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Exploring the Biological functions of vtRNA 1-1

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This thesis is submitted for the degree of Master of Research (MRes)

December 2022

Acknowledgments

Well science always evolves and changes, I had hardly ever heard of non-coding RNA before my MRes now I know that it comes in many flavours and regulates so many things. Much to my amazement molecules that were thought to be exclusively protein coding in my undergraduate teaching are now protein non-coding! Although I was exclusively a member of Fed's research group consisting of Paula, Raquel, Becky and Alex it did have a feeling of being like a community when Robs research group consisting of Barry, Charlie Sophie and Anya conducted their research and there was Dan embarking on his role as a Principal Investigator. It was clearly an exciting time to be an active part of research. Collaboration showed its true worth and as did the help and support that I received during my MRes from the afore mentioned make my prospective future brighter to which I will always be eternally grateful for plus of course the support from family,

Abstract

RNA that is transcribed from the genome mainly consists of non-coding RNA (ncRNA) which is not translated into proteins. Several important biological processes are regulated by ncRNAs, these include regulation of gene expression at the transcriptional and post transcriptional levels together with regulating protein translation. One type of ncRNA short non-coding RNA (sncRNA) vault RNA 1-1 (vtRNA 1-1) has been shown to regulate autophagy and its role in regulating specific aspects of neurodevelopment is starting to be unravelled. We also turn our attention to another type of ncRNA - the long non-coding RNA (lncRNA) MALAT1 (metastasis-associated lung adenocarcinoma transcript 1) which has also been shown to regulate specific aspects of neurodevelopment therefore we aim to provide further insight into the biological functions of vtRNA 1-1 and MALAT1. In the first instance HeLa cells were transfected to overexpress vtRNA 1-1 and a manual count to quantify P62 puncta was untaken to assess whether overexpression of vtRNA 1-1 relative to 5SRNA had any effect on a marker of autophagic flux. In a separate set of experiments primary neuronal cells were transfected to overexpress and inhibit expression of vtRNA 1-1 and MALAT1, axonal growth rates were then subsequently monitored. Overexpression of vtRNA 1-1 in HeLa cells did not reveal any effect on the levels of P62 puncta and overexpression of vtRNA 1-1 in primary neuronal cells did not demonstrate any effect upon axonal growth. Inhibition of vtRNA 1-1 expression significantly reduced axonal growth rates as did inhibiting MALAT1 expression in the same system. Collectively, we have explored the biological functions of ncRNAs in the context of axon growth and provide a foundation for others to investigate further how exactly different types of ncRNAs exerts their effect(s).

Abbreviations

BDNF-Brain derived neurotrophic factor

BL-41-Burkitt Lymphoma cell line

C6-Rat glioma cell line

DIV-Days in vitro

ERK-Extracellular-signal-regulated kinase

GFP-Green fluorescent protein

HeLa-Immortalised cervical carcinoma cell line derived from Henrietta Lacks

Huh-Immortalised hepatic cancer cell line

KD-Knock down

KO-Knock out

LC3-Microtubule-associated protein 1A/1B-light chain 3

MAPK-Mitogen activated protein kinase

mvtRNA-Mouse vault RNA

MVP-Major vault protein

NGF-Nerve growth factor

PC12-Adrenal phaeochromocytoma cell line

PSD-95-Postsynaptic density protein 95

PTEN-Phosphatase and tensin homolog

SynCAM1-Synaptic cell adhesion molecule 1

WT-Wild type

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Chapter 1-General Introduction

1.1-Non-coding RNA

RNA profiling has identified that 75 % of the human genome is transcribed into RNA (Bernard et al., 2010; Sandberg et al., 2013) but only a small percentage of these transcribed elements encode proteins (1.5-2 %) (Awan et al., 2017; Morceau et al., 2013) and over 80 % consists of non-coding RNA (ncRNA) not encoding proteins [1]. There are two classes of ncRNA classified by the length of the nucleotide sequence, short non-coding RNA (sncRNA) is less than 200 nucleotides long and long non-coding RNA (lncRNA) is greater than 200 nucleotides long (Sandberg *et al.*, 2013).

Specific types of ncRNA were first discovered in the 1950's and 1960's (Micheel et al., 2021), examples of these included transfer RNA (tRNA) (Hoagland et al., 1958), ribosomal RNA (rRNA) (Palade, 1955) and small nucleolar RNA (snoRNA) (Weinberg and Penman, 1968). Regulatory functions are performed by ncRNAs, these include the regulation of gene expression at transcriptional and post-transcriptional levels together with their involvement in remodelling chromatin complexes (Kaikkonen et al., 2011). Housekeeping roles undertaken by ncRNAs include the regulation of protein translation and alternative splicing [2].

Short non-coding RNA (sncRNA)

There are different types of sncRNAs which consist of microRNAs (miRNAs), transfer RNAs (tRNAs) and small nuclear RNAs (snRNAs) (Li et al., 2021). Next-generation sequencing (NGS) has discovered new types of sncRNAs (Liu et al., 2021) and examples of these include PIWI-interacting RNAs (piRNAs) and tRNA-derived small RNAs (tsRNAs) (Li *et al.*, 2021). Functions of sncRNAs are mainly of a regulatory nature (Inamura, 2017; Morris and Mattick, 2014) (Figure 1A) and are involved in

regulating gene expression at transcriptional and post translational levels (Gallo et al., 2022).

Role of sncRNAs in neuronal development

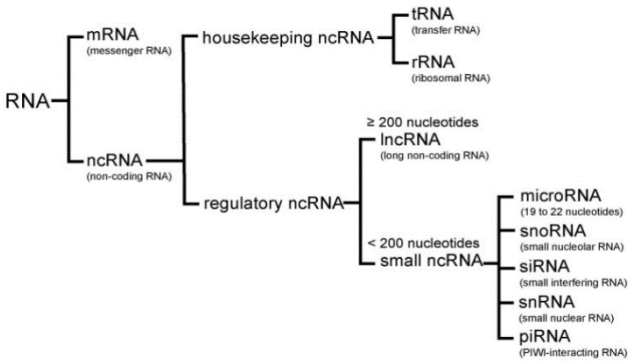
The basic morphology of a neuron is demonstrated in (Figure 1B), neurons are the components of the mammalian central nervous system (CNS) which transmit electrical signals that co-ordinate sensory information, initiate motor movement as well as being responsible for behavioural states in higher order organisms. Carefully orchestrated steps in the development of the CNS are required and one such step involves axonal growth. RNA taken from the developing axons in primary neuronal cells underwent small RNA sequencing revealed the presence of the following miRNAs - miR-434-3p, miR-151-3p and miR-92a-3p and inhibition of these specific miRNAs reduced axonal growth rates (Mesquita-Ribeiro *et al.*, 2021) (Figure 1B). Another sncRNA miR-26a was also shown to control axonal growth rates as well as playing a specific role in specifying the morphology of a neuron into the component parts axons and dendrites in a process known as polarisation (Lucci *et al.*, 2020) (Figure1B). Other miRNAs documented as playing a role in axonal development are also shown in (Figure 1B) and taken from the following review (Corradi and Baudet, 2020).

Small RNA sequencing from the RNA obtained from the axons of primary neuronal cells also revealed the presence of other specific fragments of sncRNAs which included tRNAs, small nucleolar RNAs (snoRNAs), Y RNAs as well and specifically that of vault RNA (vtRNA) (Mesquita-Ribeiro *et al.*, 2021) and the type of sncRNA that this thesis will consider is that of vtRNA 1-1.

Long non-coding RNA (lncRNA)

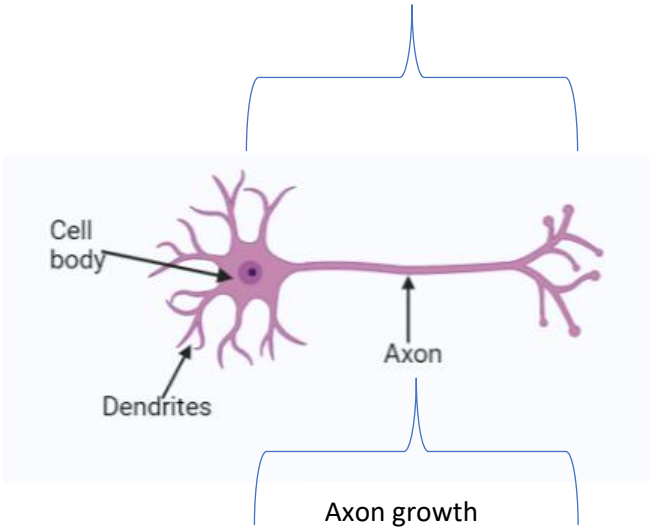
Regulatory functions are performed by lncRNAs (Figure 1A) (Inamura, 2017) and the primary structure of lncRNAs is similar to that of mRNA as both contain introns, exons, 3' poly and 5' caps (Hao et al., 2023). LncRNAs are less conserved than mRNA sequences but the promoters of lncRNAs exhibit the same amount of conservation as protein-coding genes together with having conserved exon structures, splice junctions and sequence patches (Mattick et al., 2023). Due to the relative length of lncRNAs, stable secondary structures can be formed which participate in regulation and organisation of other cells (Wang et al., 2017) and consist of modular structural domains which play a role in DNA binding, gene splicing, epigenetic silencing and mRNA stability (Mattick *et al.*, 2023). LncRNAs play a key role in regulation of the cell cycle (Rinn and Chang, 2012) and in the development of the brain, muscle, heart and lungs as revealed from high throughput loss of function genetic screens (Mattick *et al.*, 2023). The type of lncRNA that this project will focus on is MALAT1 (metastasis-associated lung adenocarcinoma transcript 1) and was first discovered as being upregulated in patients with early-stage non-small cell lung cancer (NSCLC), it is also known as nuclear enriched abundant transcript2 (NEAT 2), (Ji et al., 2003; Zhang et al., 2017).

A



miR-26a polarisation

B



Axon growth

miR-92a-3p
miR-9
miR-434-3p
miR-338
miR-29c
miR-26a
miR-19a
miR-181d
miR-151-3p
miR-132

Figure 1-How ncRNAs are classified and their roles in regulation of axonal polarisation and growth (A) basic morphology of neurons demonstrating the locations of the cell body, dendrites and axon together with the roles played by specific miRNAs in axonal growth and polarisation (B) (Taken from Inamura, 2017 and image created in Biorender)

1.2-Vault RNA (vtRNA)

Vault RNA is a type of sncRNA originally discovered through its association with vault particles, vault particles were identified as ovoid structures and as contaminants in clathrin coated vesicles (Kedersha and Rome, 1986). Ultracentrifugation to separate and purify component parts of biological material using high centrifugal forces originally identified the vault particle and its association with vtRNA (Kedersha and Rome, 1986). Vault particles consist of two main features a major vault protein (MVP) and 2 minor vault proteins. The structure of a vault particle as determined by x-ray crystallography (Casañas et al., 2012) is displayed in (Figure 2A).

Role of the vault particle

The vault particle is located in the cytoplasm and interacts with microtubules (van Zon et al., 2003) and has been shown to be transported in axons between the cell body and nerve terminals (Li et al., 1999), it's enriched in the brain and cholinergic nerve terminals of the electric torpedo ray (Herrmann et al., 1996). Important roles of the vault particle have been documented in oncology as it acts a prognostic indicator in patients undergoing radiation treatment (Lara et al., 2011) and in mediating resistance to drug treatment (Mossink et al., 2003; Steiner et al., 2006).

Association of vtRNA with the vault particle

Specific scenarios appear to drive the association of vtRNA with the vault particle, infection with Epstein-Barr virus (EBV) (Nandy et al., 2009) increased the association of vtRNA with the vault particle and drug resistant cancer cell lines demonstrated increased vtRNA association with the vault particle (Kickhoefer et al., 2002;

Kickhoefer et al., 1998; van Zon et al., 2001) potentially showing how treatment resistance in cancer drives the association of vtRNA with the vault particle.

Proteins mediating the interaction between vtRNA and the vault particle

Proteins known as telomerase associated protein (TEP1) and La RNA binding protein mediate the interactions between vtRNA and the vault particle in mice (Kickhoefer et al., 2001) and rats respectively (Kickhoefer *et al.*, 2002)

Homology and composition of the human paralogue - vtRNA 1-1

The human paralogue vtRNA 1-1 consists of the following components- A, B1 and B2 boxes which form RNA polymerase III promoter elements, the RNA polymerase III promoter elements A and B2 are conserved in mouse vault RNA (mvtRNA) as are other regulatory regions of transcription a TATA box and proximal sequence element (PSE) (Büscher *et al.*, 2020; Kickhoefer et al., 2003; Stadler et al., 2009) (Figure2B).

Human paralogues of vtRNA

There are four paralogues of vault RNA in humans - vtRNA 1-1, vtRNA 1-2, 1-3 and vtRNA 2-1 encoded on chromosome 5q31 at two different vtRNA loci (Gallo *et al.*, 2022; Hahne et al., 2021). The paralogues vtRNA 1-1, vtRNA 1-2 and 1-3 - are encoded at loci 5q31.3 (vtRNA 1 loci) (Figure 2C) between a matrin-type 2 gene and a proto-cadherin cluster and the other paralogue vtRNA 2-1 (vtRNA loci 2) is also located at loci 5q31.3 between genes coding for transforming growth factor beta 1 (TGF-β1) and SMAD family member 5 (SMAD5) (Hahne *et al.*, 2021).

Processing of vtRNA paralogues into small vtRNA and its association with the vault particle

The paralogues of vtRNA as demonstrated in (Figure 2D) can be processed into small vtRNAs in a Dicer dependent manner (3 %), full-length paralogues of vtRNA are mainly located in the cytoplasm and only a small proportion (5 %) of vtRNA paralogues associates with the vault particle (Gallo *et al.*, 2022). Mammalian vtRNA is mainly located in the cytoplasm (Kickhoefer, 2019) and 95 % of the human paralogues of vtRNA are located in the cytoplasm (Gallo *et al.*, 2022) (Figure 2E).

Importance of the secondary structure of vtRNA 1-1 and it related function(s)

The predicted secondary structure of vtRNA 1-1 displaying its stem and loop structures along with its central domain and the function of this structure are shown in (Figure 2F). The secondary structure is well conserved (Gallo *et al.*, 2022) and specific structures in vtRNA 1-1 have been shown to regulate apoptosis and autophagy, removal of the terminal stem loop structure (central domain) (Figure 2F) reduced resistance to apoptosis (Amort et al., 2015). The central domain was shown to specifically bind to the P62 receptor which is involved in initiating autophagy (Büscher et al., 2022) and the central domain of vtRNA 1-1 has also been proposed as a potential anchor point for other vtRNA 1-1 interacting proteins (Gallo *et al.*, 2022).

