydrophilic charged residue disrupting protein flexibility (Betts and Russell 2003, Hemmer et al. 1997). However, the decrease in ENPP1 activity at 48 (0.59 fold) and 72 hours (0.74 fold) is independent of *ENPP1* expression and therefore the combinatory effect of defective transport capability and reduced permanent interactions with ENPP1 due to increased recycling maybe causal for the pathology in G389R affected CMD patients.

4.2.4 – E490del

Previous studies have shown the E490del mutation to increase ePPI (Zhang et al. 2005), decrease iPPI (Pendleton et al. 2002) and decrease TNAP expression and activity (Wang et al. 2008). Our data adds more complexity to this mutant's mechanistic pathology and highlights the presence of a defective or mis-folded protein as key. However, as we were unable to measure TNAP activity we are unable to comment on the E490del mutation's

effect but we measured an increase in *TNAP* expression in contradiction to previous reports by Wang et al. (2008). Key differences in methodology may explain these contradictions, as we measured changes in expression at 24, 48 and 72 hours after transfection in non-specialised HEK293 cells but Wang et al. (2008) measured changes between days 10-23 of induced rat ATDC5 chondrocyte cells. They postulated to the presence of low molecular weight inhibitors of TNAP in their samples causing the decrease in activity. Furthermore, their study also contradicted previous results obtained by Williams et al. (2002) for the expression of *Col 10a1* and they reasoned that the single time point chosen by Williams et al. (2002) could be subjected to intrinsic compensatory feedback transcriptional down-regulation that would dampen any gain-of-function inferred by the mutation “leading to inconsistent and even contradictory results from different laboratories” (pp 28, Wang et al. 2008). In our model, the lack of E490del cell membrane expression and constant co-localisation with LC3 was pathologically compensated for by increased *ENPP1* (5.82 fold), *TNAP* (2.79 fold) and *PIT-1* (2.13 fold) expression and maybe causal in CPPDD affected patients. Mechanistically, we were unable to identify any cell membrane targeting domains at the C-terminal of ANKH, however as ANKH has been shown to modulate clathrin-mediated membrane trafficking (Seifert et al. 2016), perhaps this mutation disrupts cycling of ANKH from the TGN to cell membrane.

4.4 – Summaries of ANKH mutants' mechanism in pathology of CPPDD and CMD

We summarize the possible mechanisms in the pathogenesis of each tested mutants associated with CPPDD or CMD as follows (Figure 24):

P5L – This proline to leucine mutation may delay ANKH membrane uptake and the long-term increase in ePPi could be attributed to an overreaction from increased ENPP1 activity rather than *ENPP1* expression directly. This is likely that the rise in ePPi is not due to increased ENPP1 amounts at the cell membrane but rather to increased activity of ENPP1 that is already at the cell membrane facilitated by the P5L mutation. Since TNAP expression increased initially and has a role in reducing excess ePPi, we suggest that there should be a feedback mechanism to upregulate TNAP under conditions of high ePPi but cannot compensate for the mutation effect.

S375del – Our data suggests that deletion of serine at residue 375 completely abolishes ANKH localisation to the cell membrane and is a loss-of-function. We observed almost cytoskeletal-like localisation over 72 hours and measured a corresponding decrease in ePPi and increase in mineralization. The phenotype of S375del CMD is severe and at the molecular level even the compensatory increase in *ENPP1*, *TNAP* and *PIT-1* could not rescue the biochemical phenotype. Mechanistically, we predict the serine deletion results in inappropriate trafficking from the ER and abolish ANKH function.

G389R – This mutant displayed equivocal molecular data highlighting its variable clinical associations with both CMD and CPPDD. Its cell membrane localisation resembled the CPPDD-associated mutant P5L but we measured a sustained decrease in ePPi similar to the CMD associated mutant S375del. Early upregulation of *ENPP1* under low ePPi concentrations further supports this to be a loss-of-function mutation. Hence, since the

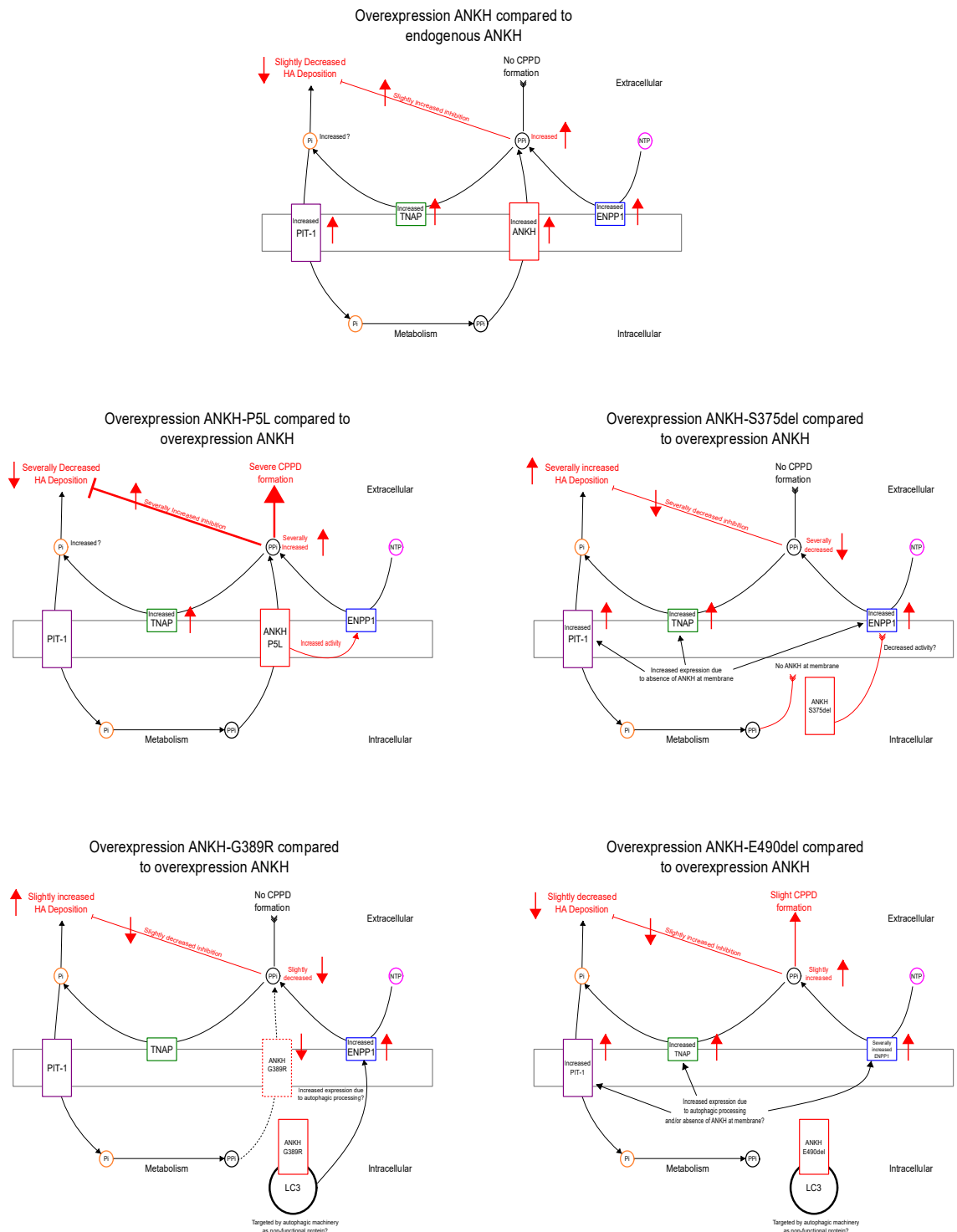


Figure 24 – Synopsis of ANKH overexpression and ANKH mutant effects on ECM biochemistry.

Red arrows = Changes in expression/activity/function/deposition. Black arrows = Generation/transport of biomolecules. Inversed black arrows = Inability to deposit. Capped red line = Inhibition.

mutant successfully localised to the cell membrane, the glycine to arginine change at residue 389 appears to cause a loss of transport channel function and decreased permanent interactions with ENPP1, which caused the decrease in ePPi and increase in mineralization, although small, reflecting its milder phenotype. The involvement of G389R in autophagy also suggests that it undergoes mis-folding or some other abnormal processing, but perhaps to a lesser degree than E490del.

E490del – The deletion of a glutamate residue at the C-terminus renders it unable to insert effectively into the cell membrane. However, this mutant resulted functions similar to wt. ANKH in regulating ePPi by increasing *ENPP1*, *TNAP* and *PIT-1* but was still pathogenic as it increased mineralization. We suggest that this mutant is recognised as a non-functional protein and is constantly recycled by the autophagy machinery, which consequently due to its loss also upregulates *ENPP1*, *TNAP* and *PIT-1* as compensation for the loss of ANKH at the cell membrane. It is noteworthy that the clinical presentation of CPPDD associated with the E490del is less severe than other forms that might be owing to some of the ANKH getting to the membrane but at lower efficiency.

4.4.1 – Final conclusions

At a molecular level the corresponding pathogenetic mechanisms for each mutant ANKH appear to be unique even between mutants causing similar phenotypes. They include effects on intracellular trafficking of ANKH, PPi transport channel function, mineralization, ENPP1 activity regulation, autophagy and guiding effects in expression level of *ENPP1*, *TNAP* and *PIT-1*, some of which could represent tractable targets for therapy. More generally, the lack of data for CMD associated mutant may arise from the difficulty of measuring minute concentrations of PPi, whereas large increases are more readily measured for CPPDD associated mutants.

4.5 – Future work

The observations can be taken forward in several important directions. PPI could be more accurately measured by using more sensitive fluorescent assays if they become available and allow for measurements in more clinically relevant cell types. Confocal imaging over longer periods would eliminate some bias attributed to specific time point selections. Use of electron microscopy would allow imaging of CH-8 and Saos2 cells without the interference of auto-fluorescent punctate bodies. Measurements of gene expression over longer periods of time needs to be investigated as gene regulation occurs in response to Pi or PPI levels (Harmey et al. 2004, Wang et al. 2009), these effects may not produce detectable changes over shorter periods. Replication in animal models would cement the gene expression and protein changes in response to the presence of mutant ANKH observed in our study. Co-immunoprecipitation of ANKH would confirm interactions with ENPP1, PIT-1 and possibly TNAP and co-localisation analysis of ANKH with ENPP1, TNAP and PIT-1 would help shore up the predicted interactions and offer insight into the clustered complex. Delineation of this complex is an important aspect in understanding the pathology behind CMD and CPPDD and would require enormous commitment that was not feasible for our study, which attempted to describe the fundamental mechanistic effects of ANKH in CMD and CPPDD. However, future studies should first focus on clearly establishing the symbiotic relationships we have alluded to but would need a crystal structure of ANKH to determine true interactions - another aspect not feasible for our study. Testing of additional ANKH mutants would invariably complicate the overall pathology of CPPDD and CMD but provide further evidence of uniqueness. Evaluating mutant effect on ANKH regulatory interactions with NF- κ B, TGF- β , HREs and JNKs pathways would provide a more complete understanding of disease progression, independent of abnormal mineralization but this would again be limited without a crystal structure for ANKH. Using methods not reliant on a crystal structure such as FRET to

determine protein interactions or advanced computer modelling may help shore up some complicating data present in the literature. Using stable models established by CRISPR technology would greatly improve reproducibility and reduce affect attributed to endogenous ANKH function when testing mutant pathogenesis.

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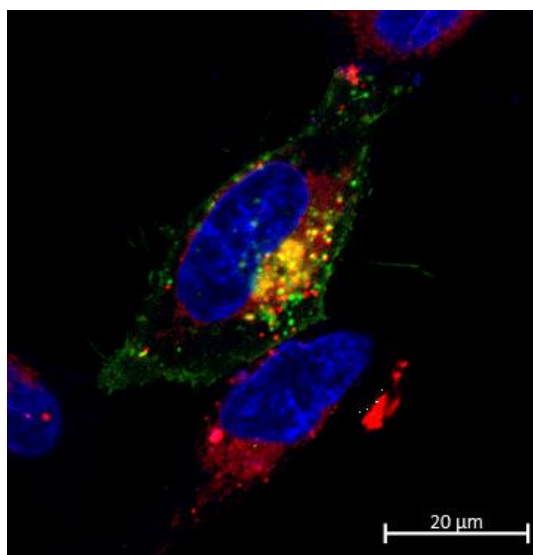
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ANKH protein and excess generation of extracellular inorganic pyrophosphate. *Arthritis and rheumatism*, 52(4), pp. 1110–1117.

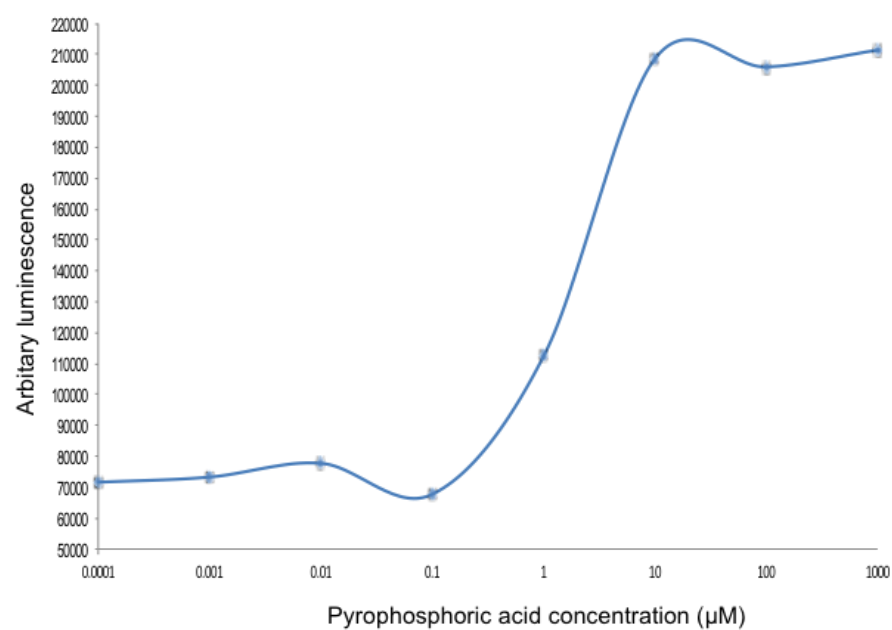
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6.1 – APPENDIX 1: ANKH supplemental data

HEK
ANKH **GFP**
ZnCL2
H33342
24 hours after
transfection



Appendix Figure 1 – ZnCl₂ staining of HEK293 cells.



Appendix Figure 2 – PPiLight Inorganic pyrophosphate assay standard curve and working range.

Plate 1 Quantification

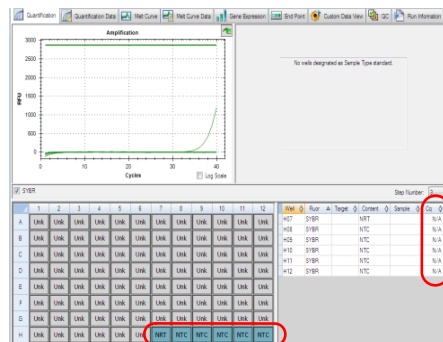


Plate 1 Melt Curve

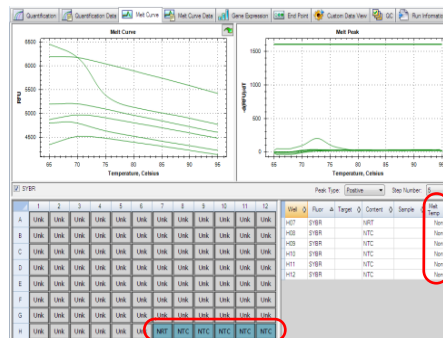


Plate 2 Quantification

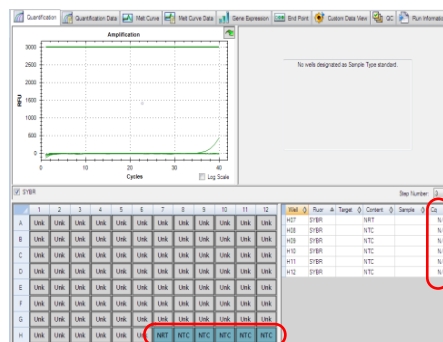
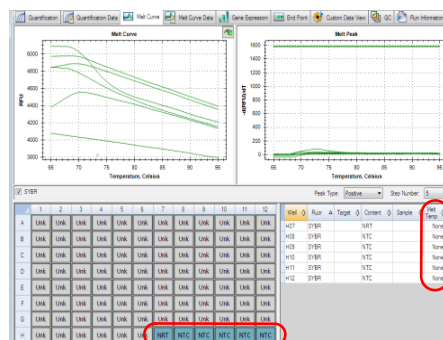


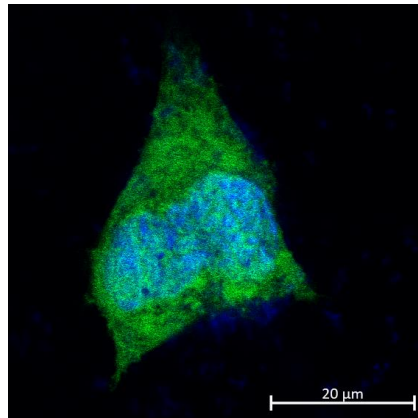
Plate 2 Melt Curve



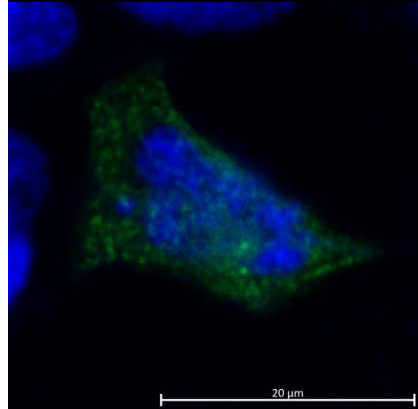
Appendix Figure 3 – Example of qPCR quality control.

NRT = No reverse transcriptase reaction. NTC = No template control. Red circles highlight corresponding amplification and melt curve results.

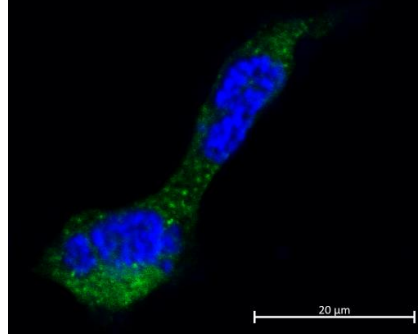
HEK
ULK4 **GFP**
H33342
3 hours after
500nM Rapamycin
induction



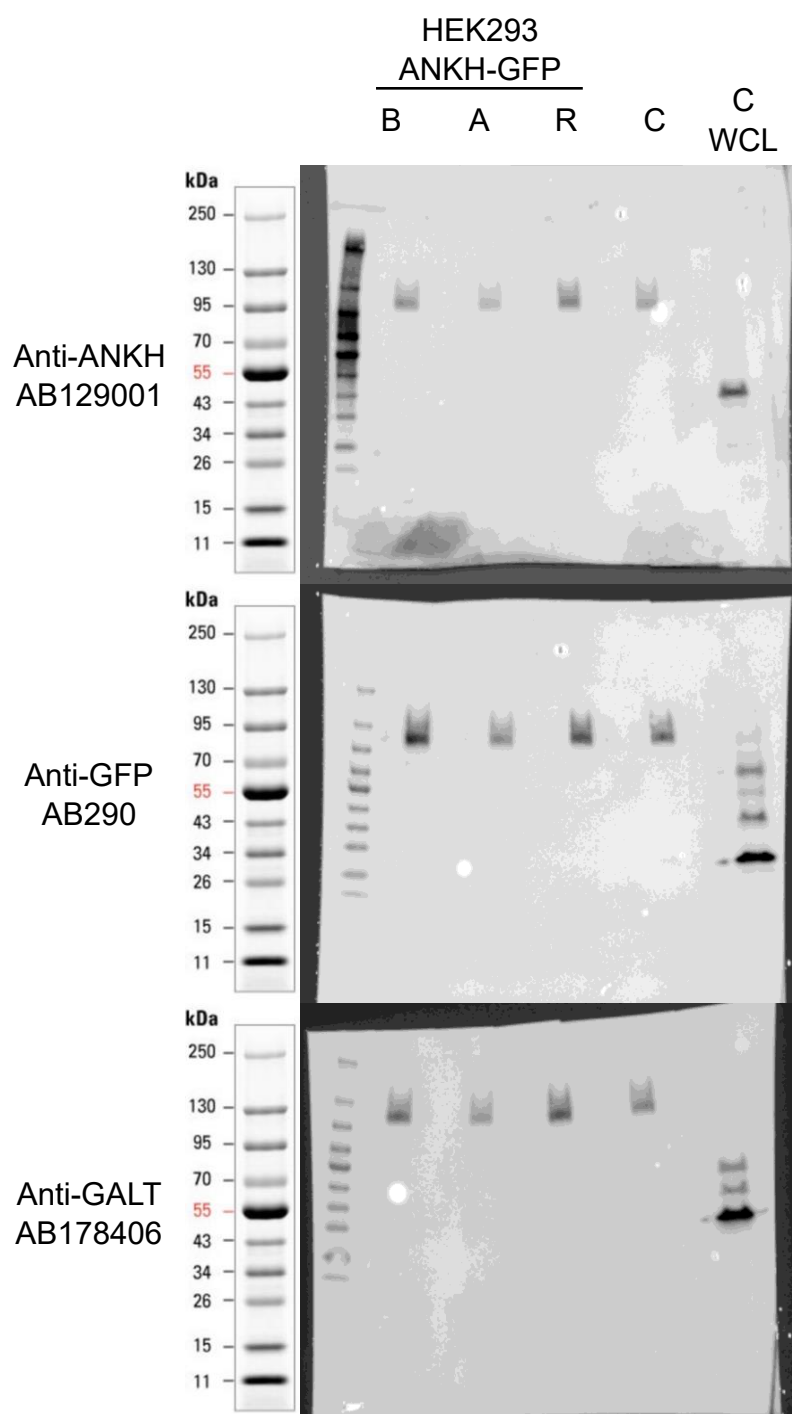
HEK
ULK4 **GFP**
H33342
3 hours after
20μM Leupeptin
induction



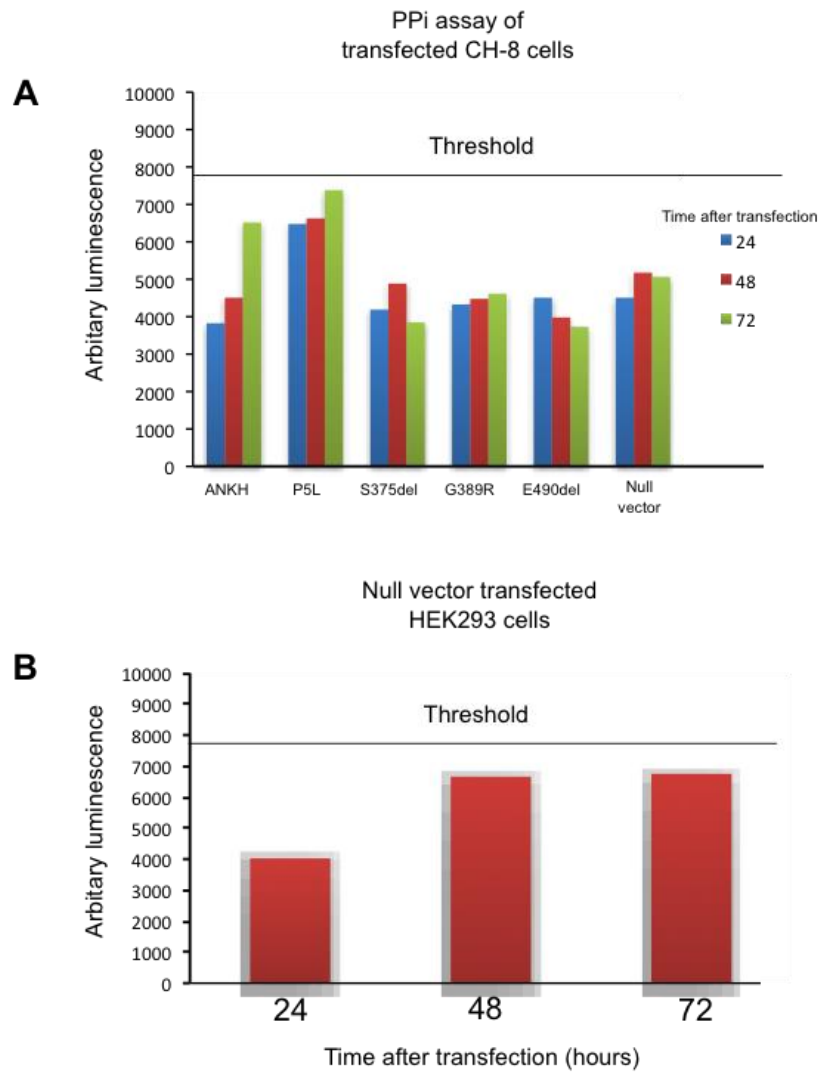
HEK
ULK4 **GFP**
H33342
3 hours after
50μM Chloroquine
induction



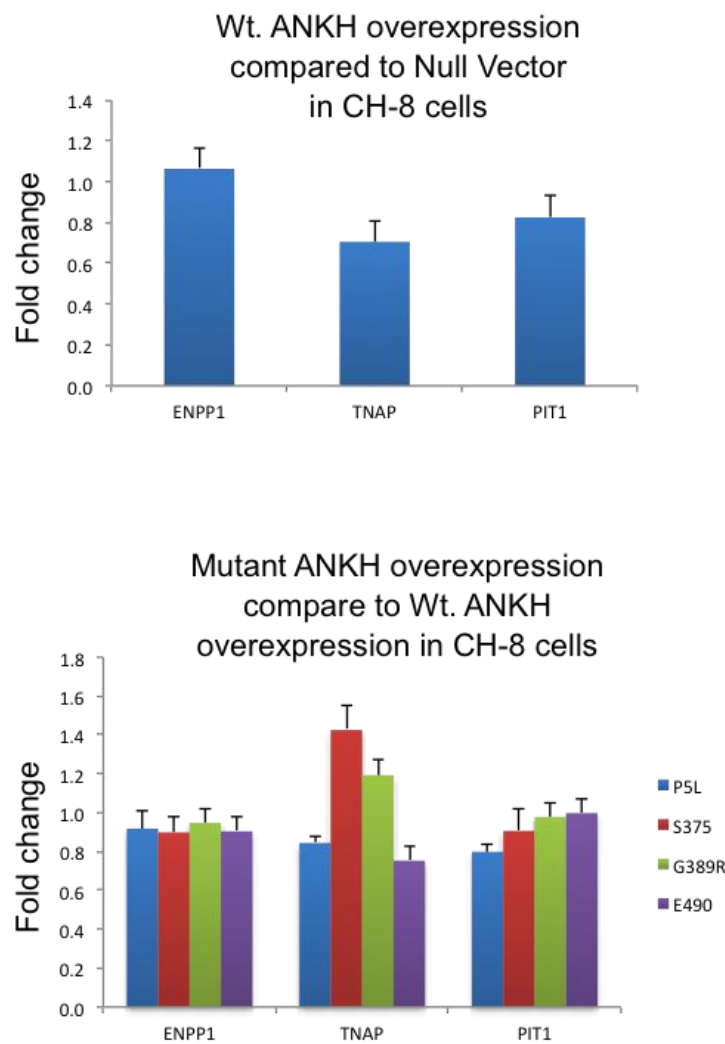
Appendix Figure 4 – Testing of rapamycin, luepeptin and chloroquine on HEK293 cells transfected with pEGFP-N3_ULK-FL.