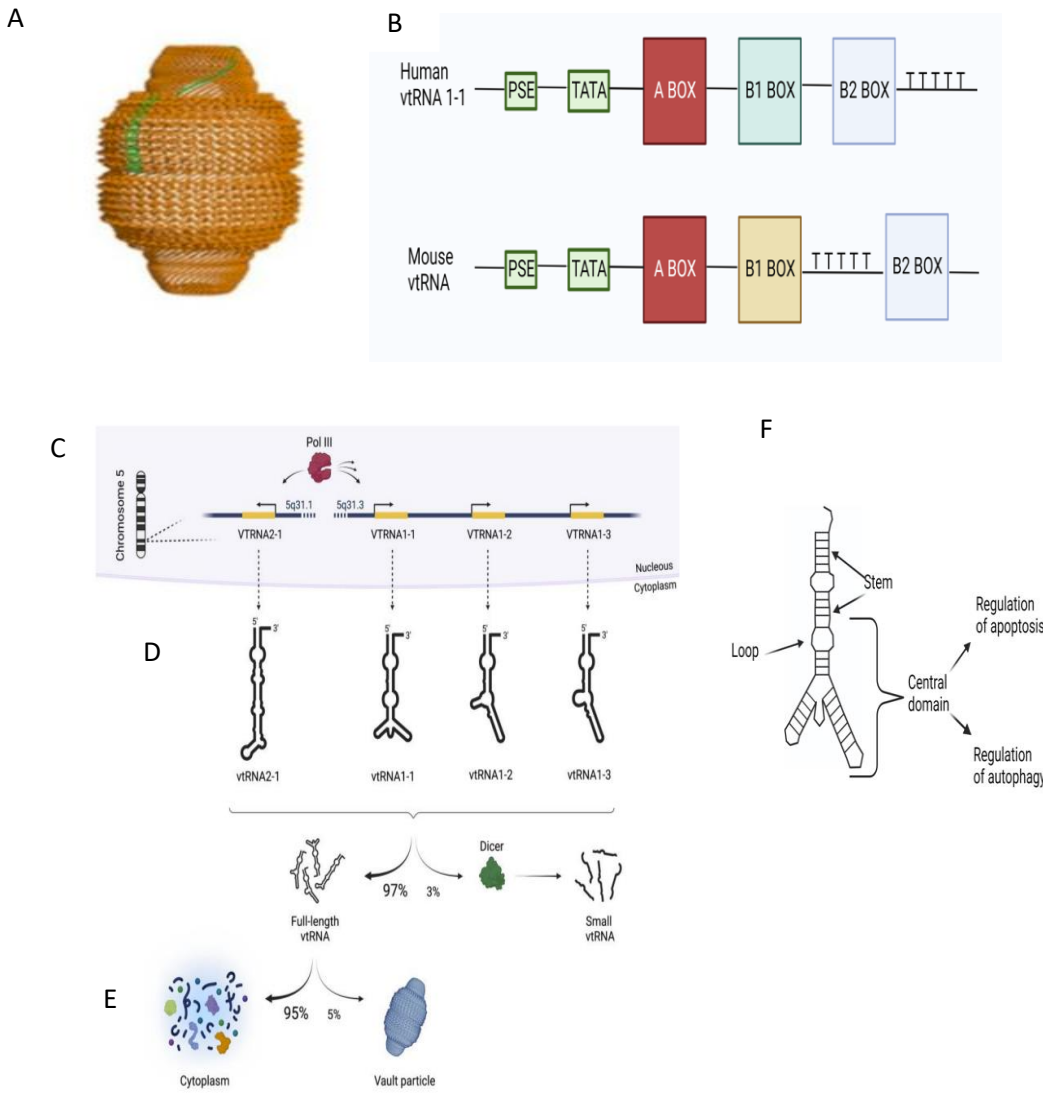


Figure 2-Vault particle, homology and composition of vtRNA 1-1, paralogues of vtRNA: processing and distribution, and structure of vtRNA 1-1 (A) Image of a vault particle structure as demonstrated with x-ray crystallography (taken from Casañas et al., 2012. RNA polymerase III promoter elements in vtRNA 1-1 - A box, B1 box, TATA box and PSE = proximal sequence elements and TTTT=Terminator. PSE, TATA, A and B2 box RNA polymerase III promoter elements in vtRNA are homologous and conserved in mouse vault RNA (same colour box denotes a conserved element). (Adapted from Kickhoefer et al., 2003) (B). Location of vtRNA loci on chromosome 5 - vtRNA 1-1, 1-2 and 1-3 paralogues are encoded on 5q31.3 and the paralogue vtRNA 2-1 is encoded on 5q31.1 (C) all of which are transcribed by RNA polymerase III (taken from Horos et al., 2022). Small proportions of each vtRNA paralogue are processed into small vtRNA in a Dicer dependent manner (taken from Horos et al., 2022) (D) Each paralogue of vtRNA is mainly free floating in the cytoplasm (95 %) and (5 %) associates with the vault particle (taken from Horos et al., 2022) (E). Predicted secondary structure of vtRNA 1-1 demonstrating the stem and loop structures, location and the proposed functions of the central domain (F) (Image of vtRNA 1-1 courtesy of Norbert Polacek and Figure 1B created in BioRender)

1.3-Roles of vtRNA 1-1

1.3.1-Regulation of autophagy

Basics of autophagy

Autophagy is a cellular homeostatic process that enables cells to dispose of potentially harmful or unwanted material (Lahiri et al., 2019) and also acts to recycle material to provide other sources of energy for organisms (Chun and Kim, 2018). Cargo that is recognised as being defective would regularly be tagged with ubiquitin and degraded by the ubiquitin-proteasomal system. Defective cargo that does not get degraded by this pathway is recognised by the P62 receptor and guided to a phagophore membrane (Figure 3A). The resultant interactions of P62 with Microtubule-associated protein 1A/1B-light chain 3 (LC3)- LC3 I and II proteins elongate the phagophore membrane (Figure 3B) to form an autophagosome (Figure 3C). The resultant cargo, P62 and LC3 proteins are then subsequently degraded through the fusion of an autophagosome and lysosome forming an autolysosomal complex. Engulfed material is then degraded through the action of lysosomal hydrolase enzymes (Figure 3D). The conversion of LC3-I to LC3-II proteins are indicative measures of autophagosome formation and degradation of P62 proteins is a known marker of autolysosomal degradation both of which act as markers of autophagic flux (Horos et al., 2019a) and are shown throughout (Figure 3). The P62 receptor is one of a number of different receptors that mediate autophagy, one specific form of autophagy acting to dispose of protein aggregates is referred to as aggrephagy (Gubas and Dikic, 2022). Protein aggregates are a common pathological feature in neurodegenerative diseases and are thought to result from defective protein homeostasis, defects in autophagy contribute to impaired proteostasis leading to increased neurotoxicity (Chua et al., 2022). Aggrephagy is also mediated

by the following receptors - Neighbour of BRCA1 gene 1 (NBR1), optineurin (OPTN), toll interacting protein (TOLLIP) and (TAX1) human T cell leukaemia virus type I binding protein 1 (TAX1BP1) (Gubas and Dikic, 2022). Recently chaperonin containing TCP1 subunit 2 (CCT2) has been shown to be another receptor involved in mediating aggrepahgy (Ma et al., 2022)

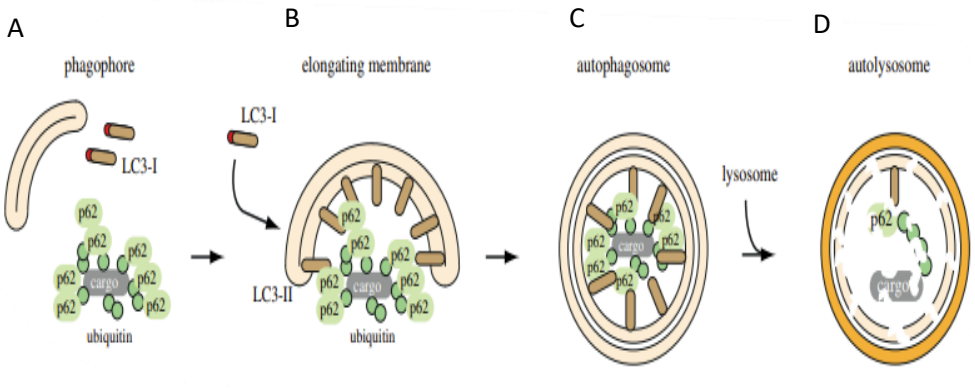


Figure 3-Autophagy, fusion of a lysosome and autophagosome leading to the degradation of material (A)- Cargo that is recognised as being defective is tagged with ubiquitin and degraded by the ubiquitin-proteasomal system. Defective cargo that does not get degraded by this pathway is recognised by the P62 receptor, P62 guides the cargo to a phagophore membrane and resultant interactions of P62 with LC3-I and II proteins result in elongating the phagophore (B) membrane to form an autophagosome (C). The resultant cargo, P62 and LC3 proteins are then subsequently degraded when an autophagosome and lysosome fuse together forming an auto lysosomal complex (Büscher et al., 2020) the engulfed material is then degraded through the action of lysosomal hydrolase enzymes (D). (Taken from Büscher et al., 2020)

Binding of vtRNA 1-1 with specific domains of the P62 receptor to inhibit autophagy

Recently (Horos *et al.*, 2019a) demonstrated that vtRNA 1-1 specifically bound to P62 and when overexpressed inhibited the oligomerisation of the PB1 domains from binding to the zinc finger domains of the P62 receptor protein (Figure 4). The PB1 domain when expressed by itself with an adjacent carboxy linker region specifically bound to vtRNA 1-1 more prominently than vtRNA 1-2, 1-3 and 2-1 (Büscher *et al.*, 2022) and the interaction between vtRNA1-1 and P62 was shown to be mediated through the central domain of vtRNA 1-1 (Büscher *et al.*, 2022) (Figure 4).

Effects mediated by vtRNA 1-1 on the markers of autophagic flux

Knock-down (KD) of vtRNA 1-1 increased the levels of LC3 proteins, reduced P62 levels and demonstrated increased autophagic flux (Horos *et al.*, 2019a; Horos et al., 2019b). Overexpression of vtRNA 1-1 increased P62 proteins levels and reduced the levels of LC3 proteins reducing autophagic flux, overexpression of other vtRNA homologues did not demonstrate these affect(s) (Horos *et al.*, 2019a).

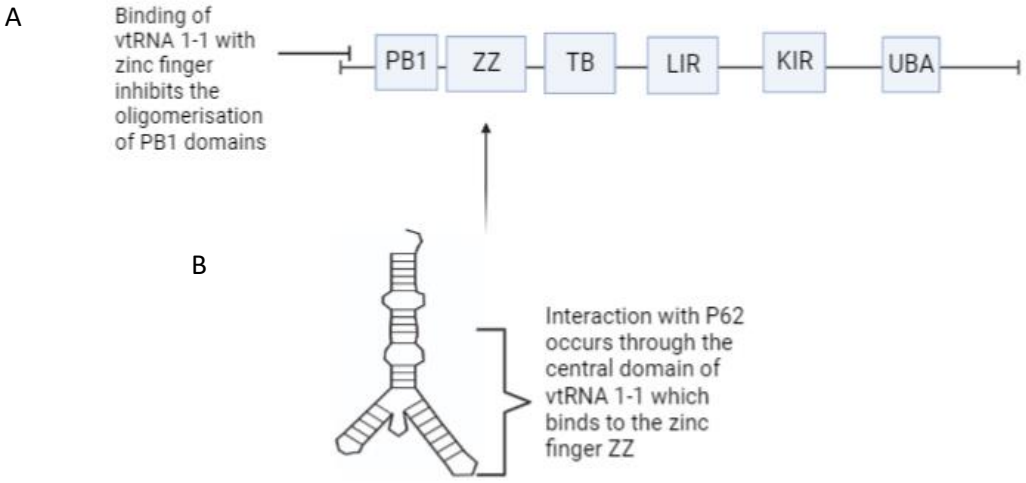


Figure 4-Domains in P62 that the central domain of vtRNA 1-1 specifically binds to in order to regulate the function of P62 Specific domains of P62 (A) (Phox and Bem1 domain= PB1, zinc finger domain=ZZ, LC3-interacting region=LIR, KEAP1 interacting region=KIR, Ubiquitin associated domain=UBA and TRAF6-binding domain =TB). Binding of vtRNA 1-1 with the zinc finger occurs via its central domain (B) and prevents oligomerisation of PB1 domains and subsequently inhibits the function of P62. (vtRNA 1-1 image courtesy of Norbert Polacek and image created in BioRender)

1.3.2-Regulation of apoptosis

The specificity of processes regulated by human vtRNA 1-1 relative to other paralogues of vtRNA is further demonstrated through regulation of apoptosis (programmed cell death) as knock down (KD) of vtRNA 1-1 increased the number of Annexin positive cells subjected to 48 hours of chloroquine treatment whereas KO of vtRNA 1-3 did not demonstrate this effect (Bracher et al., 2020). Restoration of vtRNA 1-1 reduced the number of Annexin positive cells to that of WT vtRNA 1-1 (Bracher *et al.*, 2020) in HeLa cells. Expression of vtRNA 1-1 relative to 1-2 and 1-3 reduced Annexin positive cells in response to BL-41 cells being subjected to the apoptotic Fas ligand, addition of other apoptotic agents staurosporine and etoposide demonstrated the same effects among the afore mentioned human vtRNA paralogues (Amort *et al.*, 2015).

Specific domains in a predicted secondary structure model of vtRNA 1-1 regulate apoptosis

Swapping of the central domains of vtRNA 1-1 (M5) (Figure 5A) with that of vtRNA 1-2 (M4) (Figure 5B) demonstrated reduced resistance to the apoptotic agent staurosporine whereas replacing the central domain of vtRNA 1-2 (M4) (Figure 6B) with a (M5) domain of vtRNA 1-1 (Figure 5A) conferred greater resistance to apoptosis (Amort *et al.*, 2015). Mutations generated in specific positions of the secondary structure in vtRNA 1-1, (M1-M3) represent the positions in which a single nucleotide differs in vtRNA 1-2 relative to the same nucleotide position in vtRNA 1-1 within the specific stem loop structure (Figure 5C). Incorporation of M1-M3 nucleotides from vtRNA 1-2 into vtRNA 1-1 didn't hinder vtRNA 1-1 ability to resist the apoptotic agent staurosporine, thus showing that the terminal loop structure

known as the central domain in vtRNA 1-1 specifically mediates resistance to apoptosis (Amort *et al.*, 2015). A more detailed analysis to demonstrate which subsections of the central domain regulate apoptosis was undertaken by mutating nucleotides at specific locations within the central domain (Bracher *et al.*, 2020).

Signalling cascades and role in apoptosis

The role(s) played by vtRNA 1-1 in modulating pro-survival cellular signalling cascades such as that involving phosphorylated extracellular signal related kinases 1/2 (pERK 1/2), phosphoinositide 3-kinase (PI3-kinase) and protein kinase B (AKT) haven't been investigated as is exactly how both pro-survival and apoptotic pathways are both stimulated with vtRNA 1-1, there is the possibility that apoptotic pathways overwhelm survival pathways with vtRNA 1-1 KO (Bracher *et al.*, 2020). Adding to the complexity in elucidating this could be due to the different conditions used to induce apoptosis and stimulate cell signalling cascades (Bracher *et al.*, 2020) together with the type of apoptosis under investigation (intrinsic or extrinsic) as vtRNA 1-1 expression can also affect the mediators involved in these specific types of apoptosis (Amort *et al.*, 2015). Further understanding of the role vtRNA 1-1 in mediating cell survival and apoptosis have important implications in not only understanding states of development but also that in pathophysiological state.

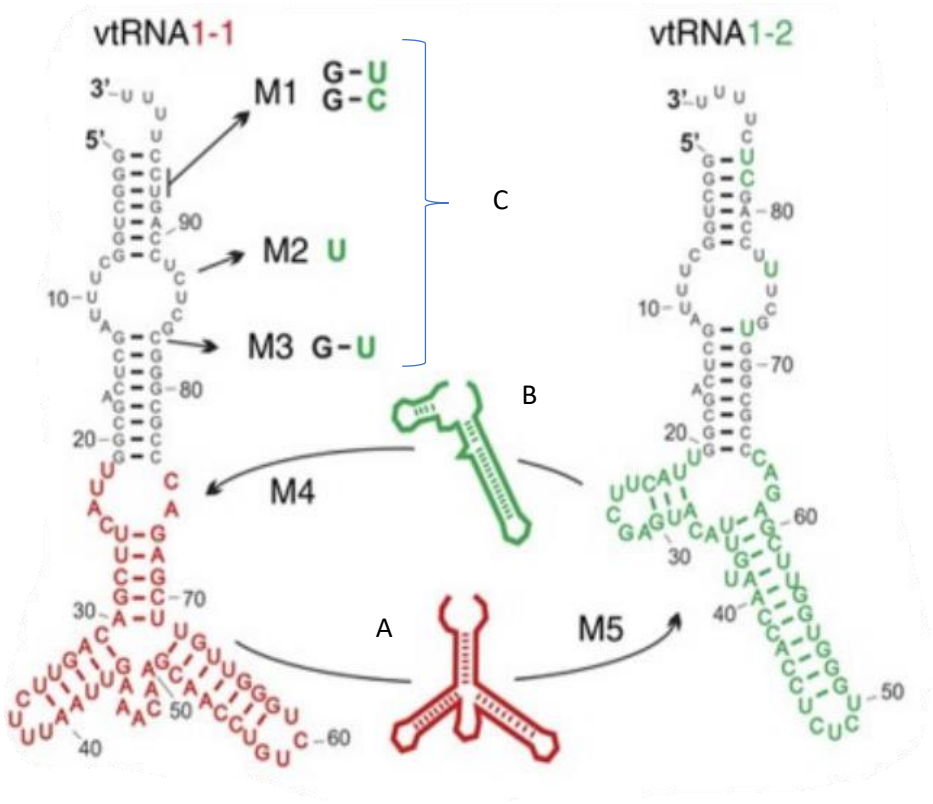


Figure 5-How specific domains within the secondary structure of vtRNA 1-1 resist apoptosis. Swapping of M5 (central domain of vtRNA 1-1) with the central domain of vtRNA 1-2 (M4) conferred resistance to apoptosis (A). Swapping of M4 (central domain of vtRNA 1-2) with the central domain position of vtRNA 1-1 (M5) reduced vtRNA 1-1 ability to resist apoptosis (B). Single nucleotide mutations generated in vtRNA 1-1 (M1, M2 and M3) (C) to recreate the single nucleotide present at the same positions in vtRNA 1-2 did not hinder vtRNA 1-1's ability to resist apoptosis (taken from Amort et al., 2015).