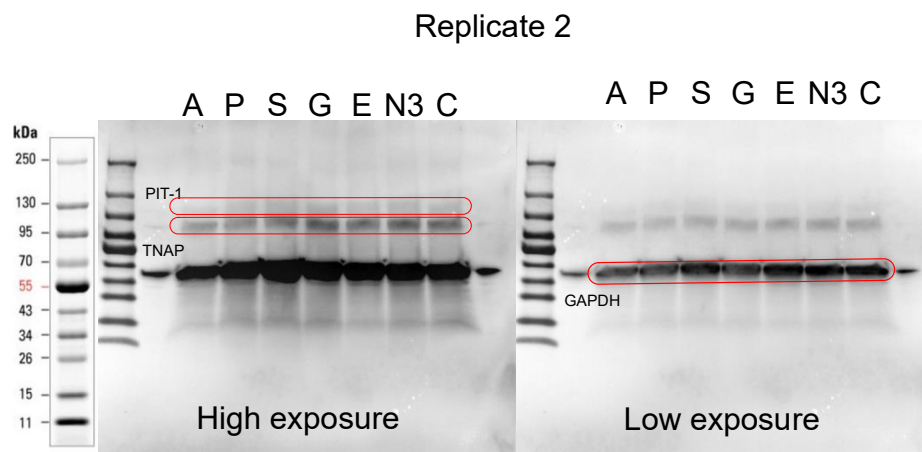
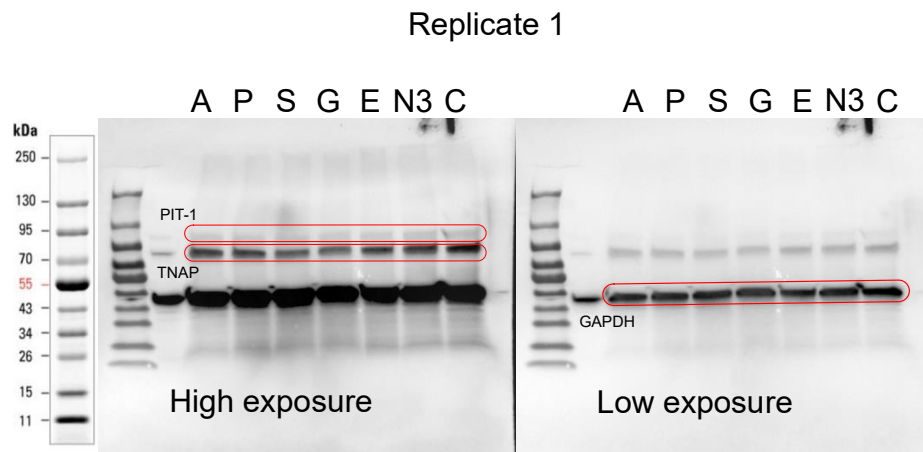
Appendix Figure 5 – Western blot analysis of Dynabeads method co-immunoprecipitation of HEK293 cells transfected with pEGFP-N3_ANKH. B = Basal condition 24 hours after transfection. A = 3 hours after autophagy induction. R = 24 hours after rescue. C = Control non-transfected HEK293 cells. C WCL = Control non-transfected HEK293 cells whole cell lysate before co-immunoprecipitation.



Appendix Figure 6 – Arbitrary luminescence measurement at 24, 48 and 72 hours transfection of PPI^{Light} Inorganic pyrophosphate assay in (A) CH-8 cells transfected with ANKH, mutants or null vector and (B) HEK293 cells transfected with null vector. Measurements are below the threshold of working range as determined by the standard curve (Appendix Figure 2).

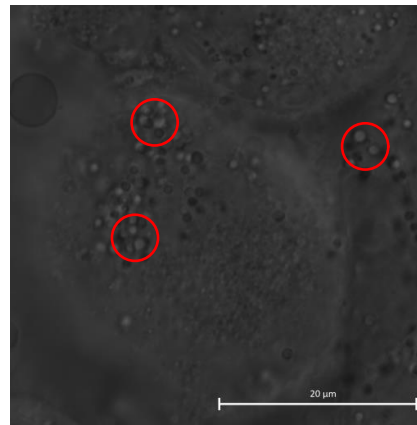


Appendix Figure 7 – qPCR analysis of *ENPP1*, *TNAP* and *PIT-1* expression fold change in CH-8 cells transfected with wt. ANKH compared to null vector (top) and mutant ANKH transfected compared wt. ANKH (bottom) normalised to β -Actin.

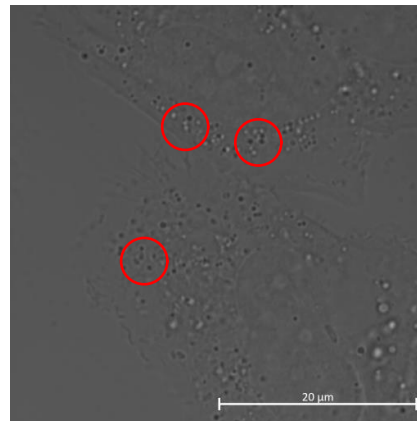


Appendix Figure 8 – Western blot analysis of wt. ANKH, mutant and null vector effect on TNAP (bottom red circle) and PIT-1 (top red circle) protein expression (High exposure). A = ANKH. P =P5L. S =S375del. G = G389R. E = E490del. N3 = Null vector pEGFP-N3. C = Non-transfected HEK293 cells. Normalised to GAPDH (Low exposure).

CH-8



Saos2



Appendix Figure 9 – Light microscopy of CH-8 and Saos2 cells displaying the round structures within the cytoplasmic regions (red circles).

6.2 – APPENDIX 2: The role and function of ULK4 in autophagy – A consolidated report of our data

Introduction

Autophagy

Autophagy, derived from the Greek words for “Self Eating”, is the major intracellular mechanism responsible for the degradation of cytoplasmic cargo in eukaryotes. It is a dynamic recycling mechanism that delivers cargo to the lysosome and produces new building blocks for cellular homeostasis.

Autophagy in human diseases

A comprehensive review published by Jiang and Mizushima, 2014, identified approximately 15 diseases associated with defective macro-, micro-, and chaperone mediated- autophagy (Peidu Jiang and Mizushima 2014). Briefly, many are causal due to single nucleotide polymorphisms (SNPs) present in *Atg* genes, such as increased risk of asthma and systemic lupus erythematosus (Martin et al. 2012, Zhou et al. 2011). Another, a missense (T300A) mutation in *ATG16L1* is associated with increased risk of Crohn’s disease (Barrett et al. 2008). SNPs in *PARK2* and *PARK6* have been associated with defective mitophagy (a sub-class of microautophagy) induction and causal for increased risk of Parkinson’s disease (Trinh and Farrer 2013).

The basics of autophagy

In eukaryotes two major degradation systems exist – proteosomal and lysosomal. Proteosomal degradation is highly selective, and requires ubiquitination of the substrate but autophagy refers only to the lysosomal mechanism. Intracellular organelles such as the endoplasmic reticulum (ER), mitochondria and ribosomes are all substrates for autophagy and the resulting essential materials produced are either used as a source of energy (starvation) or to produce new macromolecules (homeostasis) (Peidu Jiang and Mizushima 2014).

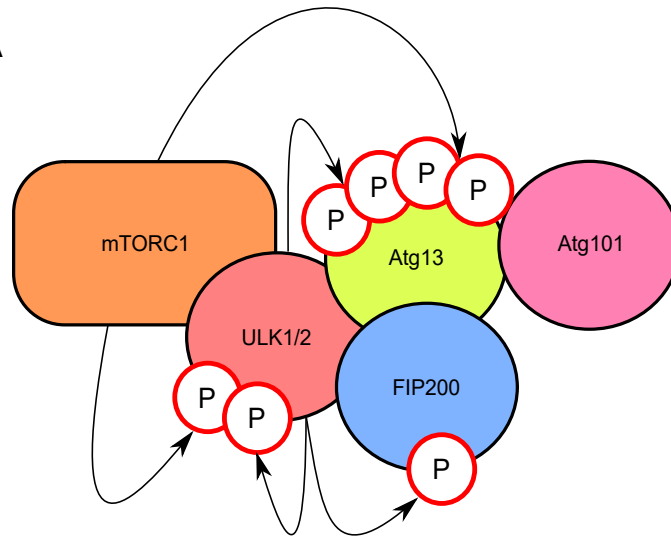
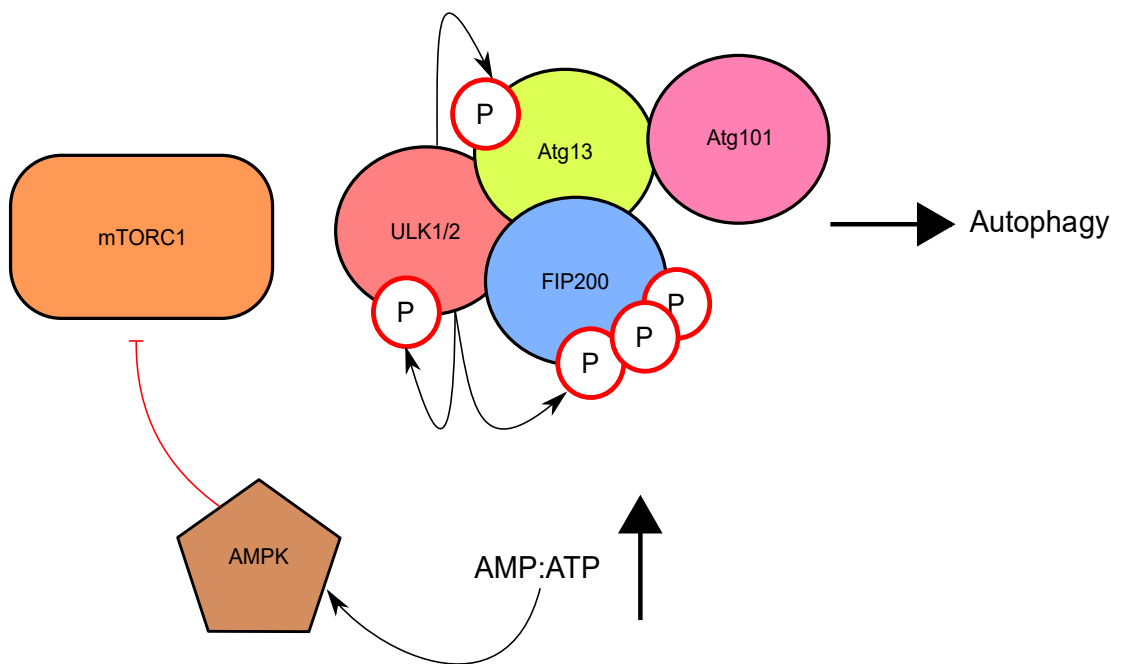
The mechanisms of autophagy vary, with three distinct forms recognized – macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA). Simply, macroautophagy (mostly referred to as “autophagy” in literature) uses the intermediate organelle autophagosome (AP). Initially, an isolation membrane termed the phagophore recruits a portion of a bi-lipid membrane, which undergoes expansion to sequester organelles bound for degradation, and hence, matures into the AP. The AP then delivers its contents to the lysosome, where acidic hydrolase enzymes breakdown the organelles to form metabolic ‘building blocks’, which are then effluxed into the cytoplasm (Mehrpour et al. 2010).

Microautophagy in eukaryotic cells is mainly non-selective, and requires the lysosome itself to engulf small compounds within the cytoplasm through the inward invagination of the lysosomal membrane (Jiang and Mizushima 2014). CMA is the only mechanism that does not involve membrane restructuring and is only observed in higher eukaryote. Chaperone proteins, such as Hsc70, and co-chaperones specifically recognize proteins with a KFERQ-like domain, unfold and deliver them to receptors on the lysosome, such as Lamp-2A, which transport the recognized proteins to the lysosomal lumen through a multimeric translocation complex (Wang and Martinez-Vicente 2010).

Regulation and induction of autophagy

Several stress-sensing pathways contribute to the induction of autophagy, but all share a common signaling node at the mammalian target of rapamycin complex 1 (mTORC1), which is the negative regulator of autophagy (Alers et al. 2012a). In nutrient rich conditions the mTORC1 complex associates with key autophagy related proteins (Atgs) ULK1/2 (Atg1 homolog), Atg13, FIP200 (RB1CC1, FAK), and Atg101. mTORC1 phosphorylates several sites on Atg13 (hyper-phosphorylation), and one on ULK1/2. ULK1/2 phosphorylates itself (self-phosphorylation) and also FIP200 and Atg13, and hence, stabilizes the complex and inhibits autophagy (Mizushima 2010) (Appendix Figure 10A).

Adenosine monophosphate activated protein kinase (AMPK) is an important stress-induced regulator of autophagy. AMPK senses fluctuations in the AMP:ATP ratio that take place during energy consumption. High levels of AMP activate AMPK, which then inhibits mTORC1. Inhibition of mTORC1 allows ULK1/2 to hyper-phosphorylate FIP200, whilst still self-phosphorylating and hence, induces the process of autophagy (Chen and Klionsky 2011) (Appendix Figure 10B).

A**B**

Appendix Figure 10 – mTORC1 inhibition of autophagy via phosphorylation of key autophagy related proteins.

(A) mTORC1 is the negative regulator of autophagy in mammals and inhibits the function of key autophagy related proteins – ULK1/2, Atg13, FIP200, and Atg101 via phosphorylation. Under nutrient rich conditions mTORC1 hyper-phosphorylates Atg13, and phosphorylates ULK1/2. ULK1/2 self-phosphorylates and also FIP200 and Atg13 and hence, stabilizes the complex and inhibits autophagy (Adapted from Chen and Klionsky, 2011). (B) Stress induced activation of AMPK and inhibition of mTORC1 leading to autophagy. Increasing levels of AMP, classically during exercise, leads to activation of AMPK, which then inhibits (red line) the function of mTORC1. Inhibition of mTORC1 “releases” the autophagy related protein complex (ULK1/2, Atg13, FIP200 and Atg101), and allows ULK1/2 to hyper-phosphorylate FIP200 and hence, the process of autophagy is activated (Adapted from Chen and Klionsky 2011).

Vesicle recruitment and Autophagosome (AP) formation

A crucial, but least understood event in AP formation is the recruitment and nucleation of the phagophore membrane that will sequester cargo for degradation. Reviewed by Chen and Klionsky, (2011), they highlight two pre-dominant models for AP formation. Firstly, one proposed by Kovacs et al. (2007), describes AP formation as *de-novo* synthesis of a nucleating structure, which expands by the addition of lipids that are transported by Atg9, a yeast cytoplasm to vacuole transport (Cvt) protein, from various sources. Secondly, described by Hayashi-Nishino et al. (2009), and Ylä-Anttila et al. (2009), a subdomain of the ER is initially used as a cradle for the formation of the phagophore that expands between the ER domains. Support for both models exist, with electron microscopy images showing a direct link between the phagophore and ER, but neither explains all the current data for AP formation as almost all lipid containing membranes have been implicated in AP formation (Chen and Klionsky 2011).

Once the ULK1/2 complex has translocated to a membrane it forms a pre-autophagosomal structure (PAS). The PAS is a dynamic assembly of Atg proteins, including ubiquitin-like (Ubl) homologs (Atg8 and Atg12) that regulate the mechanism of membrane conjugation and expansion. Atg8 is conjugated to the lipid phosphatidylethanolamine (PE), whereas Atg12 is conjugated to Atg5. The membrane then encloses around the cytosolic cargo and AP formation is complete. This process is described in much greater detail than the scope of this thesis by Mijaljica et al. (2012), and Mizushima and Komatsu (2011).

Autolysosome Formation

The mechanism that brings the AP to the lysosome is still confounding, however, cytoskeletal elements such as microtubules and actin microfilaments are thought to play a

role (Monastyrska et al. 2009). In yeast, *ATG8* is homologous to mammalian microtubule-associated protein 1 light chain 3 (MAP1-LC3 or simply LC3), and hence, utilizing the innate microtubule transport mechanism seems feasible. In mammalian cells, once delivered to the lysosome, APs can interact in two ways: (1) complete fusion that forms an autolysosome and (2) a ‘kiss-and-run’ fusion that creates an intermediate compartment. The relative contribution of either mechanism, or any related metabolic requirements are still unclear (Jahreiss et al. 2008).

ULK family

The family of unc-51-like kinases (ULKs) has been most extensively associated with autophagy, with an essential role in regulation and induction for ULK1 and ULK2 (Figure 7A and 7B). ULKs are members of an evolutionarily conserved sub-family of serine/threonine kinases (Akers et al. 2012b). Mammals possess several isoforms of the ULKs (ULK1-4 and also the serine threonine kinase STK36), and ULK1 and ULK2 have been shown to be key regulators of autophagy (Akers et al. 2012a, Mizushima 2010). Overexpression of ULK3 is shown to be sufficient to induce autophagy, but no clear mechanism has been described as yet (Young et al 2009). ULK4 has only been recently described in pathology of hypertension, multiple myeloma, and schizophrenia (Broderick et al. 2012, Ehret et al. 2011, Lang et al. 2014). However, no functional role has been reported.

ULKs, generally, have three distinct semi-conserved domains in their protein structure, a largely conserved N-terminal catalytic serine/threonine domain, a less conserved central domain, and either a serine/threonine-protein kinase C-terminal domain (CTD) (ULK1 and ULK2), a microtubule interacting and transport (MIT) CTD (ULK3), or a proline/serine rich armadillo-like (ARM) CTD (ULK4). ULK1 and ULK2 having extremely conserved

CTD facilitate their binding to Atg13 in the autophagy regulatory complex of mTORC1 (Appendix Figure 10) (Chan et al. 2009). Predicted protein structures for ULK4 show a lack of similarity at the CTD, compared to ULK1/2, and further emphasize a distinct role in autophagy. Lastly, ULK4 is found in three isoforms, a full length 1275 amino acid and shorter 732 and 580 amino acids (Lang et al. 2014). The relevance of these isoforms in autophagy is also not yet recognized.

Functional studies on ULK4

Although no studies have attempted to describe a functional role for ULK4 in initiation, regulation, recruitment or catabolic stages of autophagy some progress has been made in describing its role in neuronal diseases.

“Recurrent deletions of ULK4 in schizophrenia: a gene crucial for neuritogenesis and neuronal motility.” – Lang et al. 2014

Recently, deletions within the ULK4 gene were associated with several cases of schizophrenia and bipolar disorders. In this multi-factorial study by Lang *et al.*, they first found association of two SNPs (rs6599155 and rs17062109) within the *ULK4* gene to schizophrenia and two SNPs (rs17210774 and rs1722850) flanking the *ULK4* gene to bipolar disorder. Next, western blotting of mice brain lysates revealed a switching pattern of ULK4 isoform expression – 95,000 Da and 105,000 Da ULK4 proteins were found primarily in embryonic and post-natal brains and a larger 130,000 Da ULK4 protein was predominantly expressed in adult brain. This larger protein contained the SNPs associated with schizophrenia and suggested that ULK4 isoforms are expressed differentially during development. This was further confirmed due to increased expression of the smaller (95,000 Da) isoform when the authors treated SH-SY5Y (human neuroblastoma) cells with

pro-neuronal differentiation molecules. Histological analyses revealed expression of ULK4 was primarily located to postmitotic (hippocampal and cortex) neurons that express GABA (gamma-aminobutyric acid) receptors that have previously been shown to have implications in development of schizophrenia. Silencing of ULK4 in SH-SY5Y cells attenuated neurite “branching and elongation” due to decreased α -tubulin (required for neurite growth) and increased diffusion of tyrosinated tubulin (also required for neurite growth) within the cytoplasm and *in vitro* cell motility was significantly reduced. Furthermore, several signaling pathways were altered in ULK4 knock down SH-SY5Y cells – JNK, ERK1/2, ELK1 and p38 MAPKs showed reduced activation, whilst there was increased activation of PKC. Lastly, Lang et al. studied *Ulk4*^{-/-} mice and observed phenotypes related to attenuated neurite growth during mouse brain development.

Further research in ULK4

Therefore, clearly ULK4 has a function in the normal development of neuronal tissue (neurogenesis) and evidence of regional and patterned isoform expression is exciting, however mechanistic evidence of protein binding partners and the involvement of the autophagic process is missing. Whether ULK4 function in neurogenesis is directly mediated through its kinase properties, or whether ULK4 is a downstream effector regulated by a sensory network (like ULK1/2 via mTOR) still remains unanswered. Delineation of ULK4 function in autophagy would offer a mechanistic direction for its function in neurogenesis and hence offer realistic targets for therapeutic treatment of schizophrenia and other related neurological disorders.

Benefits of understanding ULK4 function in autophagy

Autophagic process occurs in all cell types and is a major homeostatic regulator of cell survival. There is evidence highlighting an important regulatory role for the ULK family of serine threonine kinases. ULK1/2 are key upstream initiators of the macro-autophagic process and ULK3 activates autophagic machinery when cellular stresses are present. However, there is no evidence for ULK4 in either induction or regulation of autophagy and given the clear and important role of the ULK family in autophagic processing, it would be reasonable to assume ULK4 would have a yet unknown function; perhaps one related to ossification. Recent studies have implicated the mTORC1 central node in regulating multiple aspects of cartilage development. Inhibition of Raptor, a constituent of the mTORC1 complex impaired chondrogenesis (Ming Jiang et al 2017). In addition, treatment with rapamycin was shown to reduce expression of chondrogenic markers in mouse ATDC5 chondrocytes (Phornphutkul et al 2008). Furthermore, autophagy is implicated in several diseases either as a contributing factor or a protective mechanism and delineating the function of ULK4 would provide a complete role for autophagy in these diseases, enabling better targeting of therapies.

Experimental strategy

Finally, the autophagic process is a multi-step and coordinated process, involving several key proteins at several time points and ULK4 isoforms have shown regional and patterned expression characteristics. Furthermore, there is also evidence that pathological activation of mTORC1 can reproduce osteoarthritis-like symptoms in rabbits (N-T. Cheng et al 2016), therefore we will visualise the localisation and co-localisation partners of ULK4 isoforms over time in HEK293 cells undergoing different stages of rapamycin induced autophagy and offer a novel direction for a functional role of ULK4, either in the

autophagic process or possibly ossification and finally describe differential localisation of ULK4 isoforms under varying autophagy conditions.

Materials and Methods

Generation of ULK4-FL, ULK4-580 and ULK4-732 constructs

We generated ULK4 full length (ULK4-FL) and isoforms (ULK4-580 and ULK4-732) as described in section 2.2.1. Primers and conditions are stated in Appendix Table 1. ULK4 (NM_017886.3) was provided by Dr Yun Zhang in pcDNA3.1 vector and sequenced to confirm correct nucleotide sequence that aligned to NM_017886.3 (Appendix Figure 11).

Autophagy assay

The major role of autophagy is to maintain cellular homeostasis and autophagic machinery are responsible for the sequestering and deliverance of cellular material to the lysosome for recycling. Mammalian target of rapamycin (mTOR) is the central signaling node that controls autophagy activation by phosphorylating and inactivation of ULK1/2. Rapamycin is a drug that inhibits mTOR function and activates autophagy and is commonly used study the effects of autophagy *in vitro*. In contrast Leupeptin is an autophagy inhibitor that targets lysosomal proteases to prevent catalytic degradation of sequestered cargo. Chloroquine, similar to luepeptin, prevents cargo degradation but does so by raising lysosomal pH and preventing autophagosome binding. Initial testing implemented all three drugs in elucidation of ULK4 function in the autophagic process (Figure 4 Appendix), however as ULK family is thought to initiate autophagy upstream of the lysosomal process (Alers et al. 2012b), only rapamycin was used for full-scale study. Furthermore, ULK4 function in autophagy would be better understood in conditions favoring completion of the

autophagic process and rapamycin would act to increase autophagic flux and potentiate ULK4 function in autophagy.

Determination of ULK4 localisation under autophagic conditions

HEK293 cells were grown in glass bottom dishes and co-transfected with ULK4_GFP isoforms and pmRFP_LC3 constructs (see section 2.2). 24 hours after transfection cells were visualized for ULK4 localisation and co-localisation with LC3 on the LSM 880 airyscan as described in section 2.2.4. The culture media was aspirated, cells washed once with PBS and high glucose DMEM constituted with 10% FBS and 1% penicillin-streptomycin but with essential amino acids removed and supplemented with a final concentration of 250 nM rapamycin (autophagic media) was added. After incubation for 3 hours with autophagic media cells were once again visualised for ULK4 localisation and co-localisation with LC3. Cells were rescued by aspirating the autophagic media, washing three times with PBS and incubating with growth media for 24 hours and then visualised for the final time for ULK4 localisation and co-localisation with LC3. Co-localisation was determined as described in section 2.2.4.3.

Co-immunoprecipitation and Western blot analysis

We performed co-immunoprecipitation and western blot analysis as described in section 2.2.9 and 2.2.10. Specific antibodies are listed in Table 2 along with their use conditions and optimised buffers.

ULK4 silencing

Transfection of short interfering RNA (siRNA) was used to determine ULK4 function in autophagy activation. Briefly, siRNAs bind the mRNA of the gene of interest and cause its degradation, effectively silencing gene expression. siRNA (S29925, Thermo Fisher) was chosen to silence all isoform variants of ULK4. HEK293 cells were grown and co-transfected with ULK4_GFP isoforms, RFP_LC3 and siRNA S29925 (final concentration 50 nM) as described in section 2.2.3.1 into glass bottom dishes. 24 hours after transfection cells were visualised on confocal microscope for ULK4 GFP localisation and co-localisation with RFP_LC3 as described above and a subset collected as pellet for western blot and qPCR analysis. The culture media of the remaining subset was replaced with autophagic media for 3 hours and visualised again. Subsets of cells were collected as pellets and the remaining cells were incubated for 24 hours in growth media. Cells were again visualised and the final subset collected as pellets. We checked for reduction in gene expression using qPCR as described in section 2.2.7.

Results

Early data has shown co-localization of ULK4 and LC3 in HEK293 and derived neuroblastoma SH-SY-5Y (SH) monolayer cells, cultured in starvation medium to induce autophagy, and suppression of *ULK4* by siRNA inhibition (Zhang et al. 2018, unpublished). Interestingly, ULK1 and ULK4 both interact with LC3, however they do not seem to localize to LC3 at the same stage in autophagy. Hence, a distinct role of ULK4 in autophagy regulation and induction may be possible, one that is independent of the recognized ULK1 complex (Zhang et al. 2018, unpublished). ULK4 exists in three isoforms, the full length 1275 amino acid (ULK4-FL) and two shorter 732 (ULK4-732) and 580 (ULK4-580) amino acids long.

Aims of this section

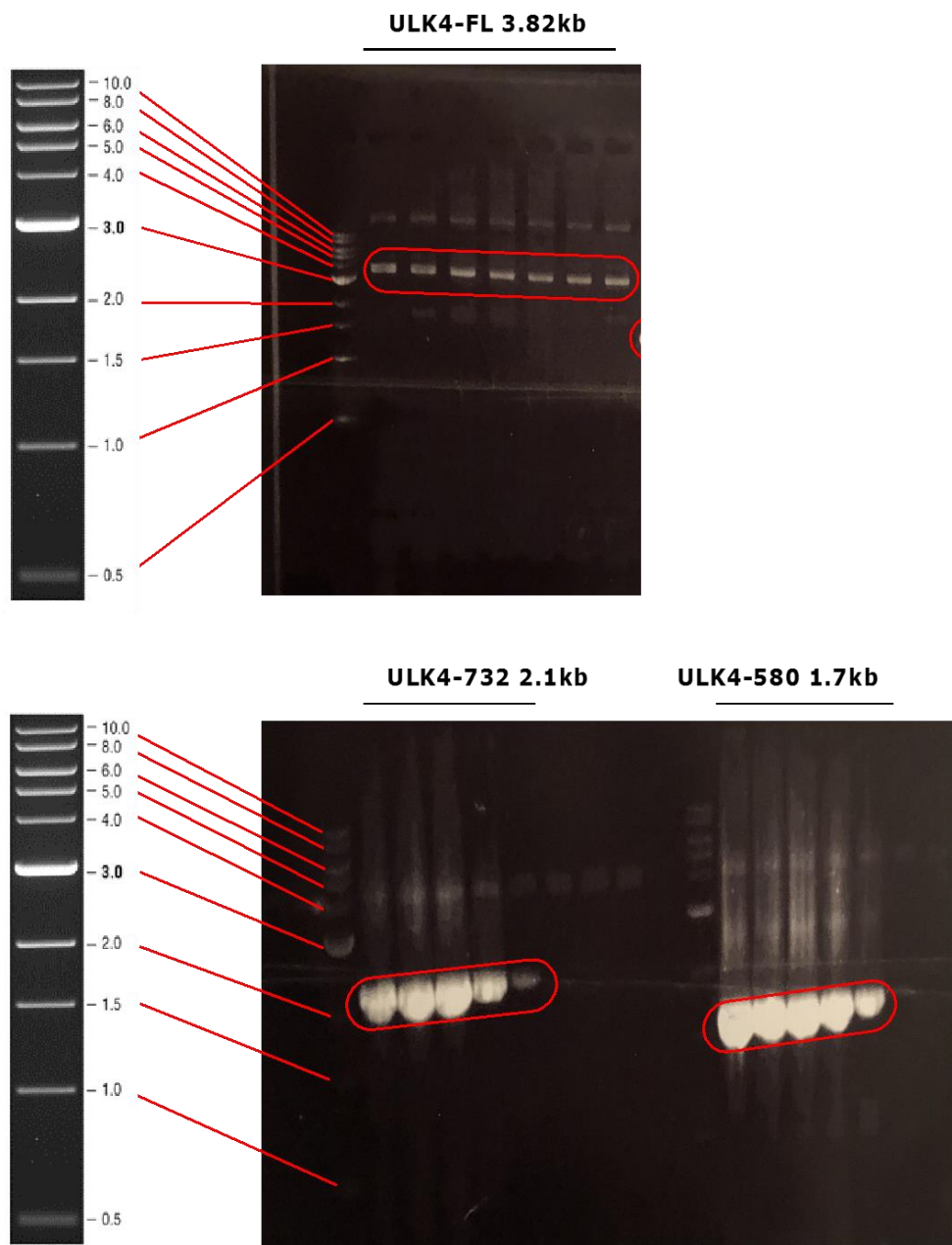
We aimed to determine whether ULK4 isoforms have a role in autophagy and visualised their intracellular localisation, co-localisation with LC3 and intracellular organelles using confocal microscopy (see section 2.2.8), and determined whether these co-localisations were a physical interaction using co-immunoprecipitation (see section 2.2.9.2) and determined if overexpression of ULK4 contributes to autophagic flux indicating a role in autophagy using western blot analysis of LC3 I/II ratio (see section 2.2.10).