1.3.3-Roles in neurodevelopment

The different morphological parts of neurons as previously shown in (Figure 1B) and their respective roles are discussed further in (Figure 6).

Role of vtRNA 1-1 in synapse formation

Human vtRNA 1-1 when transfected into primary neuronal culture increased the levels of specific synaptic markers – post synaptic density protein 95 (PSD-95), Synapsin I and increased pERK signalling (Wakatsuki *et al.*, 2021a), these effects were not shown with human paralogue vtRNA 2-1 therefore demonstrating that a specific paralogue of human vtRNA is involved in regulating synaptogenesis (Wakatsuki *et al.*, 2021a). The afore mentioned aspects of synaptogenesis where also demonstrated with mvtRNA (Wakatsuki *et al.*, 2021a; Wakatsuki *et al.*, 2021b) and (Figure 7A&B) provide a summary of the potential roles played by vtRNA 1-1 and mvtRNA in regulating pERK cellular signalling cascades that lead to the expression of the synaptic proteins PSD-95 and Synapsin I.

Role of vtRNA in axon development

Antisense oligonucleotides (ASOs) to downregulate mvtRNA demonstrated reduced the growth of newly developing axons as did short hairpin RNA (shRNA) directed against major vault protein (MVP) which was rescued with cDNA MVP resistant to shRNA (Wakatsuki *et al.*, 2021b) (Figure 7C). As demonstrated in (Figure 2B) RNA polymerase III promoter elements are conserved between vtRNA 1-1 and mvtRNA.

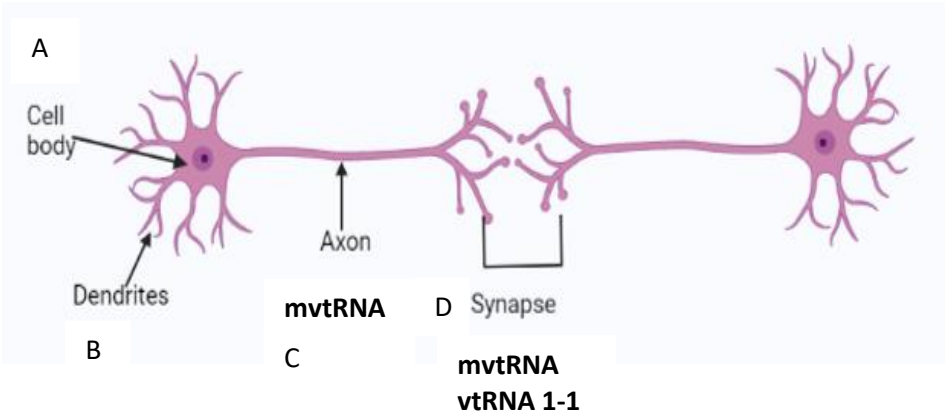


Figure 6-Morphological components of neurons and where vtRNA 1-1 and mvtRNA act within a neuron Cell body - Central organising region that integrates signals from other neurons and initiates an action potential (A). Dendrites - Small projections coming off the cell body to integrate information from other neurons (B). Axons - Long projections from the cell body that enable action potentials to travel along the neurons KD of mvtRNA reduced the growth of these components (Wakatsuki et al., 2021b) (C), Synapse - Gap between neurons that enable neurons to communicate via action potentials, overexpression of mvtRNA and vtRNA 1-1 modulated the formation of synapses (D) (Wakatsuki and Araki, 2021; Wakatsuki et al., 2021a; Wakatsuki et al., 2021b) (image created in Biorender)

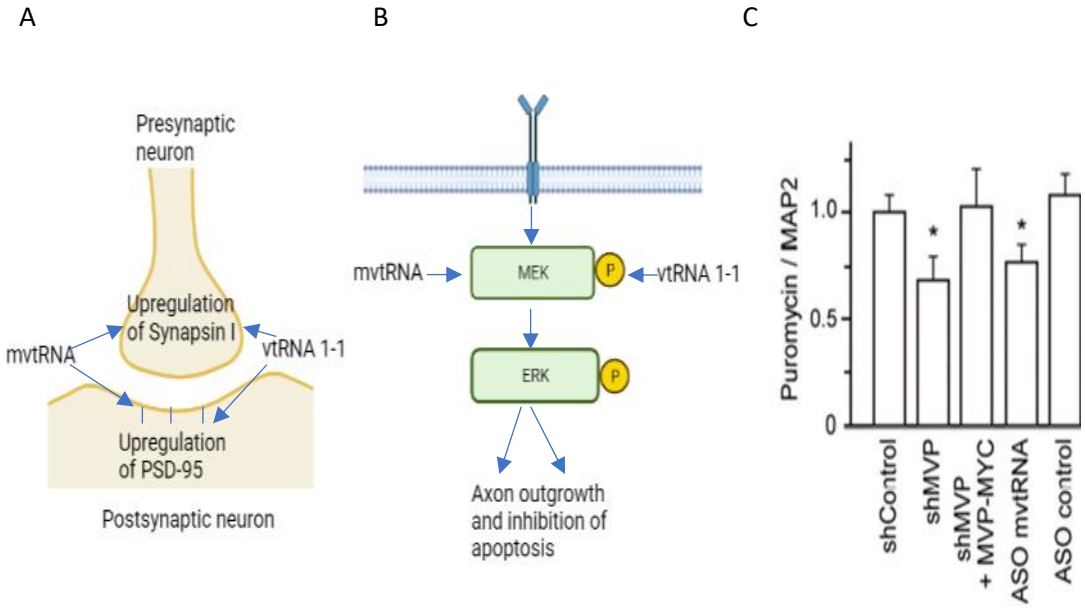


Figure 7-Mouse vtRNA and human vtRNA 1-1 regulate cell signalling cascades in synapse formation and mvtRNA is involved in axon synthesis (A) Regulation of specific sections of the MEK (mitogen activated protein kinase) ERK (extracellular signal regulated kinase) cellular signalling pathways, an extracellular signal is captured by a receptor protein which leads to the downstream activation via phosphorylation of MEK and ERK. Both vtRNA 1-1 and mouse vtRNA act to upregulate MEK and ERK signalling which lead to the upregulation of the synaptic proteins Synapsin I and PSD-95. Schematic demonstrating where mouse vtRNA and vtRNA 1-1 act pre and post synaptically to upregulate Synapsin I and PSD-95 respectively (B). Data taken from (Wakatsuki *et al.*, 2021b) (C), short hairpin directed against major vault protein (shMVP) and antisense oligonucleotides directed against mvtRNA (ASO mvtRNA) reduced the levels of puromycin (labels newly synthesised tRNA) in developing axons relative to the dendritic marker microtubule-associated protein 2 (MAP2) (*P=<0.05) (images created in BioRender) together with data taken from (Wakatsuki et al., 2021b)

1.3.4-Regulation of cellular signalling cascades - PI3-kinase and AKT and ERK 1/2

Cellular signalling pathways regulate aspects of apoptosis and cell survival; one such pathway involves PI3-kinase signalling that leads to the activation and phosphorylation of AKT (Figure 8A). Phosphorylation and activation of AKT also occurs via the Serine/Threonine kinase mammalian target of Rapamycin (mTOR) (Figure 9A). Other specific cellular signalling pathways lead to the activation of downstream mediators which involve ERK 1/2 (Figure 10A) which also regulate aspects of apoptosis and cell survival.

PI3-kinase and AKT signalling and effects of vtRNA 1-1

HeLa cells constructed with WT, KO and complementation vtRNA 1-1 (constructed like a WT vtRNA 1-1) and to stimulate the PI3-kinase signalling cascade directly each cell line underwent a period of starvation for 20 minutes and levels of the basal levels of phosphorylated AKT and phosphorylated PTEN were monitored (Bracher *et al.*, 2020). Levels of PTEN phosphorylation (inactivated state) were greater in the vtRNA 1-1 KO line relative to vtRNA 1-1 WT (Figures 8A&B) which led to the downstream activation and phosphorylation of AKT. Regulation of AKT signalling also occurs through the serine-threonine kinase mammalian target of rapamycin (mTOR) and basal levels of phosphorylated AKT were reduced in all cell lines (Figure 9A) but less so in the vtRNA 1-1 KO line (Figure 9B). Therefore, demonstrating in vtRNA 1-1KO cells that basal levels of phosphorylated AKT expression were maintained together with increasing the levels of PTEN phosphorylation a major antagonist of PI3-kinase and AKT signalling.

PI3-kinase signalling in neurodevelopment, polarity and axon growth

As demonstrated in (Figure 8A) activation of PI3-kinase leads to the accumulation of PIP3, this process has been shown to play a role in the regulating neuronal polarisation (Ménager et al., 2004) and axon elongation (Diez et al., 2016; Ménager *et al.*, 2004) as does activation of AKT (Ménager *et al.*, 2004). PI3-kinase signalling also plays a role in regulation of neuronal morphogenesis and synaptogenesis (Waite and Eickholt, 2010).

ERK 1/2 signalling cascades

HeLa cells constructed with WT, KO and complementation vtRNA 1-1 were starved for 20 minutes with chloroquine to directly stimulate ERK 1/2 signalling, the cell signalling cascade under investigation (Figure 10A) (Bracher *et al.*, 2020). The degree of phosphorylation involving ERK1/2 was monitored in each of the afore mentioned cell lines, when vtRNA 1-1 was knocked out pERK signalling was maintained (Figure 10A) and pERK signalling was reduced in vtRNA 1-1 WT and complementation vtRNA 1-1 cells lines (Figure 10B) (Bracher *et al.*, 2020).

ERK 1/2 signalling in regulation of axon growth

ERK 1/2 signalling has been shown to regulate axonal growth rates when constructed in its constitutively active form and when it's subsequent signalling has been inhibited (Dimitropoulou and Bixby, 2000; Perron and Bixby, 1999).

Taken together vtRNA 1-1 appears to regulate PI3-kinase/AKT and ERK signalling cascades, given the role that these signalling cascades have in controlling aspects of axonal elongation and polarisation we seek to investigate whether vtRNA 1-1 regulates axonal growth in a different model system and specifically in primary neuronal cells.

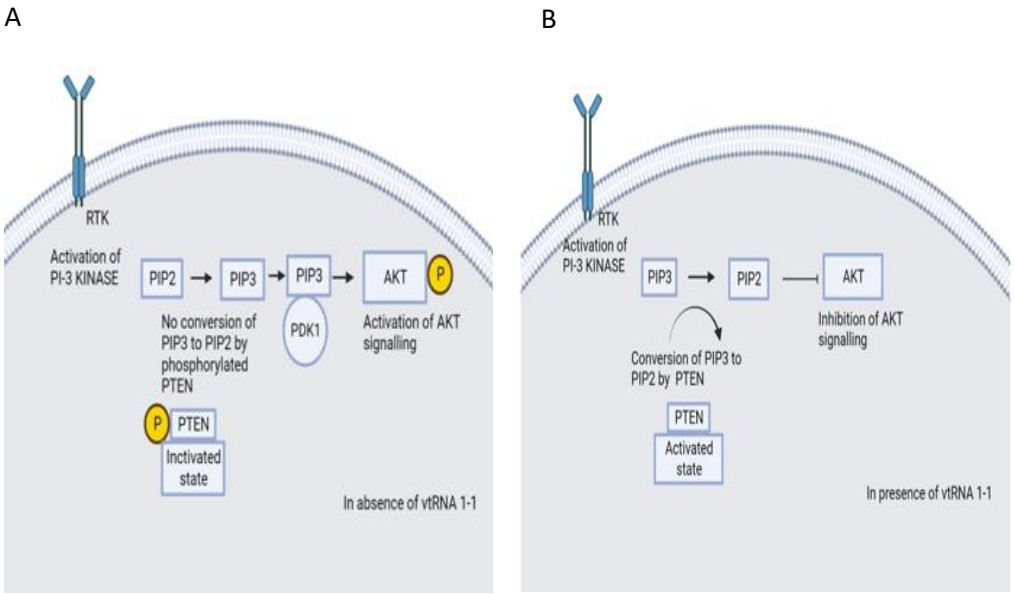


Figure 8-Regulation of PI3-kinase signalling through PTEN in the absence and presence of vtRNA 1-1 Activation of a receptor tyrosine kinase (RTK) leads to activation of PI3-kinase leading to the phosphorylation of Phosphatidylinositol 4,5-bisphosphate 2 (PIP2) to Phosphatidylinositol (3,4,5)-trisphosphate (PIP3). PIP3 mediates activation of AKT with the following kinase - Phosphoinositide-dependent protein kinase 1 (PDK1). PTEN antagonises PIP3 signalling removing a phosphate group from PIP3 and converting to PIP2. In the absence of vtRNA 1-1 PTEN's phosphorylated and inactivated state is maintained and PI3-kinase signalling triggers downstream signalling leading to the activation of AKT (A). In the presence of vtRNA 1-1, PTEN is maintained in its unphosphorylated (active state) and converts PIP3 into PIP2 thus inhibiting (PI3-kinase) signalling and reduces the activation of AKT (B). (Adapted from Bracher et al., 2020 and image created in BioRender).