ULK4 cloning

We first confirmed amplification of ULK4-FL by size determination gel electrophoresis (Appendix Figure 11). Next we confirmed the correct sequence was present by direct sequencing of the pEGFP-N3_ULK4 construct (Appendix Figure 12).

ULK4 localisation dynamics

We observed differential localisation of ULK4 isoforms under basal, autophagic and rescue conditions. HEK293 cells were transfected with ULK4-GFP isoforms and visualised under confocal microscope (see section 2.2.8) at 24 hours after transfection (basal), 3 hours after autophagy induction (autophagic) and 24 hours after rescue (rescue). Under basal conditions ULK4-FL localised primarily to the cytoplasm forming few punctate bodies with some nuclear expression. After autophagy induction ULK4-FL had reduced nuclear expression and formed more punctate bodies within the cytoplasm. After rescue ULK4-FL regained some nuclear expression and decreased the number of punctate bodies within the cytoplasm (Appendix Figure 13A). ULK4-732 had increased punctate



Appendix Figure 11 – Agarose gel images of ULK4-FL, ULK4-732 and ULK4-580 PCR products.

Red circles highlight correct sized bands excised for downstream cloning of ULK4-FL, ULK4-732 and ULK4-580. Red lines highlight corresponding 1KB DNA ladder bands.

pEGFP_N3-ULK4-FL

Homo sapiens unc-51 like kinase 4 (ULK4), transcript variant 1, mRNA
Sequence ID: [NM_017886.3](#) Length: 4697 Number of Matches: 2

Range 1: 474 to 4298 [GenBank](#) [Graphics](#) [Next Match](#) [Previous Match](#)

Score	Expect	Identities	Gaps	Strand
7064 bits(3825)	0.0	3825/3825(100%)	0/3825(0%)	Plus/Plus
Query 1	ATGGAAACCTTTATTCTGTATGAGGAGATCGGAAGAGGAAGCAAGACTGTTGCTATAAA	60		
Sbjct 474	ATGGAAACCTTTATTCTGTATGAGGAGATCGGAAGAGGAAGCAAGACTGTTGCTATAAA	533		
Query 61	GGGCGACGGAAGGGAACAATCAATTTTGTAGCCATTCTTTGTACTGATAAGTGCAAAAGG	120		
Sbjct 534	GGGCGACGGAAGGGAACAATCAATTTTGTAGCCATTCTTTGTACTGATAAGTGCAAAAGG	593		
Query 121	CCTGAAATAACCAACTGGGTCCGTCTCACCCTGAAATAAAACACAAGAAATTGTAAC	180		
Sbjct 594	CCTGAAATAACCAACTGGGTCCGTCTCACCCTGAAATAAAACACAAGAAATTGTAAC	653		
Query 181	TTTCATGAATGGTATGAAACAAGCAACCCTCTGGCTAGTGGTGGAACTCTGCACAGGT	240		
Sbjct 654	TTTCATGAATGGTATGAAACAAGCAACCCTCTGGCTAGTGGTGGAACTCTGCACAGGT	713		
Query 241	GGTTCCTTAAAAACAGTTATTGCTCAAGATGAAAACCTCCCAAGAGATGTTGTGAGAGAA	300		
Sbjct 714	GGTTCCTTAAAAACAGTTATTGCTCAAGATGAAAACCTCCCAAGAGATGTTGTGAGAGAA	773		

B

pEGFP_N3-ULK4-732

Homo sapiens unc-51 like kinase 4 (ULK4), transcript variant 1, mRNA
Sequence ID: [NM_017886.3](#) Length: 4697 Number of Matches: 1

Range 1: 474 to 2667 [GenBank](#) [Graphics](#) [Next Match](#) [Previous Match](#)

Score	Expect	Identities	Gaps	Strand
4052 bits(2194)	0.0	2194/2194(100%)	0/2194(0%)	Plus/Plus
Query 1	ATGGAAACCTTTATTCTGTATGAGGAGATCGGAAGAGGAAGCAAGACTGTTGCTATAAA	60		
Sbjct 474	ATGGAAACCTTTATTCTGTATGAGGAGATCGGAAGAGGAAGCAAGACTGTTGCTATAAA	533		
Query 61	GGGCGACGGAAGGGAACAATCAATTTTGTAGCCATTCTTTGTACTGATAAGTGCAAAAGG	120		
Sbjct 534	GGGCGACGGAAGGGAACAATCAATTTTGTAGCCATTCTTTGTACTGATAAGTGCAAAAGG	593		
Query 121	CCTGAAATAACCAACTGGGTCCGTCTCACCCTGAAATAAAACACAAGAAATTGTAAC	180		
Sbjct 594	CCTGAAATAACCAACTGGGTCCGTCTCACCCTGAAATAAAACACAAGAAATTGTAAC	653		
Query 181	TTTCATGAATGGTATGAAACAAGCAACCCTCTGGCTAGTGGTGGAACTCTGCACAGGT	240		
Sbjct 654	TTTCATGAATGGTATGAAACAAGCAACCCTCTGGCTAGTGGTGGAACTCTGCACAGGT	713		
Query 241	GGTTCCTTAAAAACAGTTATTGCTCAAGATGAAAACCTCCCAAGAGATGTTGTGAGAGAA	300		
Sbjct 714	GGTTCCTTAAAAACAGTTATTGCTCAAGATGAAAACCTCCCAAGAGATGTTGTGAGAGAA	773		

C

pEGFP_N3-ULK4-580

Homo sapiens unc-51 like kinase 4 (ULK4), transcript variant 1, mRNA
Sequence ID: [NM_017886.3](#) Length: 4697 Number of Matches: 1

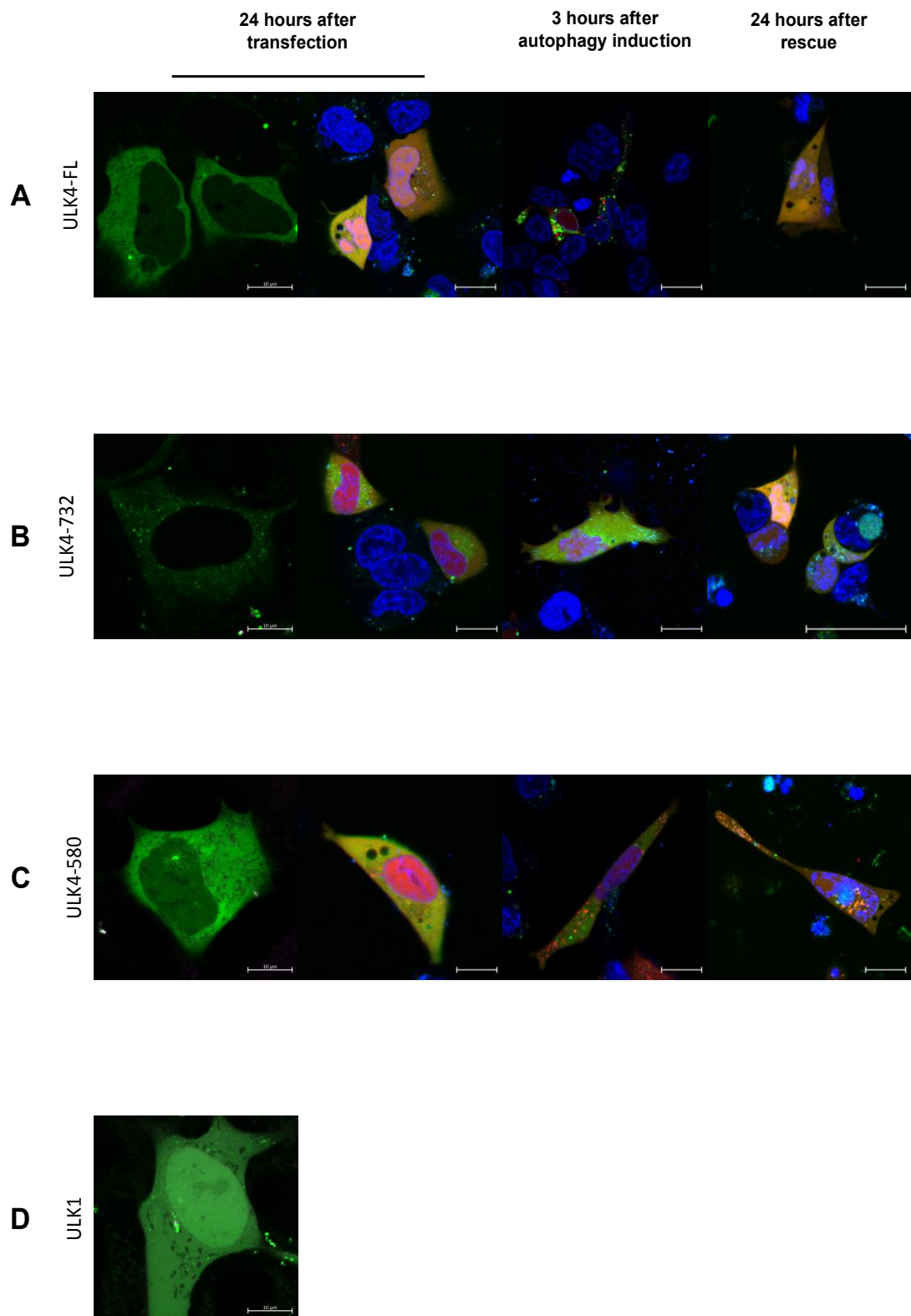
Range 1: 474 to 2213 [GenBank](#) [Graphics](#) [Next Match](#) [Previous Match](#)

Score	Expect	Identities	Gaps	Strand
3214 bits(1740)	0.0	1740/1740(100%)	0/1740(0%)	Plus/Plus
Query 1	ATGGAAACCTTTATTCTGTATGAGGAGATCGGAAGAGGAAGCAAGACTGTTGCTATAAA	60		
Sbjct 474	ATGGAAACCTTTATTCTGTATGAGGAGATCGGAAGAGGAAGCAAGACTGTTGCTATAAA	533		
Query 61	GGGCGACGGAAGGGAACAATCAATTTTGTAGCCATTCTTTGTACTGATAAGTGCAAAAGG	120		
Sbjct 534	GGGCGACGGAAGGGAACAATCAATTTTGTAGCCATTCTTTGTACTGATAAGTGCAAAAGG	593		
Query 121	CCTGAAATAACCAACTGGGTCCGTCTCACCCTGAAATAAAACACAAGAAATTGTAAC	180		
Sbjct 594	CCTGAAATAACCAACTGGGTCCGTCTCACCCTGAAATAAAACACAAGAAATTGTAAC	653		
Query 181	TTTCATGAATGGTATGAAACAAGCAACCCTCTGGCTAGTGGTGGAACTCTGCACAGGT	240		
Sbjct 654	TTTCATGAATGGTATGAAACAAGCAACCCTCTGGCTAGTGGTGGAACTCTGCACAGGT	713		
Query 241	GGTTCCTTAAAAACAGTTATTGCTCAAGATGAAAACCTCCCAAGAGATGTTGTGAGAGAA	300		
Sbjct 714	GGTTCCTTAAAAACAGTTATTGCTCAAGATGAAAACCTCCCAAGAGATGTTGTGAGAGAA	773		

D

Appendix Figure 12 – Sequence alignments of ULK4-FL, ULK4-732 and ULK4-580 constructs after insertion into the pEGFP-N3 vector.

Only the first four rows of aligned sequence are shown but we attained 100% alignment of pEGFP-N3_ULK4-FL to complete NM_017886.3 ULK4 mRNA. 100% alignment of pEGFP-N3_ULK4-732 to 1-2194 base positions of NM_017886.3 ULK4 mRNA. 100% alignment of pEGFP-N3_ULK4-580 to 1-1740 base positions of NM_017886.3 ULK4 mRNA.



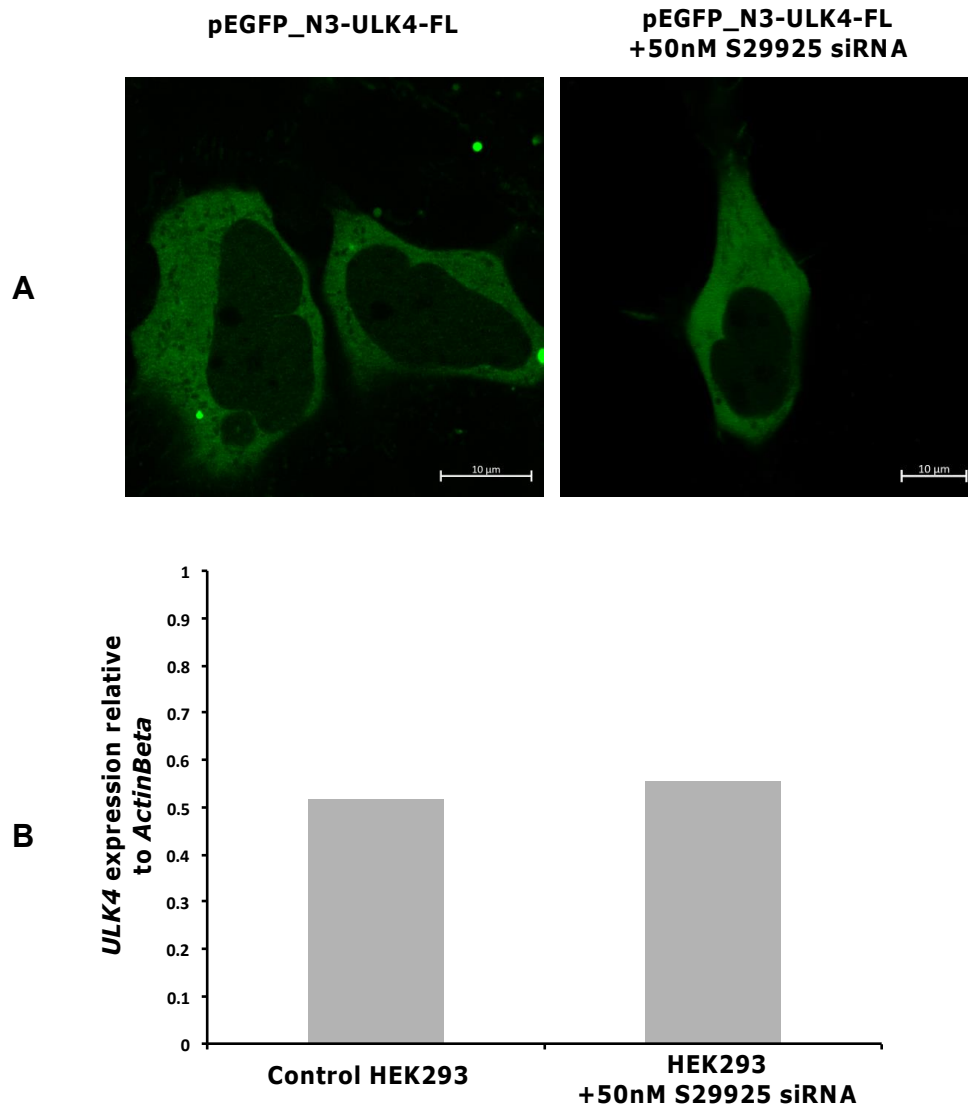
Appendix Figure 13 – ULK4 isoforms differentially localise but all co-localise with LC3.