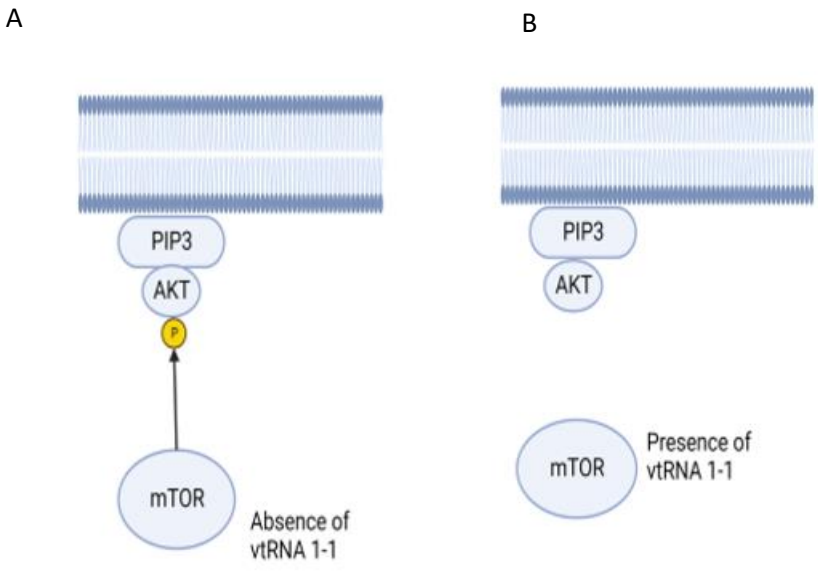


Figure 9-AKT signalling via mTOR phosphorylation in the absence and presence of vtRNA 1-1 Maintenance of phosphorylation by mTOR to activate AKT in the absence of vtRNA 1-1 (A). In the presence of vtRNA 1-1 (B) resulted in reduced activation and subsequently reduced phosphorylation by mTOR in the presence of vtRNA 1-1. (Adapted from Bracher et al., 2020 and image created in BioRender)

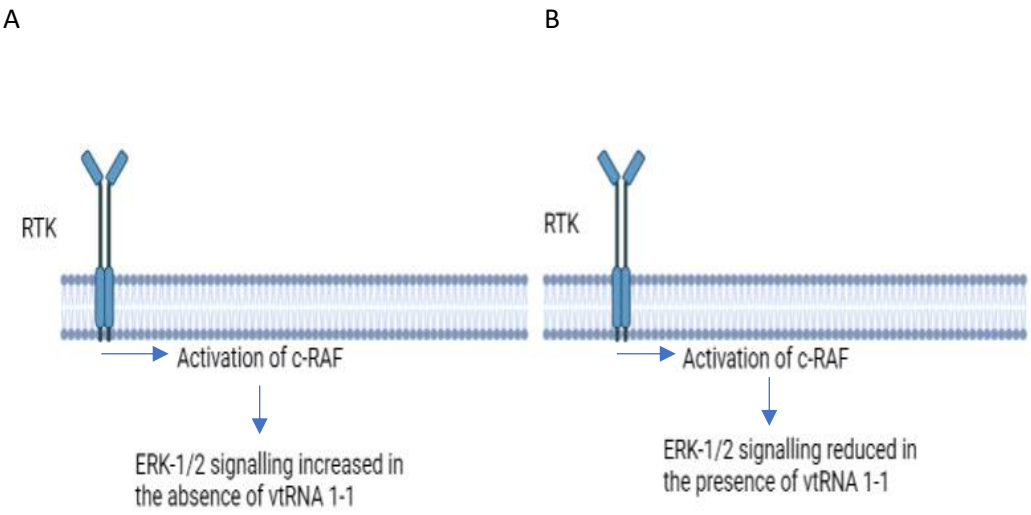


Figure 10-Subsequent effects in the presence and absence of vtRNA 1-1 on resultant ERK cell signalling cascades Activation of a receptor tyrosine kinase (RTK) leads to the activation of the serine/threonine kinase C-RAF, downstream of this activation ERK 1/2 signalling is increased. (A) In the absence of vtRNA 1-1 ERK 1/2 signalling is maintained. In the presence of vtRNA 1-1, ERK 1/2 signalling is reduced (B). (Adapted from Bracher et al., 2020 and image created in BioRender).

1.4-MALAT1 (metastasis-associated lung adenocarcinoma transcript 1)

Sequencing data from our research group (unpublished data) suggests a role for the lncRNA MALAT1 in motor neuron disease (MND), therefore we sought to investigate its function through inhibiting MALAT1 expression in primary neuronal cells and measuring subsequent axonal growth rates. The documented literature discussing the features and roles of MALAT1 in oncology, its distribution in the CNS and role in neurodevelopment are discussed below.

Features of MALAT1

MALAT1 is a lncRNA consisting of more than 8000 base pairs and like most lncRNAs it is a transcript of RNA polymerase II (Roberts et al., 2014). The human form of MALAT1 shares expressed sequence tags (ESTs) with the chimpanzee, Rhesus monkey and mouse (Ji et al., 2003) and is highly conserved (Vangoor et al., 2021; Wu et al., 2019). It is located on human chromosome 11q3 and in mice on chromosome 19qA (Wilusz, 2016; Zhang et al., 2012; Zhang *et al.*, 2017). MALAT1 is distributed in the nucleus and forms a component of nuclear speckles (Bernard *et al.*, 2010; Miyagawa et al., 2012) with a potential role in alternative splicing (Zhang *et al.*, 2017).

Role of MALAT1 in oncology

In NSCLC patients MALAT1 was first shown to be upregulated and associated with metastasis together with acting as a prognostic marker (Ji et al., 2003).

MALAT1 is upregulated in many human cancers (Hao *et al.*, 2023) which include lung, breast, liver, ovary, prostate, oesophageal and brain (Fu et al., 2020; Goyal et al., 2021; Zhao et al., 2018), one of its numerous functions in oncology comes from

acting as a regulatory hub in controlling various cascades involving different forms of cancers in humans (Fu *et al.*, 2020; Malakoti et al., 2021) together with promoting angiogenesis, metastasis along with its association with the following cellular signalling pathways ERK and PI3-kinase/AKT involved in oncology (Goyal *et al.*, 2021; Malakoti *et al.*, 2021). It has been speculated to act as a biomarker to correlate the diseases stages (Li et al., 2018) and has been considered as a therapeutic target (Amodio et al., 2018) given its likely upregulated state in human cancers.

Distribution of MALAT1 in the central nervous system

MALAT1 is shown to be largely distributed in the nucleoplasm of neurons and is highly expressed in the brains of mice (Bernard *et al.*, 2010; Chen et al., 2016; Nakagawa et al., 2012), zebrafish (Wu *et al.*, 2019) and spinal cord of rats (Meng et al., 2019) . Transcriptome analysis of primary motor neurons grown from the spinal cord demonstrated that MALAT1 was specifically expressed in the axons and dendrites together with its relative expression levels being relatively the same in each of these compartments (Briese et al., 2016) (Figure 11A). MALAT1 shows expression in the frontal and motor cortex, hippocampus, cerebellum and brainstem (Kryger et al., 2012).

Role of MALAT1 in synapse formation

KD of MALAT1 reduced the mRNA levels of Neuroligin1 (Nlgn1) and synaptic cell adhesion molecule 1 (SynCAM1) in primary neurons grown from the hippocampus (Figure 11B) (Bernard *et al.*, 2010) and overexpression of MALAT1 increased presynaptic density in the dendrites thus showing that MALAT1 acts to regulate synaptogenesis (Bernard *et al.*, 2010).

Role of MALAT1 in axon growth and neuroprotection

Inhibition of MALAT1 in N2A cells reduced axonal outgrowth and MALAT1 was thought to act through the MEK/ERK pathway to initiate axonal outgrowth and inhibit apoptosis (Chen *et al.*, 2016) (Figure 11C). Inhibition of MALAT1 resulted in reduced axonal length in primary neuronal cell cultures taken from the hippocampus (Jiang et al., 2020) (Figure 11D).

Taken together MALAT1 like vtRNA 1-1 appears to regulate specific signalling cascades that control aspects of axonal growth. Further to this MALAT1 appears to be highly expressed in the CNS and plays a role in regulating specific aspects of neurodevelopment we therefore seek to investigate how MALAT1 regulates axonal growth specifically in primary neuronal cells.

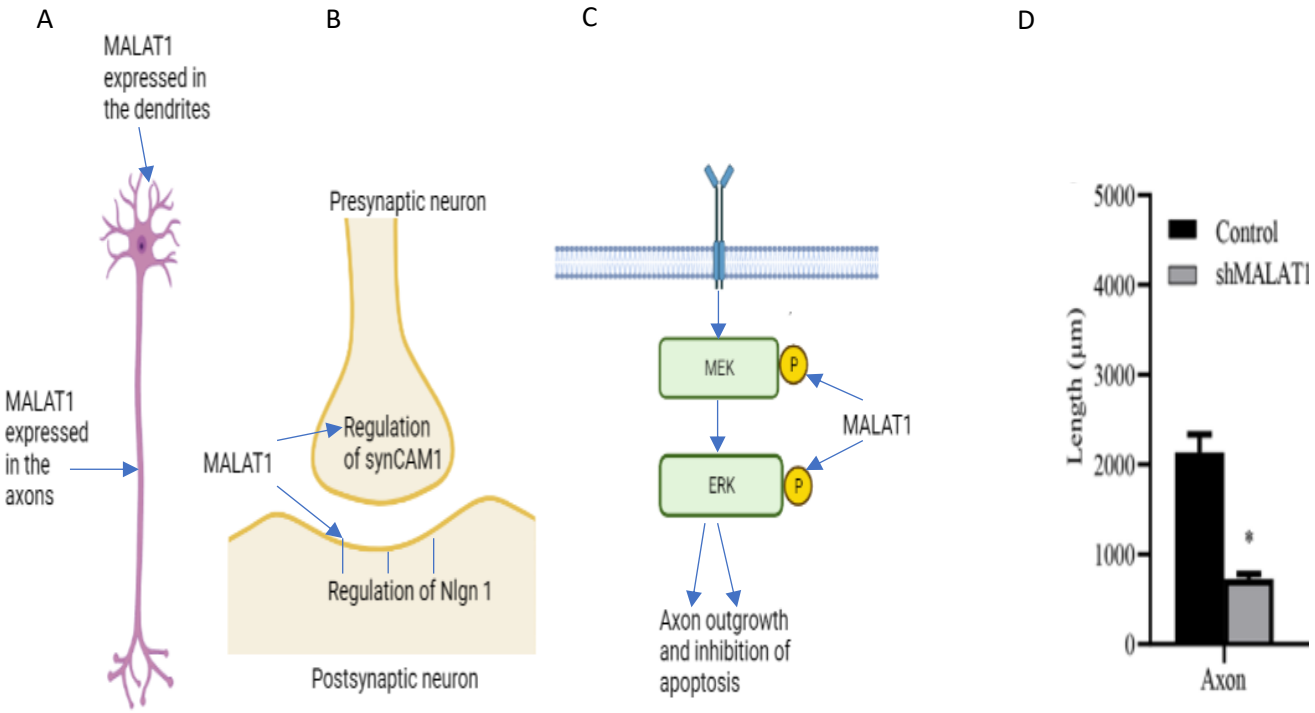


Figure 11-Expression of MALAT1 in neurons and roles of MALAT1 during specific aspects of neurodevelopment (A) MALAT1 is expressed in the dendrites and axons of neurons. Regulation of pre and post synaptic markers SynCAM1 and Nlgn1 respectively (B). Regulation of specific sections of the MEK and ERK cellular signalling pathways, an extracellular signal is captured by a receptor protein which leads to the downstream activation via phosphorylation of MEK and ERK proteins. MALAT1 acts to upregulate MEK and ERK signalling which inhibits apoptosis and promotes axonal outgrowth (C). Data taken from (Jiang et al., 2020) short hairpin directed against MALAT1 reduced axonal length (*=P<0.05) (D) (images A, B&C created in BioRender).

1.5-Aims and central questions of the thesis

The aim of this thesis is to further explore the biological functions of vtRNA 1-1, the following experimental procedures and questions will be proposed given what has been discussed about vtRNA 1-1 in regulating autophagy and neurodevelopment.

- Does overexpression of vtRNA 1-1 differentially affect levels of P62 puncta and inhibit autophagy? If so, can this be used as a positive control as demonstrated in (Horos *et al.*, 2019a) to assess whether other types of sncRNA or lncRNA differentially effect autophagy?

- Does overexpression of vtRNA 1-1 in primary neuronal cells have any effect on resultant axonal growth rates?

- Does inhibition of vtRNA 1-1 expression in primary neuronal cells have any effect on resultant axonal growth rates?

We also turn our attention to the lncRNA MALAT1, the following experimental procedure and question will be proposed given the documented role of MALAT1 in neurodevelopment.

- Does inhibition of MALAT1 expression in primary neuronal cells have any effect on resultant axonal growth rates?

Chapter 2-Materials and methods

2.1- HeLa cell transfection to overexpress vtRNA 1-1

Plasmid sequences

5SRNA (pUC57-H1-5SRNA) and vtRNA 1-1 (pUC57-H1-vtRNA 1-1) plasmids obtained were the same as those used in (Horos *et al.*, 2019a), sequences of each plasmid were obtained from the following website SnapGene | Software for everyday molecular biology and SnapGene Viewer 6.1.2 was used to obtain the sequences of each plasmid as demonstrated below.

DNA sequences of 5SRNA and vtRNA 1-1 plasmids

5SRNA

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TAGGAAAATTTAATTTTTACTTCAAAATTTAGTTAGATTTCATATATACTCATTTGAACCAGAC
TGTCAATGGTTACGAATTAGTCACTCCGTGGATAGAGTCGCTAGACAGATAAAGCAAGTAG
GTATCAACGGACTGAGGGGCAGCACATCTATTGATGCTATGCCCTCCCGAATGGTAGACCG
GGGTCACGACGTTACTATGGCGCTCTGGGTGCGAGTGGCCGAGGTCTAAATAGTCGTTATTT
GGTCGGTCGGCCTTCCCGGCTCGCGTCTTCACCAGGACGTTGAAATAGGCGGAGGTAGGTC
AGATAATTAACAACGGCCCTTCGATCTCATTCATCAAGCGGTCAATTATCAAACGCGTTGCAA
CAACGGTAACGATGTCCGTAGCACCACAGTGCGAGCAGCAAACCATACCGAAGTAAGTCGA
GGCCAAGGGTTGCTAGTTCCGCTCAATGTACTAGGGGGTACAACACGTTTTTTCGCCAATCG
AGGAAGCCAGGAGGCTAGCAACAGTCTTCATTCAACCGGCGTCACAATAGTGAGTACCAAT
ACCGTCGTGACGTATTAAGAGAATGACAGTACGGTAGGCATTCTACGAAAAGACACTGACC

ACTCATGAGTTGGTTCAGTAAGACTCTTATCACATACGCCGCTGGCTCAACGAGAACGGGCC
GCAGTTATGCCCTATTATGGCGCGGTGTATCGTCTTGAAATTTTCACGAGTAGTAACCTTTTG
CAAGAAGCCCCGCTTTTGAGAGTTCCTAGAATGGCGACAACTCTAGGTCAAGCTACATTGGG
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TTTGTCCTTCCGTTTTACGGCGTTTTTTCCCTTATTCCCGCTGTGCCTTTACAACTTATGAGTAT
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TAAACTTACATAAATCTTTTTATTTGTTTATCCCCAAGGCGCGTGTAAAGGGGCTTTTCACGG
TGGACTGCAGATTCTTTGGTAATAATAGTACTGTAATTGGATATTTTTATCCGCATAGTGCTC
CGGGAAAGCAG

5' -2921

2.1.1-Plasmid preparation and transformation

Plasmids DNA encoding vtRNA 1-1 and 5SRNA respectively underwent heat shock transformation in order to be transferred into ultracompetent XL10 e-coli cells, 100 ng of the 5SRNA and vtRNA1-1 plasmids were transferred into 50 µl of XL10 cells and left on ice for 30 minutes. Cells were then heat shocked in a waterbath at 42°C for 40 seconds and then incubated on ice for 2 minutes. 1 ml of lysogeny broth (LB) was added to the heat shocked cells and incubated for 1 hour at 37°C. Cultures were then spread onto the LB agar plate with an appropriate antibiotic (ampicillin) for selection and then incubated overnight at 37°C.

Following an overnight incubation plasmid preparation was completed according to the manufacturer's (Qiagen) recommendation.

2.1.2-HeLa cell culturing and transfection to overexpress vtRNA 1-1 and 5SRNA

HeLa cell passaging

HeLa cells were washed with 3 ml of phosphate buffer solution (PBS) in a T75 flask and 2 ml of trypsin/EDTA (Sigma) was added the flask. The trypsinisation procedure was completed when cells were incubated at 37°C supplemented with 5 % CO2 for 5 minutes. HeLa cells were supplemented with DMEM media consisting of 10 % FBS and 100 µg/ml streptomycin.

Transfection procedure

Hela cells were seeded at a density of 50,000 cells/well and six separate reactions consisting of 100 µl of Opti-MEM/well (Gibco reduced serum media) and 3 µl Trans LT-1/well (Mirus Bio) were prepared, HeLa cells were transfected with vtRNA 1-1 plasmids (1, 2 and 3 µg) and 5SRNA plasmids (1, 2 and 3 µg) respectively into a 12 well plate and transfection occurred over for a period of 48 hours in a 37°C incubator supplemented with 5 % CO2.