Confocal microscopy of ULK4 isoforms (green) and LC3 (red) co-transfected HEK293 cells revealed (A) ULK4-FL does not form many punctate bodies under basal conditions 24 hours after transfection but the few that are present are co-localised with LC3; 3 hours autophagy induction more punctate bodies were visible that co-localised with LC3; 24 hours after rescue ULK4-FL had decreased punctate bodies but remained co-localised with LC3 (B) ULK4-732 had increased punctate bodies formation under basal conditions co-localised with LC3, with minute nuclear expression; 3 hours after autophagy induction ULK4-732 had no nuclear expression and formed punctate bodies similar to ULK4-FL that co-localised with LC3; 24 hours after rescue ULK4-732 regained some nuclear expression exceeding what was observed under basal conditions retained some punctate bodies that co-localised with LC3 (C) ULK4-580 had mostly diffuse cytoplasmic expression but did not form many punctate bodies under basal conditions; 3 hours after autophagy induction ULK4-580 was completely cytoplasmic and formed punctate bodies similar to ULK4-FL and ULK4-732 and co-localised with LC3. Interestingly, after rescue ULK4-580 also had nuclear expression and decreased punctate bodies within the cytoplasm but remained co-localised with LC3 (D) ULK1 expressed diffusely within the cytoplasm and nucleus under basal conditions 24 hours after transfection.

bodies formation under basal conditions, with minute nuclear expression. Under autophagic conditions ULK4-732 had no nuclear expression and formed punctate bodies similar to ULK4-FL. After rescue ULK4-732 regained some nuclear expression exceeding what was observed under basal conditions (Appendix Figure 13B). Lastly, ULK4-580 had mostly diffuse cytoplasmic expression but did not form punctate bodies under basal conditions. After autophagy induction ULK4-580 was completely cytoplasmic and formed punctate bodies similar to ULK4-FL and ULK4-732. Interestingly, after rescue ULK4-580 also had nuclear expression and decreased punctate bodies within the cytoplasm (Appendix Figure 13C). Control ULK1-GFP expressed cytoplasmic and nuclear equally and formed very few punctate bodies under basal conditions (Appendix Figure 13D). We observed co-localisation of ULK4-FL, ULK4-732 and ULK4-580 punctate bodies with LC3 punctate bodies (Appendix Figure 13A, Appendix Figure 13B and Appendix Figure 13C).

ULK4 silencing

We observed no silencing of ULK4_GFP isoforms under confocal visualisation of GFP localisation (Appendix Figure 14A), measured no reduction in ULK4 mRNA with qPCR (Appendix Figure 14B) and hence, abandoned this method of study. Several reasons may contribute to the failure of silencing, mainly due to time constraints optimisation of the siRNA was not possible. Only one concentration was tried – 50 nM final concentration in 2 mL of transfection media, this was the recommended concentration from the suppliers. It is also recommended to try different siRNAs, as the actual sequence is proprietary and not revealed by the supplier it is almost impossible to validate a single siRNA design for every cell line. However, as the *ULK4* sequence is highly conserved perhaps alternative splicing of *Ulk4* mRNA could affect siRNA binding. Consideration to appropriate transfection reagents should also be noted; FugeneHD is specifically designed to incorporate DNA into



Appendix Figure 14 – Silencing of ULK4.

(A) We observed no reduction in fluorescent signal after treating pEGFP-N3_ULK4-FL transfected HEK293 cells with 50nM S29925 siRNA compared to untreated pEGFP-N3_ULK4-FL transfected HEK293 cells. (B) We measured no change in *ULK4* mRNA in HEK293 cells treated with 50 nM S29925 siRNA relative to β -Actin compared to untreated HEK293 cells

the cell. However, as several studies had reported using FugeneHD for chemical transfection of siRNA and Promega does not specifically state it can't incorporate RNA we decide to try with FugeneHD. Nonetheless, in the future using a transfection reagent specifically designed for siRNA and testing multiple designs would easily eliminate these above-mentioned potential problems

Discussion

Our preliminary data indicates that ULK4 isoforms are independent in their localisation dynamics but co-localise with LC3 at all stages of punctate bodies formation. Our confocal data of ULK4 isoform localisation variation somewhat replicates the switching pattern of expression shown by Lang et al. 2014. As seen for ULK1 protein that is present in large quantities within the nucleus under basal conditions but formed several punctate bodies within the cytoplasm and had decreased nuclear expression under autophagic conditions, ULK4 isoforms mimicked this translocation from the nuclear region and into the cytoplasm to form punctate bodies. However, there were subtle differences in timing and effect size between ULK4 isoforms that may indicate different timings of the autophagic flux. Perhaps, ULK1 as the master regulator is needed at the very beginning to initiate AP formation but ULK4 isoforms displayed localisation dynamics that indicated later stages of autophagic flux. Even between isoforms there were timing and localisation differences; ULK4-732 formed punctate bodies much earlier than the others and ULK4-580 was least expressed in the nucleus. ULK4-FL was an intermediate between the others, expressing in the nucleus more than ULK4-732 but also forming punctate bodies faster than ULK4-580. The co-localisation with LC3 is not surprising, however compared to ANKH mutant G389R and E490del that co-localised in sparse quantities, ULK4 isoform punctate bodies were almost always co-localised with LC3 indicating a much more prevalent role in autophagic flux, and not one limited to simple cargo.

Implications

Our preliminary data does not establish clearly a role for ULK4 in autophagy. However, as ULK4 was found co-localised with LC3 in much greater quantities compared to ANKH mutants perhaps a role in the progression of autophagy is plausible. Nonetheless, differential expression dynamics of ULK4 isoforms suggest a role in the later stages of autophagy no clear mechanism is evident.

Conclusion

An emerging role of ULK4 in neurogenesis is an exciting development, however we still do not know of a function for ULK4 in autophagy initiation and progression. ULK1 and ULK2 are known to be key initiators of autophagy, with essential upstream initiation of AP formation (Akers et al. 2012b). Increased ULK3 expression is linked to increased autophagic flux (Caballe et al. 2015). Therefore, a role for ULK4 in some stage of autophagy is expected. A non-redundant role different from ULK1/2 that are upstream initiators, to perhaps later stages is somewhat predicted by our ULK4 isoform data and previous studies in patterned neurogenesis (Lang et al. 2014).

Summary and future directions of ULK4 function in autophagy

Our preliminary data suggests ULK4 isoforms are differentially expressed during stages of autophagy. ULK4-732 formed punctate bodies much earlier than the others but ULK4-580 was least expressed in the nucleus. ULK4-FL was an intermediate between the others, expressing in the nucleus more than ULK4-732 but also forming punctate bodies faster than ULK4-580. Concordant with recent publications describing ULK4 isoforms to have a

patterned expression profile in neurogenesis (Lang et al. 2014), there seems to be a timed role for ULK4 in autophagy. Perhaps as ULK4-732 formed punctate bodies much earlier, a role in the early stages of AP formation just after ULK1/2 is possible. ULK4-FL was found more localised to nucleus suggesting a targeted response in the nuclear region. ULK4-580 remained most cytoplasmic but formed punctate bodies later suggesting a later role, perhaps during AP and lysosome fusion events. Nonetheless, as we observed co-localisation of all three isoforms with LC3 under basal, autophagic and rescue conditions there is possible redundancy in their roles. Regardless of the isoform specific function, ULK4 has recently been identified as an important modulator of neurogenesis and whether this role is in part due to its function in autophagy is an important direction for future research. Nonetheless, co-immunoprecipitation of ULK4 and testing other autophagy related protein would provide direction for ULK4 function. Silencing of ULK4 isoforms and measuring changes in autophagic flux would better differentiate their unique roles

Intracellular deviations in ANKH mutants underlie the mechanisms of biochemical changes associated to calcium pyrophosphate deposition disease (CPPDD) and craniometaphyseal dysplasia (CMD)

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Conflicts of interest

We have no conflicts of interest.

Abstract

Objective – Mutations in ANKH are associated with calcium pyrophosphate deposition disease (CPPDD) and craniometaphyseal dysplasia (CMD). This study investigated the effects of ANKH mutants in extracellular matrix biochemistry of diseases.

Methods – We generated four *ANKH* overexpression plasmids containing either CPPDD or CMD linked mutations: P5L, E490del and S375del, G389R. They were transfected into CH-8 articular chondrocyte and HEK293 cells. ANKH mutants' dynamic differential localisations were monitored by confocal microscopy and the interactions with autophagy marker LC3 were investigated. Extracellular inorganic pyrophosphate (ePPi), mineralization, ENPP1 activity, expression of *ENPP1*, *TNAP* and *PIT-1* were measured.

Results – P5L delayed cell membrane localization but once recruited into the membrane it increased ePPi (2.12 fold, $p = 0.032$), mineralization (44.64 nM, $p = 0.01$), and ENPP1 activity (3.39 fold, $p = 2.72 \times 10^{-6}$). E490del remained mostly cytoplasmic, forming punctate bodies co-localised with LC3, increased mineralization (42.79 nM, $p = 0.01$), *ENPP1* (5.82 fold, $p = 7.3 \times 10^{-5}$) and ENPP1 activity (5.33 fold, $p = 1.3 \times 10^{-5}$) with an initial but unsustained increase in *TNAP* and *PIT-1*. S375del trended to decrease ePPi (0.55 fold, $p = 0.08$), increased mineralization (52.68 nM, $p = 5.12 \times 10^{-3}$). G389R delayed cell membrane localisation, trended to decrease ePPi (0.51 fold, $p = 0.47$), increased mineralization (40.62 nM, $p = 0.001$) and co-localised with LC3.

Conclusions – Our results demonstrate a link between pathological localisation of ANKH mutants with different degrees in severity of mineralization. Furthermore, mutant ANKH functions are related to synthesis of defective proteins, PPi transport, ENPP1 activity and expression of *ENPP1*, *TNAP* and *PIT-1*.

Calcification of skeletal tissues occurs as a normal physiological event in growth plate cartilage and in bone but can also occur as a pathological event in many other tissues.

Calcification usually involves hydroxyapatite (HA) under normal conditions, but calcium pyrophosphate dihydrate (CPPD) can deposit under certain conditions. Formation of HA requires the non-enzymatic reaction of extracellular calcium and inorganic phosphate (ePi). Inorganic pyrophosphate (PPi) is a physiological regulator of calcification by inhibiting HA deposition but also contributes to the formation of CPPD crystals. At least four proteins influence PPi homeostasis in the extracellular matrix (ECM): these include the transmembrane pyrophosphate transporter ANKH (the human homologue to the mouse progressive ankylosis protein), ENPP1 (ectonucleotide pyrophosphatase 1), TNAP (tissue nonspecific alkaline phosphatase, one of the four forms of alkaline phosphatase ALPL) and PIT-1 (sodium-dependent phosphate transport 1, also named as SLC20A1) (1–6). ENPP1 readily hydrolyses extracellular adenosine triphosphate (eATP) into extracellular PPi (ePPi) and ePi, whilst ANKH exports intracellular PPi (iPPi) into the ECM, TNAP reduces ePPi and maintains ePPi/ePi ratio (4). Lastly, PIT-1 imports ePi into the cell via a sodium exchange channel to be used in metabolic pathways (7).

ANKH is mainly expressed at the cell membrane and appears to have other important intracellular functions, including localisation to primary cilia/basal body complex, facilitation of clathrin and adaptor mediated membrane trafficking from the *trans*-Golgi-network (TGN) to endosome surface (5,8,9).

ANKH mutations cause two distinct conditions - calcium pyrophosphate dihydrate deposition disease (CPPDD [MIM118600]) and craniometaphyseal dysplasia (CMD [MIM123000]). CPPDD typically presents with destructive arthritis, including chondrocalcinosis in about 50% of cases; it may mimic rheumatoid arthritis, gout or osteoarthritis and is the commonest form of inflammatory monoarthritis in the elderly, occurring in up to 40% of those over 65 years of age (10). Among individuals undergoing total hip replacement for the clinical diagnosis of osteoarthritis, about 50-60% patients had evidence of CPPDD (11). In contrast, CMD is a rare disorder characterised by

hyperostosis/sclerosis of the skull and abnormal modelling of the long bones (12). Individuals with severe forms of CMD can have reduced life expectancy as a result of compression of the foramen magnum. CMD is caused by decreased ePPi, which allows increased HA deposition and altered bone modelling via chondrogenesis, osteoblastogenesis and osteoclastogenesis (13,14). There is currently no specific treatment for CPPDD and CMD.

The *ANKH* mutations associated with CPPDD or CMD are mainly within the transmembrane domains (10,15–17). Interestingly, mutations near either end of *ANKH* are mostly associated with CPPDD while mutations in the middle of *ANKH* have been reported to cause CMD. However, the biological effect and cellular function of these mutant variants remain largely unexplored. Mutant P5L (p.Pro5Leu, NM_054027.4:c.14C>T) caused increased expression of ANKH (18) and was further reported to increase expression and activity of ENPP1 (19–21). Mutant E490del (p.Glu490del, NM_054027.4:c.1468_1470delGAG) deregulated TNAP activity but was highlighted to be involved with other unknown factors (22). A follow-up study described the inability of mutant M48T (p.Met48Thr, NM_054027.4:c.143T>C) to interact with PIT-1 and also the complex interplay of ANKH, TNAP and PIT-1 in contributing to the deregulated PPi/Pi levels underlying CPPD crystal deposition in joints (23). CMD related mutants have largely been restricted to clinical case studies. One case report of mutant S375del (p.Ser375del, NM_054027.4:c.1123_1125delTCC) showed a decrease in ePPi that was consistent with the predicted loss-of-function (24). Mutant G389R (p.Gly389Arg, NM_054027.4:c.1165G>A), reported in several cases of CMD and recently also in CPPDD, was predicted to be a loss-of-function variant (25–27).