2.1.3-Immunofluorescence to quantify P62 puncta

Transfected HeLa cells were fixed with 4 % paraformaldehyde (Thermofisher) for 15 minutes and washed for 5 minutes with PBS, cells were permeabilised with the following blocking buffer consisting of 0.2 % Triton (Fisher Bioreagents) and 0.5 % bovine serum albumen (BSA) (Sigma) in PBS for 1 hour. Mouse primary antibody p62 (3/P62 LCK LIGAND cat no 610833) (BD Biosciences) (1:600) was made up in the afore mentioned blocking buffer and left to incubate overnight. Following overnight incubation of the primary antibody the cells were washed in PBS for 5 minutes and the secondary antibody Alexa Fluor 488 (1:1000 Thermofisher) was added and left to incubate for 1 hour in the blocking buffer solution. Cells were then further washed for 5 minutes in PBS and 200 µl 4',6-diamidino-2-phenylindole (Dapi) (Roche Diagnostics) was added to each coverslip to visualise the nuclei. Coverslips were then mounted on glass slides left overnight and viewed using the following wide-field fluorescent microscope - (Axiovert 200M, Zeiss coupled with a CCD camera). Image analysis was performed using ImageJ https://imagej.nih.gov/ij/.

2.1.4-Manual count to quantify P62 puncta

All cells present within an individual Field of View (FOV) with puncta present were manually counted with the multipoint function in imageJ. The total number of puncta manually counted was divided by the proportion of Dapi positive cells to obtain an average number of cells across each of the experimental conditions, number of Dapi positive cells (5SRNA=494) and (vtRNA 1-1=355).

2.2-Primary mouse cortical cultures and vtRNA 1-1 overexpression

2.2.1-Culturing conditions

C57/BL6 whole mouse embryonic brains were removed and separated from the meninges at embryonic day 16.5 (E16.5) through the use of a dissection microscope as described in (Lucci *et al.*, 2020; Mesquita-Ribeiro *et al.*, 2021). Primary neuronal cell cultures were seeded at the following density ($1.75x10^5$ cells $/cm^2$) in 6-well plates as described in (Lucci *et al.*, 2020; Mesquita-Ribeiro *et al.*, 2021). Neuronal transfections were performed 24 hours after plating using 250 µl/well of opti-MEM reduced-serum media (Thermofisher Scientific) and 5 µl/well of Lipofectamine (Thermofisher Scientific) according to the manufacturers instruction, vtRNA 1-1 was overexpressed with 1 µg vtRNA 1-1 relative to 1 µg 5SRNA, both vtRNA 1-1 and 5SRNA plasmids were co-transfected with 1 µg pmaxFP-Green-C (Lonza) hereafter referred to as GFP in order to visualise the axons according to the manufacturers instruction.

2.2.2-Immunofluorescence to visualise axons

Following a 48-hour period of transfection primary neuronal cells were washed with 1x PBS and fixed with 4 % Paraformaldehyde (Thermofisher) for 30 minutes. Cells were washed further with 1x PBS and coverslips were mounted with Vectashield Hardset Mounting Media. (Axiovert 200M, Zeiss coupled with a CCD camera). Image analysis was performed using ImageJ https://imagej.nih.gov/ij/.

2.2.3-Determining axon length in vtRNA 1-1 overexpression experiments

(Figures 12A&B) demonstrate how the length of an axon was determined using ImageJ using the freehand line function. In order to quantify axon length, axons were defined as a neurite which was three times longer than any other neurite and measured from the distal portion of the cell body to most distal portion of the axon (Lucci *et al.*, 2020). In each respective field of view three axons were measured, to allow for randomisation individual images were randomly selected for the afore mentioned analysis.

A B

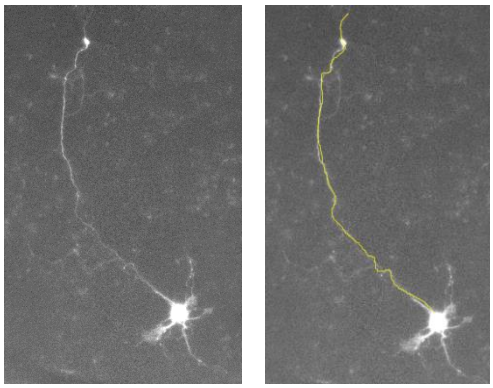


Figure 12-Image of neuron (A) and quantification of axon length using the freehand function as shown in (B) using ImageJ

2.3-Primary mouse cortical cultures and loss of function in vtRNA 1-1 and MALAT1 experiments

2.3.1-Culturing conditions

C57/BL6 whole mouse embryonic brains were removed and separated from the meninges at embryonic day 16.5 through the use of a dissection microscope as described in (Lucci *et al.*, 2020; Mesquita-Ribeiro *et al.*, 2021). Primary neuronal cell cultures were seeded at a density of $3.5x10^5$ /well in a 12 well plate (Lucci *et al.*, 2020; Mesquita-Ribeiro *et al.*, 2021). Primary neuronal cell cultures were transfected with the following antisense oligonucleotides locked nucleic acids (LNAs) each at a concentration of 100 nM - Gapmer control, vt1-1 (1) sequence 5'-TTAAAGAACTGTCGAA-3', vt 1-1 (3) -5'TTAAAGAACTGTCGA-3' and MALAT1 5'-CGTAACTAGGTTA-3' according to the manufacturers protocol (Qiagen). Two wells were left untreated and acted as the control sample(s)

2.3.2-Immunofluorescence to visualise axons

Following a 72-hour period of transfection, primary neuronal cells were washed with 1x PBS and fixed with 4 % Paraformaldehyde (Thermofisher) for 30 minutes. Cells were washed for a further 5 minutes with (PBS/10 µM Glycine; Sigma) and incubated for 30 minutes in PBS/Glycine/Triton (1x PBS, 10 mM glycine, 0.2 % Triton X-100; Sigma). Cells were then washed for a further 5 minutes in PBS/Triton (0.1 %) and then incubated in 3 % BSA (BSA; Sigma) in PBS for 30 minutes. The primary antibody anti-acetylated tubulin (1:300, C6-11B-1, Cat no. T7451; Sigma-Aldrich) was mixed with 3 % BSA was added to the cells and left for an overnight incubation. Following incubation of the primary antibody, cells were washed in PBS/Triton. The secondary antibody Alexa Flour 488 (Molecular Probes 1:300) and Dapi stain (Vertorlabs 1:300) were mixed with 3 % BSA added to the cells and incubated for 2 hours and further

washed for 5 minutes in PBS/Triton (0.1 %). Coverslips were mounted with Vectashield Hardset Mounting Media. (Axiovert 200M, Zeiss coupled with a CCD camera). Image analysis was performed using ImageJ https://imagej.nih.gov/ij/.

2.3.3-Determining axon length in vtRNA 1-1 and MALAT1 inhibition experiments

Methodology used to determine axon length was the same as that discussed in section 2.2.3.

2.4-Statistical analysis

All statistical analysis was undertaken in GraphPad Prism 9 and the number of independent experiments carried out was given as an n number. In order to obtain the best statistical power 5 independent experiments were carried out, data was presented as mean +/- standard error of the mean (SEM). Tests to establish whether the data was normally distributed and a specific statistical test based on this was selected accordingly. When comparing two separate experimental groups an unpaired t-test was used and when comparing more than two separate experimental groups an analysis of variance (ANOVA) approach was utilised.

2.4.1-Experiments involving vtRNA 1-1 overexpression on P62 puncta

Tests for normality were undertaken using Prism and the appropriate statistical test was selected on this basis. Values of $P<0.05$ were considered statistically significant and experimental data was presented as mean +/- the standard error of the mean.

2.4.2-Overexpression of vtRNA 1-1 experiments in primary neuronal cells

The accumulated data involving axon lengths were gathered from 5 independent experiments (n=5) involving GFP and from transfection with 1 µg of 5SRNA and 1 µg vtRNA 1-1 plasmids, the total number of axons measured from each experimental condition were as follows - (GFP=169, 5SRNA=192 and vtRNA 1-1=177). 5SRNA and

vtRNA 1-1 axon lengths were normalised to the mean axon length involving the GFP plasmid only. In each independent experiment (n=5) axon lengths were quantified across each of the experimental conditions and were expressed as a percentage of the mean length of axons from the 1 µg GFP experimental control condition (Lucci *et al.*, 2020; Mesquita-Ribeiro *et al.*, 2021).

Tests for normality of the normalised data were undertaken and then an appropriate statistical test was undertaken and p values of $P<0.05$ were considered statistically significant. Experimental data was presented as mean +/- the standard error of the mean.

2.4.3-Inhibition of vtRNA 1-1 experiments in primary neuronal cells

The accumulated data involving axon lengths were gathered from 5 independent experiments (n=5) involving the following antisense oligonucleotides vtRNA 1-1 (1) (n=5), vtRNA 1-1 (3) (n=5), Gapmer control (n=5) and control (n=5). The total number of axons measured from each experimental condition were as follows (Gapmer control = 600), (vtRNA 1-1 (1) =600), (vtRNA 1-1 (3)=540) and (control=540). In each independent experiment (n=5), axon lengths were quantified across each of the experimental conditions and were expressed as a percentage from the mean axon length of the control experimental condition (Lucci *et al.*, 2020; Mesquita-Ribeiro *et al.*, 2021).

Tests for normality of the normalised data were undertaken and then an appropriate statistical test was undertaken and p values of $P<0.05$ were considered statistically significant. Experimental data was presented as mean +/- the standard error of the mean.

2.4.4-Inhibition of MALAT1 experiments in primary neuronal cells

The accumulated data involving axon lengths were gathered from 4 independent experiments (n=4) involving the following antisense oligonucleotides MALAT1 (n=4) Gapmer control (n=4) and (control n=4). The total number of axons measured from each experimental condition were as follows, (MALAT1=480) and (Gapmer control=480) relative to (control=480). In each independent experiment (n=4), axon lengths were quantified across each of the experimental conditions and were expressed as a percentage from the mean axon length of the control experimental condition (Lucci *et al.*, 2020; Mesquita-Ribeiro *et al.*, 2021).

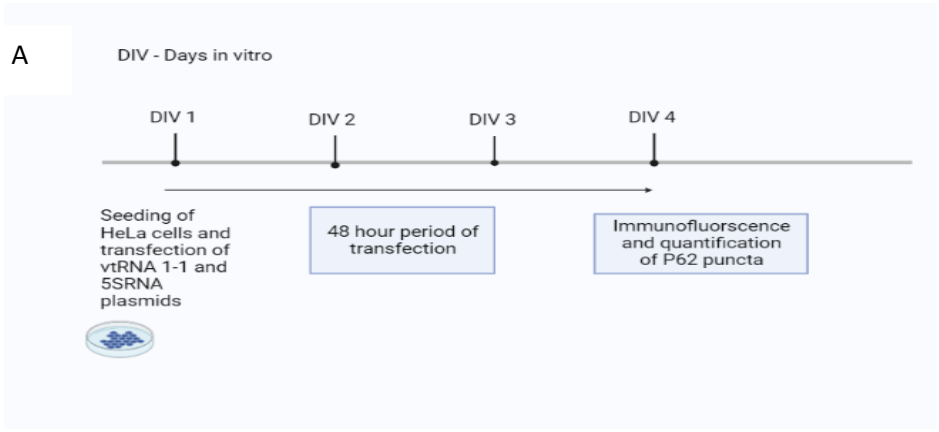
Tests for normality of the normalised data were undertaken and then an appropriate statistical test was undertaken and p values of $P<0.05$ were considered statistically significant. Experimental data was presented as mean +/- the standard error of the mean.

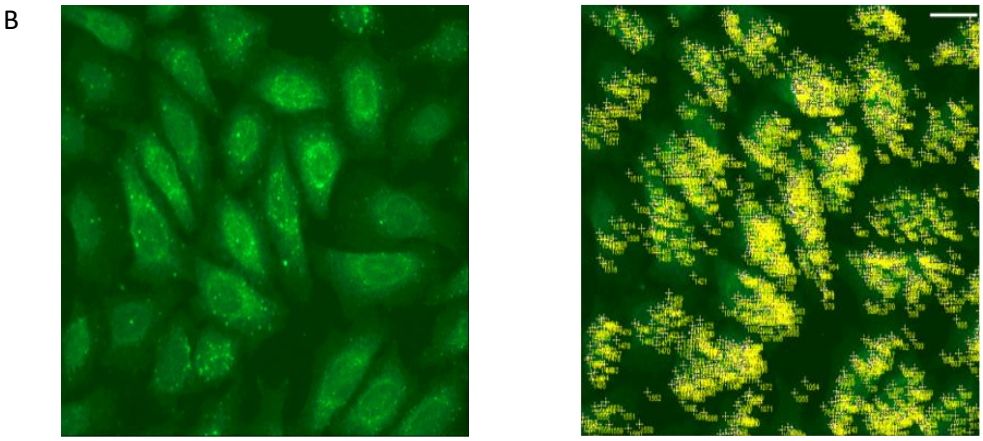
3.0-Results

3.1-Overexpression of vtRNA 1-1 does not increase P62 puncta in HeLa cells

Manual count to quantify P62 puncta

(Horos *et al.*, 2019a) previously demonstrated that vtRNA 1-1 overexpression resulated in increased levels of P62 protein and knock down of vtRNA 1-1 reduced the levels of P62 protein thus demonstrating that overexpression of vtRNA 1-1 had an inhibitory effect on autophagic flux. For the afore mentioned reasons we devised an assay to assess whether overexpression of vtRNA 1-1 had any effect upon autophagic flux through quantifying the levels of P62 puncta, HeLa cells were seeded and transfected with the following plasmids encoding vtRNA 1-1 (1 µg, 2 µg and 3 µg) and 5SRNA (1 µg, 2 µg and 3 µg) (Figure 13A). A manual count of P62 puncta (Figure 13B) did not reveal any significant increase in the levels of P62 puncta (Figure 13C) as was previously reported (Horos *et al.*, 2019a) with vtRNA 1-1 overexpression. However previous studies have demonstrated that inhibition of vtRNA 1-1 in HeLa cells did not demonstrate any significant effect on P62 protein levels (Bracher *et al.*, 2020). It is thus possible to speculate that as a consequence of the cell line the reported effects of vtRNA 1-1 on P62 levels (Horos *et al.*, 2019a) might not be possible to replicate in HeLa cells. Overexpression of vtRNA 1-1 possibly affects several other specific pathways other than autophagy (Bracher *et al.*, 2020) and previous studies to investigate the effects of vtRNA 1-1 and autophagy involved HuH cells (Büscher *et al.*, 2022; Gallo *et al.*, 2022; Horos *et al.*, 2019a). Therefore to further investigate the effects of vtRNA 1-1 expression on autophagy the cell line used needs to be carefully considered.





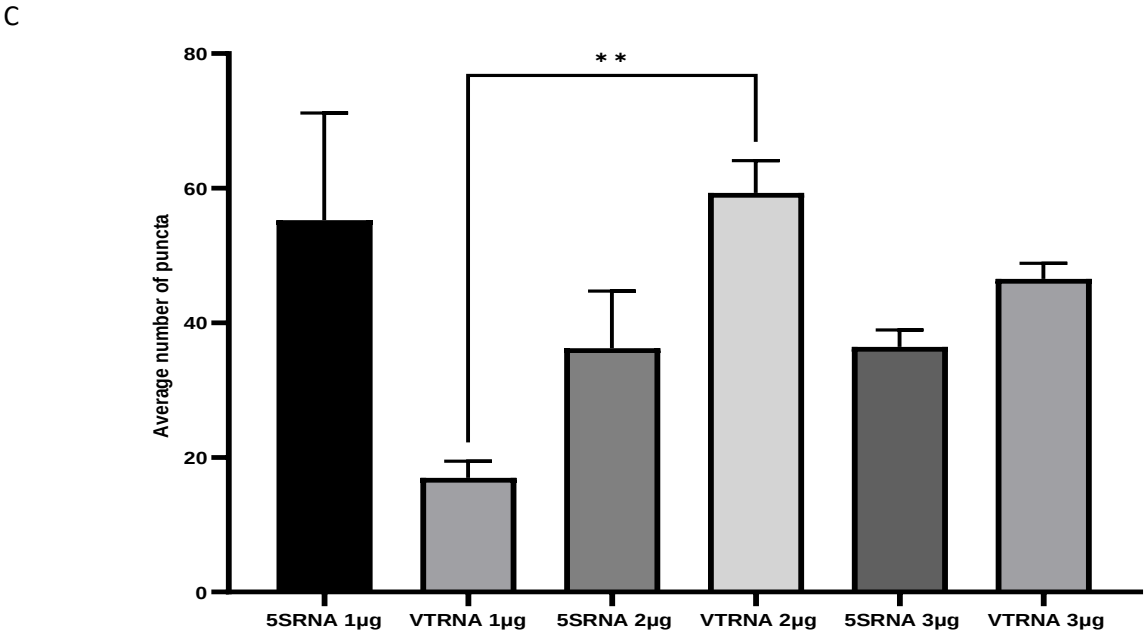


Figure 13-Manual count of HeLa cells demonstrating that vtRNA 1-1 overexpression has no effect on the levels of P62 puncta (A)- Experimental protocol followed to seed, transfect and undertake immunofluorescence to quantify P62 puncta in vtRNA 1-1 and 5SRNA transfected HeLa cells (image created in BioRender). Schematic images showing how P62 puncta was quantified in transfected HeLa cells with the multitool function of ImageJ used to manually count the P62 puncta present (Scale bar =20 µm) (B). Manual quantification of P62 puncta is displayed in (C) with the bar graph presented as mean +/- the standard error of the mean. We employ a Kruskal-Wallis Dunn's multiple comparisons test to manually quantify P62 puncta across each of the experimental conditions (**P<0.028). Number of Dapi positive cells (5SRNA=494) and (vtRNA 1-1=355).

Two unsucessful automated attempts were made to quantify P62 puncta in transfected HeLa cells which are discussed in the appendix section of this thesis

In order to further asses the biological functions of the vtRNA 1-1 transfection experiments to overexpress vtRNA 1-1 were undertaken in primary neuronal cell cultures.

3.2-Overexpression of vtRNA 1-1 does not affect axonal growth

Primary neuronal cells are grown from the brains of embryonic mice at embryonic day 16.5 (E16.5) (Lucci *et al.*, 2020; Mesquita-Ribeiro *et al.*, 2021), as neurons develop projections are sent from the cell body and the length of one of these specific projections known as an axon as shown in (Figures 1B and 6) can be measured (Figure 12). Axonal growth assays can be used to demonstrate whether overexpressing or inhibiting cellular mechanisms/components such as that of sncRNAs can differentially effect axonal growth rates (Lucci *et al.*, 2020; Mesquita-Ribeiro *et al.*, 2021). Examples of specific sncRNAs that have been shown to differentially affect axonal growth rates (Lucci *et al.*, 2020; Mesquita-Ribeiro *et al.*, 2021) and affect neuronal polarisation (Lucci *et al.*, 2020) are demonstrated in (Figure 1B).

Primary neuronal cells were transfected with vtRNA 1-1, 5SRNA and GFP and the protocol used to transfect and undertake image analysis is demonstrated in (Figure 14A), transfection with 1 µg of vtRNA 1-1 had no significant effect on axonal growth relative to primary neuronal cell cultures transfected with 1 µg of 5SRNA normalised to 1 µg GFP (Figure 14B).

Overexpression of vtRNA 1-1 didn't exert a significant effect on axonal growth, the relative expression levels of vtRNA 1-1 are possibly likely to be at a high level subsequently specific pathways that vtRNA 1-1 interact with are allready funtioning

and exerting their specific effect(s). As a result overexpression of vtRNA 1-1 is likely to not have any effect upon axonal growth, given this likely scenario we chose to inhibit vtRNA 1-1 expression in primary neuronal cells and subsequently assess axonal growth in order for more potential insight into the physiological functions of vtRNA 1-1.

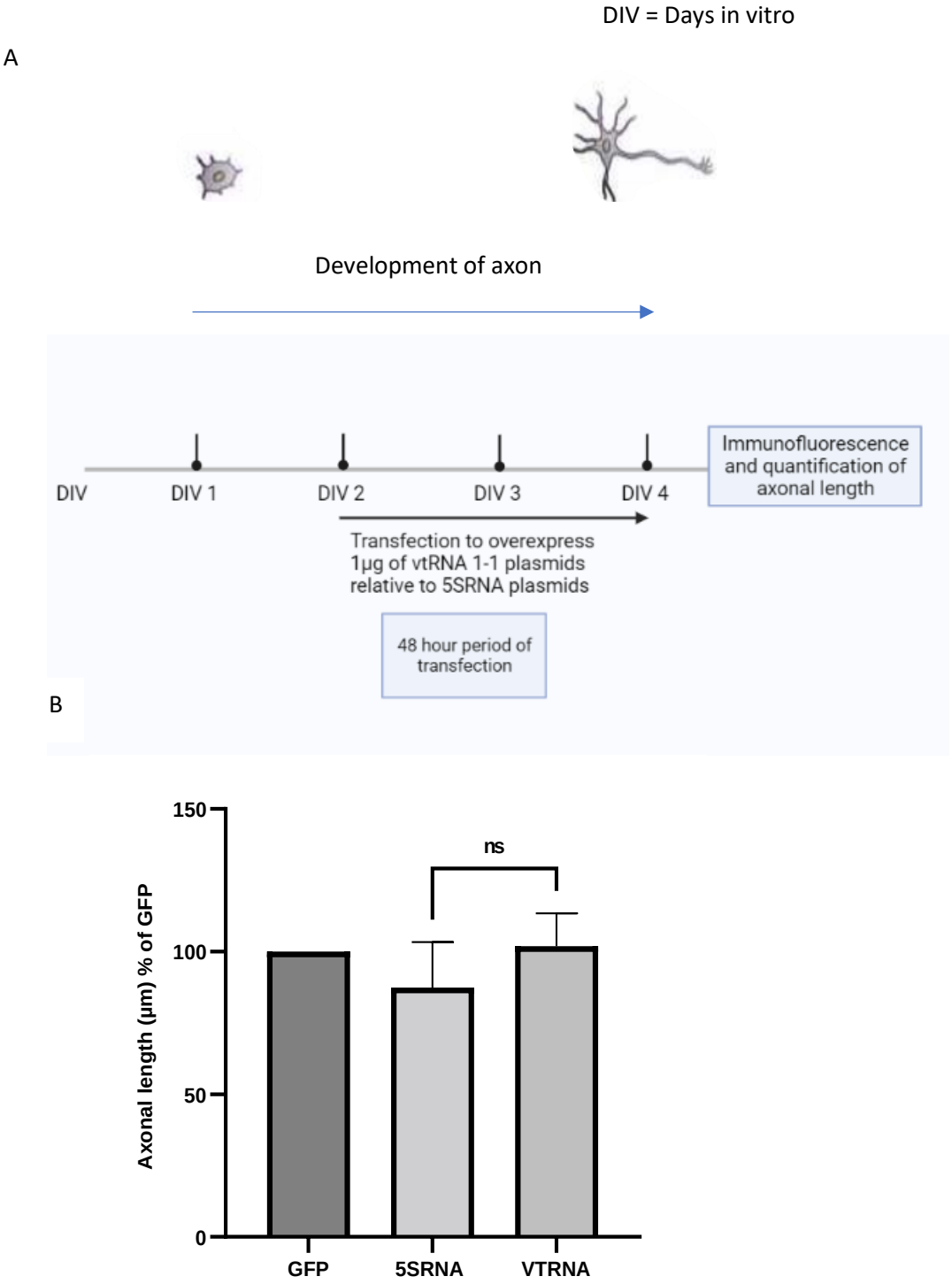


Figure 14-Overexpression of vtRNA 1-1 relative to 5SRNA in primary neuronal cells did not significantly affect axonal growth rates. (A) Experimental protocol used to seed, transfect and overexpress 1 µg vtRNA 1-1 and 5SRNA in primary neuronal cells (image created in Biorender and neuron images from (Lucci et al., 2020; Mesquita-Ribeiro et al., 2021). Quantification of the length of a single axon in primary cortical neurons following transfection with 1 µg of 5SRNA and vtRNA 1-1 and expressed as a percentage of the mean length of axons from the 1 µg GFP group. Manual quantification of axonal length is presented in (B), with the bar graph presented as mean +/- the standard error of the mean. An unpaired test did not reveal anything statistically significant between vtRNA 1-1 and 5SRNA overexpression on axonal growth rates (P>0.5048). (GFP=169, 5SRNA=192 and vtRNA 1-1=177).

3.3-Gapmer oligonucleotides to inhibit vtRNA 1-1 expression in primary neuronal cell cultures and investigate whether axonal growth is affected

Having completed investigations into whether overexpression of vtRNA 1-1 differentially effected axonal growth rates, we next sought to understand the endogenous functions of vtRNA 1-1 using Gapmers to target vtRNA 1-1. Gapmers oligonucleotides used to knock down and subsequently reduce vtRNA 1-1 expression were added to primary neuronal cell cultures and subsequent axonal growth rates were monitored.

Gapmer oligonucleotides are short stretches of DNA that are cell permeable and bind specifically to RNA targets which include lncRNA and mRNA without the requirement for any transfection reagents [3], this binding is recognised in the nucleus by RNAse H1 enzymes and cleaves the RNA targets which are then subsequently broken down by exonucleases. The small stretches of oligonucleotides are flanked on two sides by structures known as locked nucleic acids which are high affinity RNA analogues and are resistant to nuclease activity. Regions of oligonucleotides that do not consist of locked nucleic acids are known as Gapmers and these are the regions which are recognised by RNAse H1 enzymes [3].

To attempt to further understand the biological roles of the sncRNA - vtRNA1-1, Gapmer oligonucleotides were used to knock down and reduce expression of vtRNA 1-1 in primary neuronal cell cultures and axonal growth rates were monitored.

3.4-Gapmers used to inhibit vtRNA 1-1 reduce axonal growth

The protocol used to seed and transfect primary neuronal cell cultures with each of the Gapmer oligonucleotides and quantify the length of axons using immunofluorescence is shown in (Figure 15A). Gapmer oligonucleotide vtRNA 1-1 (1) has the same nucleotide sequence as vtRNA 1-1 (3) but has an additional adenine nucleotide located at the 3' of the oligonucleotide (see methodology section 2.3.1 for sequence data). Both vtRNA 1-1 Gapmers demonstrated a significantly reduced level of axonal growth following transfection into primary neuronal cells (Figures 15B&C). The Gapmer vtRNA 1-1 (1) had a statistically significant greater effect than Gapmer vtRNA 1-1 (3) in reducing axonal length relative to control neuronal cells.

Only one study appears to have investigated the effects of inhibiting a vtRNA paralogue upon axonal growth (Wakatsuki *et al.*, 2021b), inhibition of the mouse paralogue mvtRNA in mouse primary neuronal cells significantly reduced the levels of newly developing axons in mouse primary neuronal cells taken from primary neuronal cells which were grown between the following time periods (DIV 7-10) (Figure 7C) (Wakatsuki *et al.*, 2021b). What is not noted in (Wakatsuki *et al.*, 2021b) is the time period of when the antisense oligonucleotides were added to the primary neuronal culture and the duration in how long the cells were transfected so a time period may have been identified when to add oligonucleotides to inhibit axonal growth with another sncRNA together with those identified by (Lucci *et al.*, 2020; Mesquita-Ribeiro *et al.*, 2021) (Figure 1B).

The paralogue mvtRNA has some homology with that human vtRNA 1-1 regarding the conservation of RNA polymerase III promoter elements (Büscher *et al.*, 2020; Kickhoefer *et al.*, 2003; Stadler *et al.*, 2009) (Figure 2E) which may also explain why inhibition of mvtRNA reduced axonal growth rates.

To the best of our knowledge this is the first instance demonstrating that inhibition of the human paralogue vtRNA 1-1 reduced axonal growth rates through measuring the length of individual axons in a primary neuronal cell culture during a particular time period of when axonal development is at its most prominent (Banker, 2018; Dotti et al., 1988; Kim et al., 2020; Roqué et al., 2014), despite this significant advance the mechanisms that underpin this cellular process remain to be elucidated.

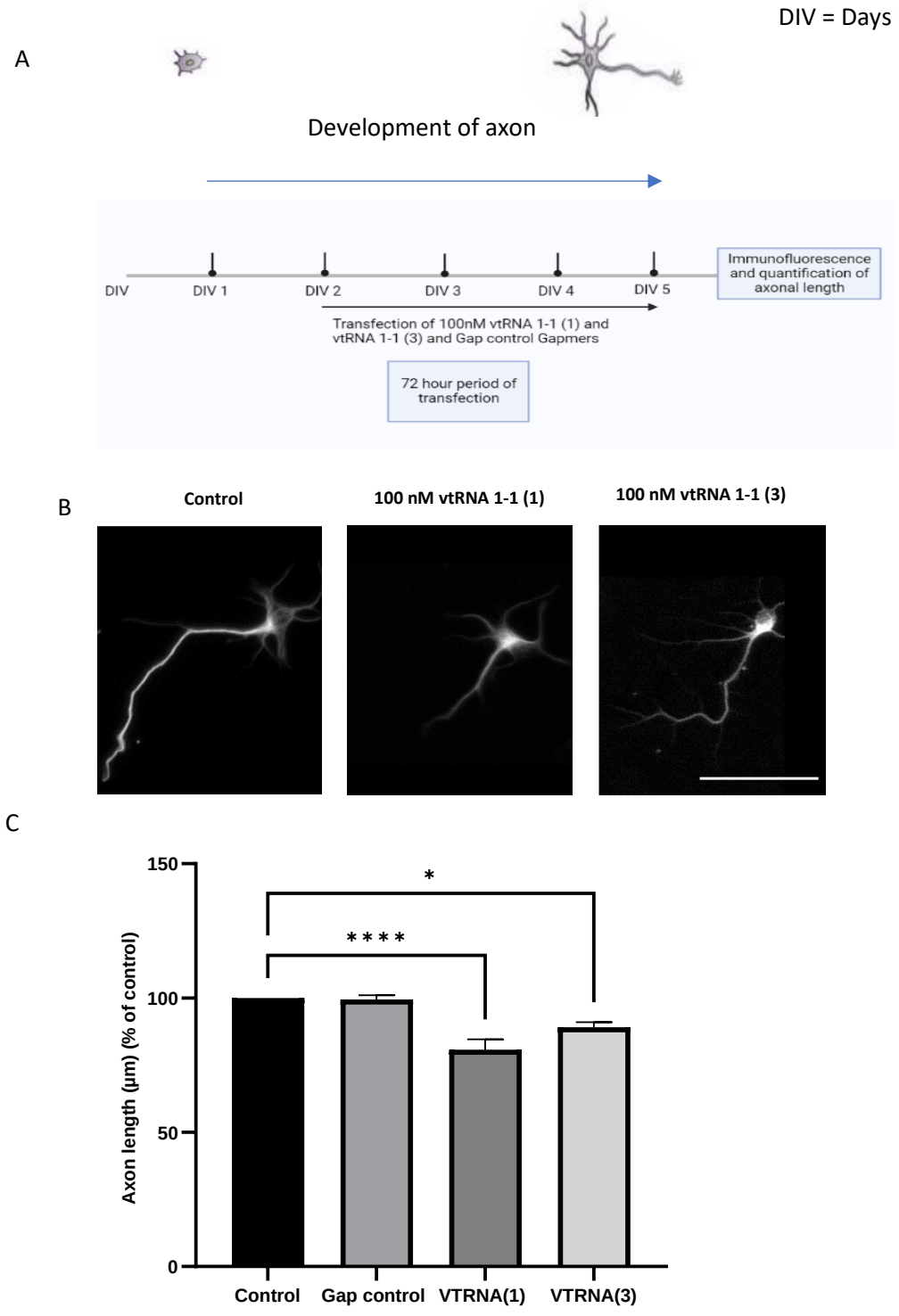


Figure 15-Inhibition of vtRNA 1-1 in primary neuronal cells significantly reduce axonal growth (A) Protocol used to seed, transfect and inhibit vtRNA 1-1 using Gapmer oligonucleotides in primary neuronal. Representative images of primary cortical neurons (DIV5) following transfection with 100 nM of vtRNA 1-1 (1) and vtRNA 1-1 (3) Gapmers relative to control (scale bar = 50 µm) (B)**.** Quantification of the length of a single axon in primary cortical neurons following transfection with 100 nM of vtRNA 1-1 (1) and vtRNA 1-1 (3) Gapmers and expressed as a percentage of the mean length of axons from the control samples. Manual quantification of axonal length is presented in (C), with the bar graph presented as mean +/- the standard error of the mean. We employ a one-way ANOVA with Dunnett's multiple comparisons test to compare axonal growth rates in primary neuronal cells treated with the following Gapmers across 5 separate experiments (n=5), (vtRNA 1-1 (1) =600), (vtRNA 1-1 (3) =540) and (Gapmer control=600) relative to (control=540). We reveal a significant alteration in vtRNA 1-1 (1) (**** = P<0.0001) and vtRNA 1-1 (3) (* = P<0.0116) treated cells versus controls.