Autophagy is a dynamic catabolic mechanism that recycles damaged organelles and non-functional proteins and maintains cellular homeostasis (28). There are three sub-types of

autophagy – chaperone mediated autophagy (CMA), microautophagy, and macroautophagy. CMA refers to the chaperone-dependant selection of cytosolic components and direct translocation across the lysosome membrane. Microautophagy is the direct engulfment of cytoplasmic cargo by the lysosome itself. Macroautophagy, the most common and herein referred to simply as autophagy, utilises the intermediate double membrane organelle termed the autophagosome (AP) to sequester cargo and deliver to the lysosome (29). Previous studies have highlighted the importance of autophagy in maintaining healthy chondrocytes in the formation of cartilage and preventing degeneration during osteoarthritis (30). Osteoarthritis related gene expression has shown to be modulated via autophagy in human chondrocytes (31).

To investigate the pathogenic mechanisms of ANKH in CPPDD and CMD, we generated four disease-associated ANKH mutants containing the first reported mutations associated with relatively severe clinical phenotypes: two are terminal CPPDD associated P5L and E490del (32,33), two are central CMD associated S375del and G389R (24–27,34). We used confocal imaging to identify each ANKH mutant differential cell localisation dynamics, measured ePPi concentrations and altered mineralization level, evaluated each ANKH mutant effect on the function of ENPP1 and gene expression of *ENPP1*, *TNAP* and *PIT-1*, we also investigated the involvement of autophagy for potential mutated ANKH protein recycling in the pathogenesis of CPPDD and CMD.

Material and methods

ANKH construct and mutant generation

Standard restriction cloning was used to create NM_054027.4:ANKH-eGFP_N3 (wt. ANKH) overexpression construct. A Q5 Site-Directed mutagenesis kit (New England BioLabs, Massachusetts, USA) was used to create the desired overexpression mutants with

the following PCR primers, P5L: 5'- GTGAAATTCCTGGCGCTCACG, 3' – CATGGAATTCGAAGCTTGAGC; E490del: 5' – AATGAAAATGGATCCATCGC, 3' – CTCTCTCATTTCCACGATG; S375del: 5' – TTCTTCCCAGTTCCAGTC, 3' – GAAGATCCGCAAAGGAAC; G389R: 5' – GCATCTCACCAGGTGGCTGATG, 3' – GCCCTCACTGTGACTGGA. Colonies were checked for the correct insert via sequencing.

Cell culture and plasmid transfection

CH-8 articular chondrocyte (CH-8) and HEK293 cells were cultured in high glucose DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. Transfections were performed using FugeneHD (Promega, Wisconsin, USA) as following; per 2ml of growth media 95µl OPTIMEM (Thermo Fisher, Massachusetts, USA), 4.5µl FugeneHD and 5µg plasmid DNA was incubated together at room temperature for 10 mins and added to the cells for the appropriate time.

Confocal Imaging

CH-8 and HEK293 cells were cultured on 35mm high glass bottom u-dishes (iBidi, Martinsried, Germany) and transfected with appropriate plasmids for 24, 48 and 72 hours. Before visualisation, cells were washed with PBS and fresh growth media was added, nuclear DNA was stained with Hoechst 33342 (5ug/ml) (Thermo Fisher) for 10 mins. Confocal imaging was performed on a Zeiss LSM 880 with Airyscan using 405 nm, 488 nm and 561 nm lasers and 40x, 63x and 100x oil immersion lens with a temperature and CO₂ controlled stage and chamber. Images were processed on proprietary Zen 2.1 and output as .CZI, .TIFF or .GIF (Zeiss, Jena, Germany). Autophagy assay was performed on HEK293 cells grown to 60% confluence and then co-transfected with *pmRFP_LC3* (autophagy marker, red) and wt. or mutant *ANKH_eGFP* separately. After 24 hours, the cells were visualised under confocal microscope. Autophagy was induced for 3 hours by

replacing culture media with high glucose DMEM constituted with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin but with essential amino acids removed and supplemented with a final concentration of 250 nM rapamycin. Cells were examined again. Finally, cells were rescued for 24 hours in normal growth media and imaged again. Intensity over distance was measured for co-localisation of mutants G389R and E490del with LC3 by simply drawing a straight line through overlapping puncta and obtaining raw arbitrary intensity values which were then plotted against distance (35). Cell counting was performed via tiling an 8x8 (1.68mm²) area and counting transfected expressed cells (n>100) and recorded the numbers of either only cell membrane expression or cytoplasmic expression. The results were reported as a percentage of the cells with only cell membrane expression.

Inorganic Pyrophosphate Assay

Culture media from transfected and control CH-8 and HEK293 cells were collected every 24 hours. The concentration of PPi was measured using the PPLight Pyrophosphate Detection Kit (Lonza, Basel, Switzerland) following the manufacturer protocol. Arbitrary luminescence was recorded on a MicroBeta TriLux 1450 (Perkin Elmer, Massachusetts, USA) reader using a 0.1s integrated read time (36,37). Results were normalised to the native non-cellular fluctuation in media only and concentration was deduced by plotting a standard curve.

Alizarin Red S Mineralization Assay

CH-8 cells were cultured to 60% confluence and were transfected with wt. ANKH, mutants or GFP only plasmid. The media was replaced with mineralization media consisting of high glucose DMEM supplemented with 10% FBS, 50 ug/ml Ascorbic acid, 10 mM *B*-glycerol phosphate (BGP) and 100 nM dexamethasone 24 hours after transfection and the cells were incubated for another 48 hours. Cells were washed twice

with PBS for 5 min, fixed with 3.7% PFA for 15 min and then stained with 1ml alizarin red S solution (pH 4.2) for 30 min at room temperature, then further 5 times washes with PBS for 5 min until a bright red colour was observed. Cells were then imaged using a Zeiss AxioPlan light microscope. To quantify the staining, 500µL 10% acetic acid was added to the cells and incubated at room temperature under constant agitation. Cells were scraped, collected, vortexed for 30 sec and heated at exactly 85°C for 10 min. Lysates were allowed to cool in ice for 5 min and then centrifuged at 17,000g for 20 min. 500µL of the supernatant was collected into a new tube and 150µL of 10% ammonium hydroxide pH 4.3 was added. 150µL was aliquot into 96-well plates and absorbance was read at 405 nM. Results were normalised to Alizarin Red S standard curve.

Quantitative PCR

Transfected CH-8 and HEK293 cells were harvested as pellets via scraping at 24, 48 and 72 hours, RNA was extracted by using an RNeasy Kit (Qiagen, Hilden, Germany) following manufacturer protocol, cDNA synthesised using an iScript cDNA Kit (Bio-Rad, California, USA) following manufacturer protocol and qPCR was performed using SYBR Green Kit (Bio-Rad) and the following primers, *ENPP1*: 5' – GGTGGACTTCTTCCTGTTATTA, 3' – GGTGACAATGCTGTAGTGA; *TNAP*: 5' – GCTTGACCTCCTCGGAAGAC, 3' – GGGGCCAGACCAAAGATAGA; *PIT-1*: 5' – GACTGGCGTCTCTTTCGTA, 3' – ATGATGGCAGCACTGATAAC.

ENPP1 enzyme activity assay

Transfected CH-8 cells were harvested as pellets via scraping at 24, 48 and 72 hours. The colorimetric substrate *p*-nitrophenyl thymidine 5'-monophosphate (Sigma) was used to assess the ENPP1 enzyme activity following a published protocol (38).

Co-immunoprecipitation (co-IP) and western blotting

CH-8 and HEK293 cells were cultured to 60% confluence and then co-transfected with wt. ANKH or mutant plasmids and *pmRFP_LC3* for autophagy experiments. At 24 hours one set of cells were collected as pellets by trypsinisation. Autophagy was induced for 3 hours by replacing culture media with high glucose DMEM constituted with 10% FBS and 1% pen-strep but with essential amino acids removed and supplemented with a final concentration of 250 nM rapamycin and a subset of lysates collected again. Finally, cells were rescued for 24 hours in normal growth media and a final subset of lysates collected. Co-IP was performed using GFP-Trap magnetic agarose beads that implement a unique camelid nanobody (Chromotek, Martinsried, Germany) following manufacturer protocols. SDS PAGE was performed on the lysates and pull downs in either 4-20% TGX or 12% TGX precast protein gels (Bio-Rad) and transferred to a PVDF membrane using the Trans-Blot turbo system (Bio-Rad) using the Mixed MW protocol (25V, 2.5A, 7 min). Membrane blots were blocked for 1 hour in 5% milk in tris buffered saline with 0.1% tween 20 (TBST). ANKH-eGFP was probed using a 1:2500 dilution of rabbit anti-GFP (NB600-308, R&D Systems, Abingdon, UK) antibody and LC3 was probed using a final concentration of 1 µg/ml rabbit anti-LC3 (NB100-2220, R&D Systems) overnight at 4°C in 5% milk TBST. Goat anti-rabbit IgG-HRP (AB97051, Abcam, Cambridge, UK) 1:5000 was used to label bound antibodies for 1 hour in 5% milk TBST at room temperature and detected using Clarity ECL western substrate (Bio-Rad) following manufacturer protocol on a ChemiDoc XRS+ (Bio-Rad).

Statistical analysis

All experiments were repeated a minimum of three times. For the chemical data analysis, wt. ANKH effect was compared to null vector control whilst mutant effect was compared to wt. ANKH. Assuming equal variance, 2-tailed *t*-test was used to evaluate significance for normally distributed data, whilst a Mann-Whitney *u*-test was performed on non-normally distributed data. An *F*-test was performed to check equal and unequal variance.

Statistical significance was set at $p < 0.05$. We use # to highlight the data $0.05 < p < 0.1$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0005$.

Results

We found that ANKH mutations altered cellular localisation dynamics and led to biochemical changes at different levels by comparing with wt. ANKH. Our findings are summarized in Table 1 and the details are described as below.

Wt.ANKH localisation to the cell membrane was non-uniform and directly influenced the expression levels of ENPPI, TNAP and PIT-1

Wt.ANKH showed clear localisation to the cell membrane and perinuclear area in HEK293 cells as reported in other cell types such as osteoblastic MC3T3-E1, human adult fibroblasts (HAF), adenocarcinomic human alveolar basal epithelial cells (A549), HeLa and monkey Cos7 cells (Figure 1A) (5,9). We observed this specific cell membrane localisation in transfected cells ($n > 100$) at 24 (57%), 48 (66%) and 72 (66%) hours after transfection. Further clarification by using 3D confocal Z-stack rotating rendering also showed the localisation was non-uniform but general to the cell membrane (Supplemental Movie 1). CH-8 cells displayed auto-fluorescence and showed large punctate bodies like structures that masked overexpression of control eGFP and wt. ANKH-eGFP (Supplemental Figure 1 and supplemental Movie 2). Overexpressing wt. ANKH in HEK293 cells increased ePPi at 48 (2.6 μ M) and 72 (3.2 μ M) hours after transfection (Figure 1B). Interestingly, wt. ANKH overexpression in CH-8 cells significantly decreased mineralization (37.15 nM, $p = 5.2 \times 10^{-4}$) compared to GFP only control (44.26 nM) (Figure 1C).

Overexpressing wt. ANKH in HEK293 cells showed decreased gene expression of *ENPP1* at 24 hours (0.485 fold, $p = 0.059$) but increased *ENPP1* at 48 (2.4 fold, $p = 0.032$) and 72 hours (1.6 fold, $p = 0.035$) (Figure 1D). A similar, but non-significant trend, was measured for *TNAP* at 24 (0.56 fold, $p = \text{NS}$), 48 (1.2 fold, $p = \text{NS}$) and 72 (0.61 fold, $p = \text{NS}$) hours after transfection (Figure 1E). *PIT-1* increased significantly at 72 hours (2.00 fold, $p = 0.018$) (Figure 1F). ENPP1 activity in CH-8 cells overexpressing wt. ANKH mimicked the drop in *ENPP1* expression at 24 hours (0.70 fold, $p = 2.02 \times 10^{-5}$) and increase at 48 (1.93 fold, $p = 7.9 \times 10^{-5}$) but returned to baseline at 72 hours (Figure 1G). Lastly, wt. ANKH-eGFP does not co-localise with pmRFP-LC3 under autophagic or rescue conditions (Supplemental Figure 2).

P5L ANKH slowed membrane recruitment and increased ePPi and mineralization via increased ENPP1 activity

We observed diffuse expression of mutant P5L within the cytoplasm with perinuclear aggregation and formation of vesicle like structures at 24 hours, with only 34% of HEK293 cells displaying specific cell membrane localisation. At 48 and 72 hours, cells displaying specific cell membrane localisation reached 64 % and 73% and resembled the specific cell membrane - non-uniform localisation dynamics observed in wt. ANKH (Figure 2A). In Mutant P5L significantly increased ePPi (2.12 fold, $p = 0.032$) at 72 hours in HEK293 cells and mineralization (44.64 nM, $p = 0.01$) in CH-8 cells compared to wt. ANKH (Figures 2B and 2C). It also increased *TNAP* in HEK293 cells at 24 (1.72 fold, $p = 0.044$) and 72 hours (1.39 fold, $p = 0.055$) but did not change *ENPP1* or *PIT-1* (Figures 2D, 2E and 2F). Most interestingly in CH-8 cells we observed increased ENPP1 activity at 24 (1.92 fold, $p = 7.56 \times 10^{-4}$), 48 (1.54 fold, $p = 5.1 \times 10^{-5}$) and 72 hours (3.39 fold, $p = 2.72 \times 10^{-6}$) (Figure 2G).