3.5-Gapmers used to inhibit MALAT1 also reduce axonal growth

As a result of axonal growth being reduced from inhibition of the sncRNA vtRNA 1-1 and the documented roles of the lncRNA MALAT1 in regulating axonal development we turned our attention into investigating the role of MALAT1 in axonal growth through inhibiting expression in primary neuronal cells. The protocol used to seed the primary cortical neurons, transfect the cells with each respective Gapmer oligonucleotide 100 nM) together with the time periods of transfection and when immunofluorescence and quantification of axonal length were carried out are demonstrated in (Figure 16A).

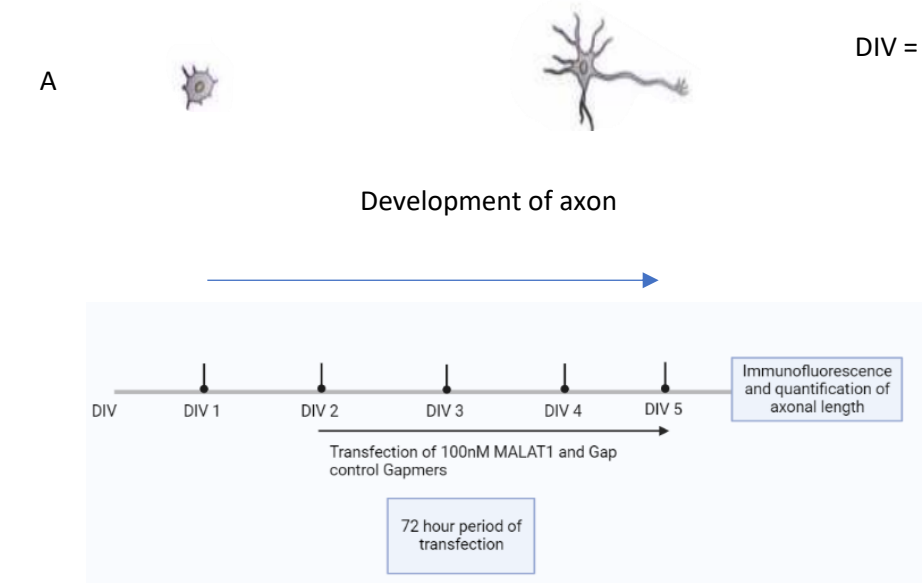
The Gapmer MALAT1 oligonucleotide significantly reduced axonal growth rates (Figures 16 B&C) which is similar to that reported by (Jiang *et al.*, 2020). As shown in this recent study inhibition of MALAT1 expression reduced axonal growth rates in hippocampal primary neuronal cells (Jiang *et al.*, 2020) (Figure 11D) and also acted in an axis with miR-30 and Spastin to regulate axonal growth (Jiang *et al.*, 2020). Also noticeable from the (Jiang *et al.*, 2020) study was that that the primary neurons were cultured for a shorter period of time at (DIV3) and transfection to inhibit MALAT1 occurred over a period of 2 days (DIV5) therefore any effect from inhibiting a ncRNA could have occurred during known periods of axon elongation (Banker, 2018; Dotti *et al.*, 1988; Kim *et al.*, 2020; Roqué *et al.*, 2014). Relative to vtRNA 1-1, MALAT1 has more documented roles in neurodevelopment and has a high level of expression in axons (Briese *et al.*, 2016) (Figure 11A). The trophic effects of MALAT1 were seen in N2A cells as it promoted axonal outgrowth together with decreasing rates of apoptosis (Chen *et al.*, 2016) through acting on the MEK/ERK signalling pathway (Figure 11C). MALAT1 demonstrated that it has a trophic effect in Alzheimer's

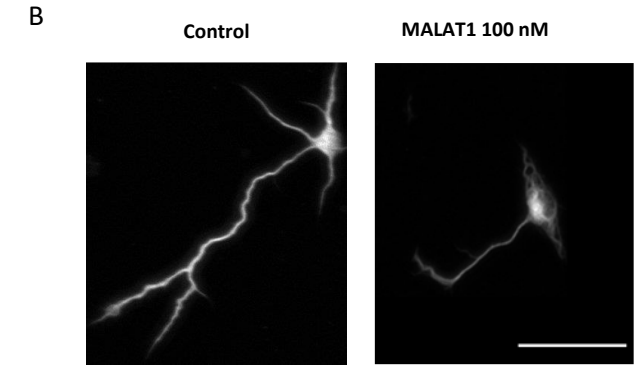
primary neuronal and cell line models as overexpression of it reduced the rate of apoptosis and increased neurite outgrowth (Ma et al., 2019).

Other trophic effects exerted by MALAT1 were shown from its overexpression in PC12 and C6 cells exposed to Amyloid β25-35, this alleviated neuronal injury together with reducing levels of apoptosis and improving cellular viability (Li et al., 2020).

Albeit in different contexts and under pathological conditions MALAT1 has also been shown to regulate the rate of apoptosis and protect against neuronal injury in primary and cell line models of Alzheimer's (Li et al., 2020; Ma et al., 2019) thus providing further evidence in how it mediates a trophic effect not just in a developmental state but also in a pathological state.

In contrast to vtRNA 1-1 more potential clues have been provided into the potential mechanisms in how MALAT1 regulates axonal growth.





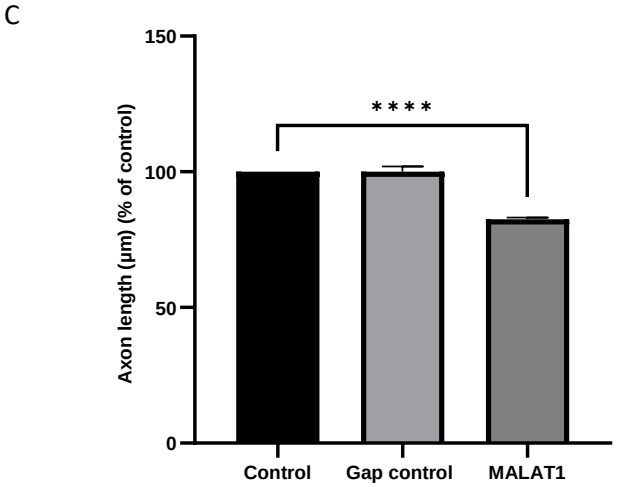


Figure 16-Inhibition of MALAT1 in primary neuronal cells significantly reduce axonal growth. (A) Protocol used to seed, transfect and inhibit MALAT1 using Gapmer oligonucleotides in primary neuronal cells images of neurons from (Lucci et al., 2020; Mesquita-Ribeiro et al., 2021) and image created in BioRender days in vitro (DIV). (B) Representative images (DIV5) to demonstrate how the length of axons were reduced in primary cortical neurons following transfection with 100 nM of MALAT1 (scale bar = 50 µm). (B) Quantification of the length of a single axon in primary cortical neurons following transfection with 100 mM of MALAT1 Gapmers and expressed as a percentage of the mean length of axons in the control samples. Manual quantification of axonal length is presented in (C), with the Bar graph presented as mean +/- the standard error of the mean. We employ a one-way ANOVA with Dunnett's multiple comparisons test to compare axonal growth rates in primary neuronal cells treated with the following Gapmers across 4 separate experiments (n=4), (MALAT1=480) and (Gapmer control=480) relative to (control=480). We reveal a significant alteration in MALAT1 Gapmer treated cells **** = ($P<0.0001$) versus control.

4-Discussion

Overexpression of vtRNA 1-1 had no effect on autophagy and axonal growth

In this thesis we set out to find more about the biological functions of vtRNA 1-1 and MALAT1, we first tried to establish whether vtRNA 1-1 overexpression in HeLa cells have the same effect in inhibition of autophagy as has been reported (Horos *et al.*, 2019a). Overexpression of vtRNA 1-1 in HeLa cells did not lead to a concomitant increase in P62 puncta, and this might be a reflection of the fact that vtRNA 1-1 does not have any effect on P62 protein levels in Hela cells as has been reported (Bracher *et al.*, 2020). We then attempted to see whether overexpression of vtRNA 1-1 in a different biological system such as in primary neuronal cells had any effect on axonal growth. As shown in the results section overexpression of vtRNA 1-1 had no effect on axonal growth and it is possible that the effects of vtRNA 1-1 might not be seen until the later stages of neurogenesis as has been specifically been shown with synaptogenesis (Wakatsuki and Araki, 2021; Wakatsuki *et al.*, 2021a; Wakatsuki *et al.*, 2021b). As mentioned previously overexpression of vtRNA 1-1 did not exert a significant effect as its expression levels were likely to already be at a high level and further overexpression of vtRNA 1-1 is not likely to have exerted a significant effect, therefore in order to fully assess the physiological effects of vtRNA 1-1 its expression was reduced and knocked down.

Inhibition of vtRNA 1-1 inhibited axonal growth

To the best of our knowledge only one study has investigated the effects of inhibiting a vtRNA paralogue on newly developing axons, inhibition of mvtRNA reduced the

development of axons (Wakatsuki *et al.*, 2021b) (Figure 7C) but not axonal length was not used as a quantitative measure as was the case in this study. Further still the rate of axon development (Wakatsuki *et al.*, 2021b) and the addition of an oligonucleotide to reduce mvtRNA expression did not appear to be assessed during periods of known axon elongation (Banker, 2018; Dotti *et al.*, 1988; Kim *et al.*, 2020; Roqué *et al.*, 2014) (Figure 19). The results obtained by (Wakatsuki *et al.*, 2021b) (Figure 7C) compared the rate of axon development relative to that of the dendritic marker microtubule-associated protein 2 (MAP2) and given that the cells were cultured for a longer time period more maturation of dendrites may have occurred (Figure 17).

Homology is shared with vtRNA 1-1 and mvtRNA (Kickhoefer *et al.*, 2003; Stadler *et al.*, 2009) (Figure1E) that may go somehow into explaining how vtRNA 1-1 exerts an effect on axonal growth.

<u>Possible mechanisms for how inhibition of vtRNA 1-1 reduced axonal growth</u>

An interaction between mvtRNA and MVP was shown to be mediated by the kinesin superfamily protein (KIF5) (Wakatsuki and Araki, 2021; Wakatsuki *et al.*, 2021b). Silencing MVP also led to reduced levels of mvtRNA in the axons but not in the cell bodies, thus demonstrating how relative levels of MVP differentially effect levels of mvtRNA in specific compartments of neurons and may aid in the transport of mvtRNA in axons (Wakatsuki and Araki, 2021; Wakatsuki *et al.*, 2021b). What could therefore be affecting the rate of axonal growth is the potential interaction between the vault particle and that of vtRNA 1-1. As an independent entity the vault particle was shown to be highly expressed in the microtubules of the growth cones of developing axons and associated with the following axonal related proteins Tau and

tubulin together with that of the dendritic associated protein (MAP2) (Paspalas et al., 2009).

The association of vtRNA paralogues with vault particles has been demonstrated (Kickhoefer *et al.*, 1998; van Zon *et al.*, 2001). High speed fractionation used to purify vault particles (Kedersha and Rome, 1986; Kickhoefer *et al.*, 1998; van Zon *et al.*, 2001) demonstrated the association of the paralogue vtRNA 1-1 with the vault particle (Kickhoefer *et al.*, 2002; Lee et al., 2011) whereas vtRNA 1-2 and 1-3 do not (Kickhoefer *et al.*, 2002) as does the other paralogue vtRNA 2-1 (Kickhoefer *et al.*, 1998; Lee *et al.*, 2011) demonstrate that only human vtRNA 1-1 associates with the vault particle in a drug resistant myeloma cell line and overexpression of vtRNA 1-1 in BL-41 cells demonstrated a high level of MVP (Amort *et al.*, 2015). Whether vtRNA 1-1 association with the vault particle is furthered or lessened during states of axonal growth remains to be elucidated.

What was not investigated during this study was the effect of overexpressing or inhibiting vtRNA 1-1 upon cellular signalling pathways involving axonal growth. PI3-kinase signalling has been shown to be regulate axonal growth, inhibition of PI3-kinase reduced the number of cells projecting long axons following treatment with NGF (Kimura et al., 1994) and inhibition of PI3-kinase reduced the number of axonal abors in retinotectal projections in embryonic zebrafish (Schmidt et al., 2014). As demonstrated in (Figures 9&10) vtRNA 1-1 does effect PI3-kinase/AKT signalling cascades but again under specific conditions and in an immortalised cell line. Given that inhibition of PI3-kinase reduced axonal growth rates (Kimura *et al.*, 1994; Schmidt *et al.*, 2014) investigation as to whether vtRNA 1-1 modulates/effects this specific signalling pathway is warranted.

Axonal growth in primary retinal neurons from chick embryos demonstrated increased levels of axonal growth when treated with constitutively active forms of ERK (Dimitropoulou and Bixby, 2000) and inhibition of ERK reduced axonal growth rates (Perron and Bixby, 1999). MEK and ERK signalling cascades were shown to be upregulated during synaptogenesis when mvtRNA and vtRNA 1-1 was overexpressed (Wakatsuki *et al.*, 2021a; Wakatsuki *et al.*, 2021b). However the effects of vtRNA 1-1 expression on MEK/ERK signalling during axonal growth require further understanding as ERK signalling cascades have been shown to be modulated by vtRNA 1-1 (Figure 10) (Bracher *et al.*, 2020) albeit under specific conditions and in a specific cell line and not in a primary neuronal cell culture. In primary neuronal cell cultures overexpression of vtRNA 1-1 and mvtRNA has only been shown to upregulate synaptogenesis through increasing MEK/ERK signalling and specific synaptic proteins (Figures 7A&B) therefore substantiating the need to find out how vtRNA 1-1 and mvtRNA specifically in regulating axonal growth in primary neuronal cell cultures.

Another potential reason in how vtRNA 1-1 may regulate axonal growth is through its regulation of apoptosis particularly as knocking down vtRNA 1-1 expression reduced the resistance to apoptosis (Amort *et al.*, 2015; Bracher *et al.*, 2020) and specifically the central domain of vtRNA 1-1 regulates this (Amort *et al.*, 2015) (Figure 5). How vtRNA 1-1 regulates apoptosis during axonal growth is warranted given the importance of apoptosis in neural development and in refining neural connections (Cavallaro, 2015).

Potential mechanisms in how inhibition of MALAT1 affects axonal development

Given that inhibition of the sncRNA vtRNA 1-1 reduced axonal growth rate we turned our attention to the lncRNA MALAT1 to see whether inhibition of this reduced axonal length in primary neuronal cells. MALAT1 inhibition did have a significant effect on axonal growth (Figure 16C) and inhibition of MALAT1 has been documented as reducing axonal growth rates (Jiang *et al.*, 2020).