S375del completely abolished ANKH localisation to the cell membrane and decreased ePPi but increased mineralization

Mutant S375del completely rendered ANKH unable to enter the HEK293 cell membrane and remained diffusely expressed within the cytoplasm at 24, 48 and 72 hours after transfection (Figure 3A) with 0% of transfected cells displaying specific cell membrane localisation. We measured a trending decrease in ePPi at 72 hours (0.55 fold, $p = 0.08$) in HEK293 cells and observed a significant increase in mineralization (52.68 nM, $p = 5.12 \times 10^{-3}$) in CH-8 cells compared to wt. ANKH (Figures 3B and 3C). In HEK293 cells we measured non-significant increases in *ENPP1* (1.62 fold, $p = 0.089$) and *TNAP* (1.95 fold, $p = 0.052$) at 24 hours and found a significant increase in *PIT-1* (1.877 fold, $p = 0.04$) at 48 hours (Figures 3D, 3E and 3F). *ENPP1* activity in CH-8 cells was found to increase at 24 hours (1.14 fold, $p = 9.63 \times 10^{-5}$) but decrease at 48 hours (0.65 fold, $p = 3.25 \times 10^{-5}$) (Figure 3G).

G389R slowed ANKH to insert in the membrane and trended to decrease ePPi, increased mineralization and was likely targeted as a non-functional protein

Mutant G389R expressed mostly within the cytoplasm with just 28% of transfected HEK293 cells displaying specific cell membrane localisation at 24 hours after transfection. At 48 and 72 hours the mutant protein was recruited into the cell membrane and also formed vesicle like puncta (Figure 4A) with 66% and 72% of transfected cells, respectively, displaying specific cell membrane localisation. In HEK293 cells we found a non-significant decrease in ePPi at 48 (0.51 fold, $p = 0.47$) and 72 hours (0.79 fold, $p = 0.68$), suggesting a slower trending recovery, nonetheless in CH-8 cells we measured a significant, if small, increase in mineralization (40.62 nM, $p = 0.001$) (Figures 4B and 4C). Mutant G389R overexpressed in HEK293 cells was found to significantly increase *ENPP1* at 24 hours (2.28 fold, $p = 0.02$) and did not significantly change *TNAP* or *PIT-1* at any time (Figures 4D, 4E and 4F). Similar to *ENPP1* expression we measured an increase in

ENPP1 activity in CH-8 cells at 24 hours (1.93 fold, $p = 1.21 \times 10^{-8}$) but decreased activity at 48 (0.59 fold, $p = 1.73 \times 10^{-4}$) and 72 (0.74 fold, $p = 6.99 \times 10^{-4}$) hours (Figure 4G). Intriguingly, we also observed co-localisation of G389R-eGFP with RFP-LC3 under autophagic and rescue conditions in HEK293 cells (Figure 4H, zoomed and intensity over distance plots and supplemental Movie 3). However, this co-localisation was not a physical interaction, as we did not observe co-immunoprecipitation of LC3 with mutant G389R (Figure 4I).

E490del mutated ANKH remained mostly cytoplasmic and formed punctate bodies like structures that co-localised with LC3, and increased mineralization through increased expression of ENPP1, TNAP and PIT-1

Mutant E490del localised primarily to the cytoplasm at 24, 48 and 72 hours with respectively only 35%, 7% and 29% of HEK293 cells displaying specific cell membrane localisation and also formed vesicle like puncta at 48 and 72 hours (Figure 5A); we found a non-significant decrease in ePPi at 48 hours (0.41 fold, $p = 0.57$) with a return to baseline at 72 hours. In CH-8 cells we observed a significant increase in mineralization (42.79 nM, $p = 0.01$) (Figures 5B and 5C). Strikingly in HEK293 cells, mutant E490del increased expression of *ENPP1* (5.82 fold, $p = 7.3 \times 10^{-5}$), *TNAP* (2.79 fold, $p = 0.001$) and *PIT-1* (2.13 fold, $p = 0.045$) at 24 hours and trended to increase *PIT-1* at 48 hours (1.77 fold, $p = 0.058$) compared to wt. ANKH (Figures 5D, 5E and 5F). ENPP1 activity in CH-8 cells overexpressing mutant E490del resembled *ENPP1* expression at 24 hours with a remarkable increase (5.33 fold, $p = 1.3 \times 10^{-5}$) (Figure 5G). Similar to G389R we observed co-localisation of E490del-GFP with RFP-LC3 under autophagic and rescue conditions (Figure 5H, zoomed and intensity over distance plots and supplemental Movie 4). However, this co-localisation was not a physical interaction, as we did not observe co-immunoprecipitation of LC3 with mutant E490del (Figure 5I).

Discussion

This study highlights the differential localisation dynamics of mutant ANKH in the pathogenesis of CPPDD and CMD. Previous studies showed observation of increased ePPi associated with CPPDD ANKH mutants and there have been no report on the converse reduction of ePPi for CMD mutants. Although altered intracellular trafficking of one CMD mutation was described, the molecular mechanism was unclear (39). Our results demonstrate that ANKH terminal CPPDD and central CMD associated mutations regulate ePPi differently due to altered ANKH cellular localisation; furthermore, we provide mechanistic evidence of altered ENPP1 activity and gene expression of *ENPP1*, *TNAP* and *PIT-1*.

We initially discovered ANKH localisation to the cell membrane to be non-homogenous (Figure 1A and Supplemental Movie 1). We observed a striking distinction between the localisation dynamics of each ANKH mutants. Mutants P5L and G389R both localised more slowly but specifically to the cell membrane (Figures 2A and 4A). Interestingly, mutant S375del did not localise specifically to the cell membrane at any time and while mutant E490del showed some cell membrane localisation it was mostly cytoplasmic (Figures 3A and 5A). These fragmented localisation dynamics, even between mutants causing similar clinical features, highlights the complex and divisive function of ANKH in the pathogenesis of CPPDD and CMD. ANKH function at the cell membrane to transport PPi into the ECM is well established, however contrasting mechanistic evidence of mutated ANKH altered intracellular trafficking and localisation has not been previously reported.

We then quantified each ANKH mutant effect on ePPi concentration and mineralization. The concentrations of ePPi in CH-8 cells were below threshold (data not shown), and it

may be evident that chondrocytes maintain ePPi at lower levels than in cells such as non-chondrocyte HEK293 cells. Perhaps due to their specific function, ePPi is more strictly controlled within the ECM of chondrocytes as well as expression and function of proteins related to ePPi homeostasis resulting in lower levels of ePPi. As expected, in HEK293 cells, overexpression of wt. ANKH increased ePPi level compared to endogenous ANKH (Figure 1B). The CPPDD linked gain-of-function P5L mutant caused a remarkable increase in ePPi compared to wt. ANKH but only once a majority of the mutant had inserted into the cell membrane (Figure 2B) at 72 hours, suggesting a slowed response to ANKH changes in the pathophysiology. Conversely, both CMD linked loss-of-function mutants S375del and G389R trended to decrease ePPi (Figures 3B and 4B). Of interest, there was no significant change in ePPi for CPPDD mutant E490del but just a moderate increase (Figure 5B). In the ECM ePPi plays a key role in inhibiting HA accretion and bone mineralization (40). In CMD, there is thought to be abnormal skeletal modelling due to excessive HA mineralisation because of the decrease in ePPi (41), a phenotype confirmed by our novel molecular data. In contrast, increased ePPi leads to excessive CPPD mineralization in the ECM of those suffering with CPPDD. Our data highlighted all tested mutants had pathologically increased mineralization (Figures 2C, 3C, 4C and 5C). Furthermore, the quantity of mineralization ranks the mutations in their severity of clinical phenotypes with S375del being the most severe (52.68 nM); second P5L (44.64 nM), third E490del (42.79 nM) and fourth G389R (40.62 nM). Taken together, our data suggest a direct and novel link between differential cellular localisation, altered ePPi concentration and mineralization associated with mutated forms of ANKH.

As bone mineralization is regulated by the ratio of $[Pi]/[PPi]$ in the growth plate ECM, a synergistic relationship between ANKH, ENPP1, TNAP and PIT-1 exists to control physiological vs. pathophysiological mineralization (7,40,42). We have explored some of the reported interactions of ANKH with ENPP1, TNAP or PIT-1 in regulating ePPi

(16,22,24,26,27,43) and discerned the complexity of these relationships especially in the context of disease progression and causality. Increased *ENPP1* and *PIT-1* of wt. ANKH corresponded to the increase in ePPi, and the reduction in *TNAP* was concordant with decreased mineralization (Figures 1D, 1E and 1F). Therefore, simply increasing the quantity of ANKH does not replicate a gain-of-function phenotype. This is likely due to saturation of the ANKH - ENPP1 interaction, where ENPP1 is thought to provide most of the ePPi present in the ECM (3,38,44). The decrease in mineralization can be attributed to the mild increase in ePPi, enough to further inhibit HA accretion but lacking for CPPD formation, representing an overall decrease.

Mutant P5L increased expression of *TNAP* at 24 hours as a response to increased ePPi, but not sustained; however the level of *ENPP1* and *PIT-1* were similar to wt. ANKH (Figures 2D, 2E and 2F). Interestingly, we measured an increase in ENPP1 activity at 24 (1.92 fold), 48 (1.54 fold) and 72 hours (3.39 fold) (Figure 2G) and therefore conclude that P5L raised ePPi via increased ENPP1 activity rather than increased ANKH function or increased *ENPP1* expression – enlightening the previous observation showing P5L to increase activity of ENPP1 (21). Furthermore, the increased ePPi and non changed *PIT-1* indicated that the increased mineralization caused by P5L was specific to CPPD formation rather than HA. Inability of mutant S375del to insert into the membrane was so severe that even increased expression of *ENPP1*, *TNAP*, *PIT-1* and corresponding ENPP1 activity as compensation was not enough to rescue the phenotype (Figures 3D, 3E, 3F and 3G). S375del showed by far the largest mineralization profile and decrease in ePPi over time indicated the mineralization to be likely HA rather than CPPD. In the long term, reduced ANKH membrane presence would exacerbate a condition reliant on extracellular ePPi control and this explains the severe phenotype. The milder phenotype mutant G389R increased expression and corresponding activity of ENPP1 but did not change *TNAP* and *PIT-1* (Figures 4D, 4E, 4F and 4G). Interestingly, as mutant G389R was recruited to the

cell membrane similar to wt. ANKH but still increased ENPP1 activity and expression as compensation for decreasing ePPi levels, this indicated a severe reduction in transport capability for G389R. Mutant E490del drastically increased early expression of *ENPP1*, *TNAP* and *PIT-1* and corresponding ENPP1 activity (Figures 5D, 5E, 5F and 5G) as compensation for lack of ANKH at the cell membrane, however as this mutant did not completely abolish ANKH localisation and hence, the compensatory effect seemed to be overpowered and might indeed be causal in the pathogenesis of CPPDD in sufferers affected by the E490del mutation.

Abnormal or defective proteins are often targeted by autophagic machinery for recycling (45). This homeostatic mechanism is present in all cell types and offers a quick and simple method in identifying mis-folded and non-functional proteins reliant on the organisms own stringent selection criteria (45). Hence, we described co-localisation of mutant ANKH with the autophagy marker LC3 as strongly suggesting defective protein processing. Co-localisation of autophagy marker with mutant E490del under normal (data not shown), autophagy induction and rescue conditions, and with mutant G389R under autophagy induction and rescue conditions suggested that these mutated proteins were targeted as non-functional or defective proteins (Figures 4H, 5H, supplemental Movies 3 and 4). We also checked for direct physical interaction of ANKH with LC3, which perhaps might indicate a role in the autophagy process, however we did not observe co-immunoprecipitation of these mutants with LC3 (Figures 4I and 5I), confirming that G389R and E490del were targets of autophagy as non-function or defective proteins rather than facilitators in the autophagic process. The absence of co-localisation for mutants P5L and S375del was not surprising as protein recycling is not limited to autophagy. The Endoplasmic-reticulum-associated protein degradation (ERAD) ubiquitination pathway that is preferential for insoluble aggregates within the cytoplasm would explain S375del

not co-localising with LC3 and being preferentially recycled via a different mechanism (45).

We summarize the possible mechanisms in the pathogenesis of each tested mutants associated with CPPDD or CMD as follows:

P5L – This proline to leucine mutation may delay ANKH membrane uptake and the longer term increasing in ePPi could be attributed to an overreaction from increased ENPP1 activity rather than *ENPP1* expression directly. This is likely that the rise in ePPi is not due to increased ENPP1 amounts at the cell membrane but rather to increased activity of ENPP1 that is already at the cell membrane facilitated by the P5L mutation. Since TNAP expression increased initially and has a role in reducing excess ePPi, we suggest that there should be a feedback mechanism to upregulate TNAP under conditions of high ePPi but can not compensate for the mutation effect.

S375del – Our data suggests that deletion of serine at residue 375 completely abolishes ANKH localisation to the cell membrane and is a loss-of-function. We observed almost cytoskeletal-like localisation over 72 hours and measured a corresponding decrease in ePPi and increase in mineralization. The phenotype of *S375del* CMD is severe and at the molecular level even the compensatory increase in *ENPP1*, *TNAP* and *PIT-1* could not rescue the biochemical phenotype. Mechanistically, we predict the serine deletion results in inappropriate trafficking from the ER and abolish ANKH function.

G389R – This mutant displayed equivocal molecular data highlighting its variable clinical associations with both CMD and CPPDD. Its cell membrane localisation resembled the CPPDD-associated mutant P5L but we measured a sustained decrease in ePPi similar to the CMD associated mutant *S375del*. Early upregulation of *ENPP1* under low ePPi

concentrations further supports this to be a loss-of-function mutation. Hence, since the mutant successfully localised to the cell membrane, the glycine to arginine change at residue 389 appears to cause a loss of transport channel function, which caused the decrease in ePPi and increase in mineralization, although small, reflecting its milder phenotype. The involvement of G389R in autophagy also suggests that it undergoes misfolding or some other abnormal processing, but perhaps to a lesser degree than E490del.

E490del – The deletion of a glutamate residue at the C-terminus renders it unable to insert effectively into the cell membrane. However, this mutant resulted function similar to wt. ANKH in regulating ePPi by increasing *ENPP1*, *TNAP* and *PIT-1* but was still pathogenic as it increased mineralization. We suggest that this mutant is recognised as a non-functional protein and is constantly recycled by the autophagy machinery, which consequently due to its loss also upregulates *ENPP1*, *TNAP* and *PIT-1* as compensation for the loss of ANKH at the cell membrane. It is noteworthy that the clinical presentation of CPPDD associated with the E490del is less severe than other forms that might be owing to some of the ANKH getting to the membrane but at lower efficiency (10).

In conclusion, the corresponding pathogenetic mechanisms for each mutant ANKH appear to be unique even between mutants causing similar phenotypes. They include effects on intracellular trafficking of ANKH, PPi transport channel function, mineralization, ENPP1 activity regulation, autophagy and guiding effects in expression level of *ENPP1*, *TNAP* and *PIT-1*, some of which could represent tractable targets for therapy.

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