MALAT1 was shown to regulate axonal growth from its interaction with miR-30 and acted as a miRNA sponge as inhibition of MALAT1 increased miR-30 levels. Subsequent overexpression of miR-30 inhibitors in MALAT1 depleted primary neuronal cultures rescued the axonal growth defects showing that MALAT1 initiated axonal growth by supressing miR-30 expression (Jiang *et al.*, 2020).

There are other potential pathways in how MALAT1 regulates axonal growth which involve the microtubule severing protein spastin, miR-30 acted to reduce spastin levels which were replenished with MALAT1. In primary neuronal cells the axonal growth defects from MALAT1 inhibition were also rescued from overexpression of spastin and miR-30 inhibitors, therefore demonstrating another instance of MALAT1 acting within an axis to regulate axonal growth (Jiang *et al.*, 2020).

As demonstrated in (Figure 11C) MALAT1 also acts to upregulate MEK and ERK signalling cascades to promote neuronal outgrowth in N2A cells and reduce apoptosis, it remains to be established whether MALAT1 acts on this pathway to upregulate MEK and ERK signalling in primary neuronal cells during axonal growth MALAT1 also has a role in regulating synaptogenesis (Bernard *et al.*, 2010; Keihani et

al., 2021; Lyu et al., 2019; Rusconi et al., 2020) (Figure 11B) and MALAT1 has also been shown to be upregulated in differentiated human induced pluripotent stem cell neurons (Lin et al., 2011). There is therefore the possibility that MALAT1 exerts specific effects during all stages of neurodevelopment.

MALAT1 and its trophic role in pathological states

Some further potential clues into how MALAT1 regulates axonal growth have been provided from studies looking at how MALAT1 expression mediates a neuroprotective effect.

Vitamin B1 and B12 provided a neurotrophic effect in N2A cells subjected to oxygen glucose deprivation/reoxygenation (OGD/R) conditions and in an axis consisting of MALAT1/miR-1/BDNF/PI3-kinase/Akt the neuroprotective effects of vitamin B and B12 (Li et al., 2019) was shown to be in part mediated through MALAT1 expression, vitamin B1 and B12 increased the levels of brain derived neurotrophic factor (BDNF) which were reduced when MALAT1 expression was inhibited and rescued through miR-1 inhibition.

In another part of the afore mentioned axis the neuroprotective effect of vitamin B1 and B12 was shown to act through the PI3-kinase/AKT signalling cascade in regulating BDNF levels, BDNF levels were reduced in N2A cells subjected to oxygen glucose deprivation/reoxygenation (OGD/R) conditions and restored with vitamin B1 and B12. Inhibition of MALAT1 reduced PI3-kinase/AKT signalling which was rescued through miR-1 inhibition (Li *et al.*, 2019), thus providing evidence that MALAT1 acts through the PI3-kinase/AKT signalling cascade in mediating the neurotrophic effects of vitamin B1 and B12.

In-vivo data from rats with induced cerebral palsy demonstrated that downregulation of MALAT1 reduced the neuroprotective effects of vitamin B1 and B12 through reducing BDNF levels together with impaired performance pertaining to aspects of motor co-ordination and memory as assessed through behavioural assays (Li *et al.*, 2019). The findings from (Li *et al.*, 2019) suggest that MALAT1 doesn't act alone in mediating trophic support for neurons acting to supplement the neuroprotective effect conferred by vitamin B1 and B12 within the MALAT1/miR-1/BDNF/PI3-kinase/Akt axis.

MALAT1 overexpression in PC12 and C6 cells did alleviate neuronal injury when cells were exposed to Amyloid β25-35 together with reducing levels of apoptosis and improving cellular viability (Li et al., 2020). Like (Jiang *et al.*, 2020) who speculated that MALAT1 acted like a sponge for miR-30 also acted as a sponge for miR-30b. Levels of apoptosis increased, and neuronal injury was aggravated through expression of miR-30b, miR-30b acted in a pathway to inhibit cannabinoid receptor 1 (CNR1) which also reduced neuronal injury and apoptosis when overexpressed.

Other instances of MALAT1 acting as a potential sponge in pathophysiological states were demonstrated when it reduced the levels of miR-125b in both PC12 and primary Alzheimer's neuronal models (Ma *et al.*, 2019). Transfection of miR-125b reduced neurite outgrowth in PC12 and primary Alzheimer's neuronal models overexpressing MALAT1 (Ma *et al.*, 2019) therefore showing that miR-125b can resist the sponging effects of MALAT1.

The neuroprotective effect mediated by MALAT1 and CNR1 upon exposure to Aβ25-35 was potentially shown to mediated by the PI3-kinase/AKT signalling pathway as

inhibition of MALAT1 increased the levels of apoptosis together with inhibiting PI3-kinase/Akt signalling (Li *et al.*, 2020). Some clues have therefore been potentially provided into how MALAT1 regulates axonal growth via PI3-kinase signalling albeit in pathological states and not that during neurodevelopment.

Limitations of the study

A limitation of this study is that the expression levels of vtRNA 1-1 and MALAT1 have not been determined during each transfection procedure involving the HeLa cell line and primary neuronal cell cultures (Figures 14A, 15A and 16A), this would need to be determined with quantitative PCR (qPCR) to show when vtRNA 1-1 had been overexpressed or knocked down (as also the case with MALAT1). In order to ascertain whether overexpression of vtRNA 1-1 or knock down of vtRNA 1-1 and MALAT1 has occurred in primary neuronal cells the relative expression levels of MALAT1 and vtRNA 1-1 would first need to be determined during specific stages of primary neuronal development (Figure 17) (Lucci, 2019) and as further discussed (Banker, 2018; Dotti *et al.*, 1988; Kim *et al.*, 2020; Roqué *et al.*, 2014). The relative and temporal expression levels of vtRNA 1-1 and MALAT1 are not known during the development of primary neuronal cells and only (Bernard *et al.*, 2010) appear to demonstrate the relative and temporal expression of MALAT1 during postnatal stages of neurodevelopment, the approach taken by (Lucci *et al.*, 2020) who demonstrated the relative and temporal expression levels of miR-26a levels in primary neuronal cells needs to be utilised for vtRNA 1-1 and MALAT1. Timings of when the transfections have occurred and when to monitor the subsequent expression levels of vtRNA 1-1 and MALAT1 in primary neuronal cells need to also be considered. Transfection of vtRNA 1-1 and mvtRNA has been shown to occur in primary neuronal cells, whilst the relative levels of expression have been monitored

and determined in the context of synaptogenesis and in axon synthesis (Wakatsuki and Araki, 2021; Wakatsuki *et al.*, 2021b) the timings of when the transfections have occurred and subsequently monitored are not accounted for. Only one paper appears to describe the time periods in which MALAT1 transfection occurred in primary neuronal cells (Jiang *et al.*, 2020) and this is not mentioned in (Bernard *et al.*, 2010) in the context of synaptogenesis. A finding from (Bracher *et al.*, 2020) showed that inhibition of vtRNA 1-1 in HeLa cells had no effect on P62 levels, we demonstrated that vtRNA 1-1 overexpression in HeLa cells also had no effect on P62 levels. However we did not demonstrate the expression levels of vtRNA 1-1 when overexpressed in HeLa cells, consequently further experiments are required to fully demonstrate that vtRNA 1-1 has no effect on autophagy specifically in HeLa cells relative to those documented such as Huh cells (Horos *et al.*, 2019a).

The relative and temporal expression of vtRNA 1-1 from transfection has been shown in specific cell lines, this has been demonstrated 1-1 in Huh cells (Horos *et al.*, 2019a), BL-41 cells (Amort *et al.*, 2015) and in HEK293, A549 cells and HeLa cells through the use of CRISPR-Cas (Bracher *et al.*, 2020).

The relative and temporal expression levels of MALAT1 from transfection have also been demonstrated in specific cell lines, these have included N2A (Chen *et al.*, 2016), HT22 and COS1 cells (Jiang *et al.*, 2020), together with being shown in primary neuronal and PC12 models of Alzheimers disease (Ma *et al.*, 2019) and in PC12 and C6 cells although the temporal levels of expression of MALAT1 were not considered (Li *et al.*, 2020). Having shown that transfection to alter the expression levels of vtRNA 1-1 and MALAT1 has occured in specific cell lines further experiments are required to measure the specific temporal and relative levels of each entitity in

primary neuronal cells in order to devise a suitable protocol to assess whether a transfection has occurred.

To confirm the translational potential of the results obtained, human model systems would need to be used in future studies such as that of neurons produced from human induced pluripotent stem cells.

Future directions

Time window in which to monitor whether sncRNAs and lncRNAs exert their effect upon specific stages of neurodevelopment?

The stages in which morphological components of primary hippocampal neurons develop are shown in (Figure 17) (Lucci, 2019) and further discussed (Banker, 2018; Dotti *et al.*, 1988; Kim *et al.*, 2020; Roqué *et al.*, 2014). Axon development mainly occurs between stages 3 and 4 and this may represent a window or time frame in which to assess whether inhibition of sncRNAs or lncRNAs affect axonal growth. (Figure 17) provides a summary of the experimental findings regarding vtRNA 1-1 and MALAT1 together with those of other sncRNAs from (Lucci *et al.*, 2020; Mesquita-Ribeiro *et al.*, 2021) in regulating axonal growth vtRNA 1-1, miR-434-3p, miR-151-3p, miR-92a-3p, miR-26a and the lncRNA MALAT1.

The stages with which vtRNA 1-1, the mouse paralogue mvtRNA and MALAT1 may act to regulate synaptogenesis are also displayed at stage 5 (Figure 17) (Bernard *et al.*, 2010; Wakatsuki and Araki, 2021; Wakatsuki *et al.*, 2021a; Wakatsuki *et al.*, 2021b) potentially showing that there is further scope in which to investigate whether other ncRNAs regulate the later stages of neurodevelopment. As outlined in

(Figure 17) designated windows may exist in which to assess whether sncRNAs effect specific aspects of neurodevelopment. It may therefore be possible to utilise assays to monitor the levels of apoptosis or specific components of cellular signalling cascades as discussed in (Figure 18B) and establish whether vtRNA 1-1 and MALAT1 differentially effect these during axonal growth (stages 3 and 4) (Figure 17).

The experimental approach(es) needed to assess how vtRNA 1-1 and MALAT1 regulate axonal growth via specific cellular signalling pathways need to be carefully considered because as demonstrated in (Figures 8, 9&10) regulation of PI3-kinase/AKT and ERK 1/2 signalling cascades consist of a complex set of pathways, each constituent part of a signalling cascade has many potential other points of regulation. If vtRNA 1-1 and MALAT1 and their subsequent effects on apoptosis during axonal growth were to be investigated the type of apoptosis (intrinsic or extrinsic) under investigation needs to be carefully considered given also how PI3-kinase/AKT and ERK 1/2 signalling cascades regulate apoptosis and provide trophic support to neurons as discussed.

Further underestanding of the relative and temporal expression levels of vtRNA 1-1 and MALAT1 during the developmental stages of primary neurons (Figure 17) provides not only a method to assess the efficacy of a transfection procedure it also potentially provides an avenue in order to understanding how key stages in neuronal development are effected by apoptosis and specific cell signalling cascades together with how other ncRNAs potentially effect axonal polarity as was demonstarted with miR-26a (Lucci *et al.*, 2020).

We have been able to demonstrate that 2 ncRNAs both a sncRNA and lncRNA have a function in regulating axonal growth, in the case of the human paralogue vtRNA 1-1 and to best of our knowledge this has not been previously demonstrated. Inhibition of MALAT1 also reduced axonal growth rates replicating previous findings of others and may also provide some clues into potential mechanisms in how vtRNA 1-1 mediates axonal growth.

Whilst this study sheds some light upon the functions of vtRNA 1-1 and further insight into the functions of MALAT1 in axonal growth (Figure 18A) what is still not clear is the mechanisms through which vtRNA 1-1 and MALAT1 exert their effect(s) in regulating axonal growth in primary neuronal cell cultures (Figure 18B).

What are the relative and temporal levels of expression in vtRNA 1-1 and MALAT1 during stages 1-5?

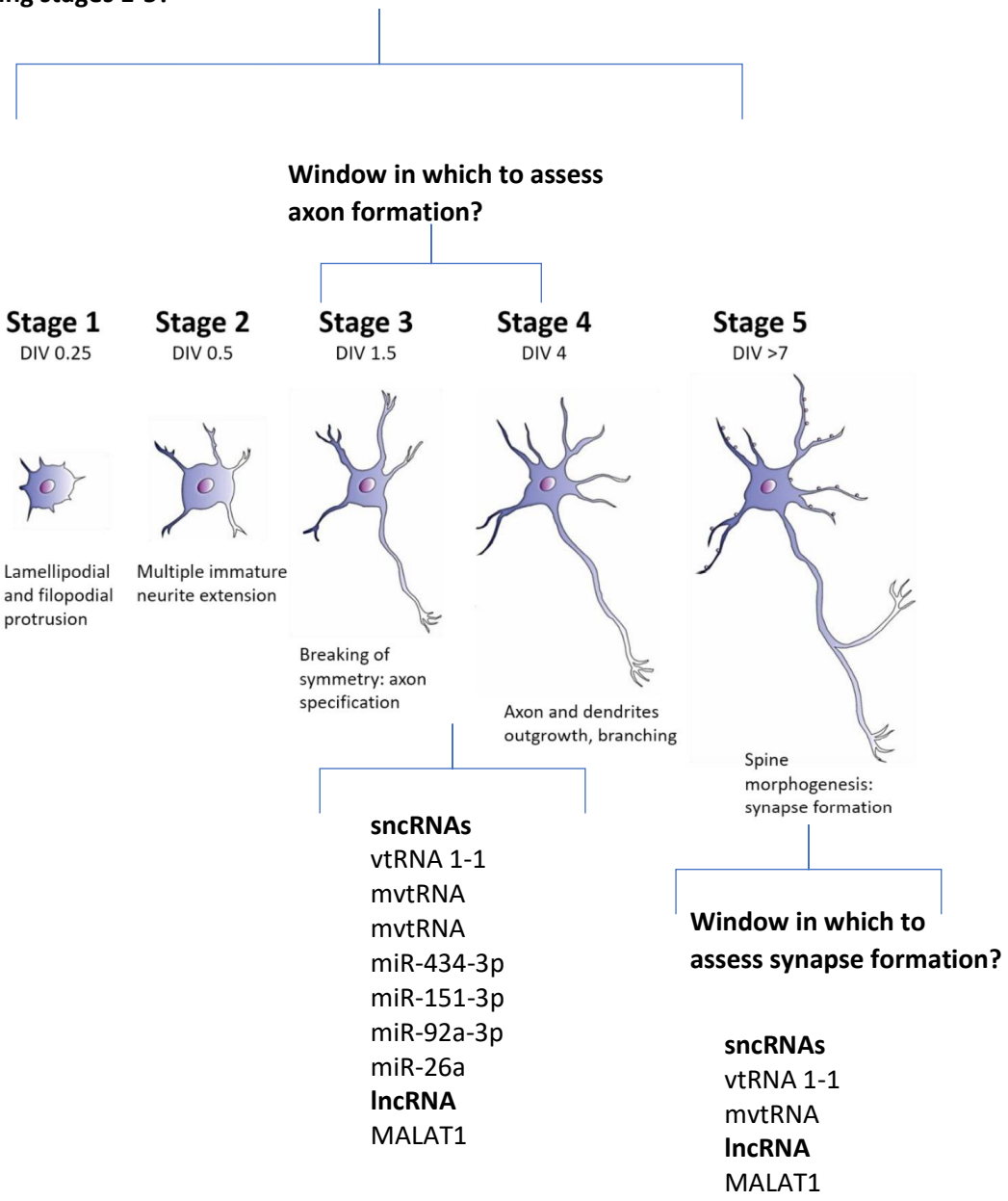


Figure 17-Stages of when sncRNAs and lncRNAs may exert their effects during specific stages of neurodevelopment in primary hippocampal neurons (Taken and adapted from Lucci, 2019)

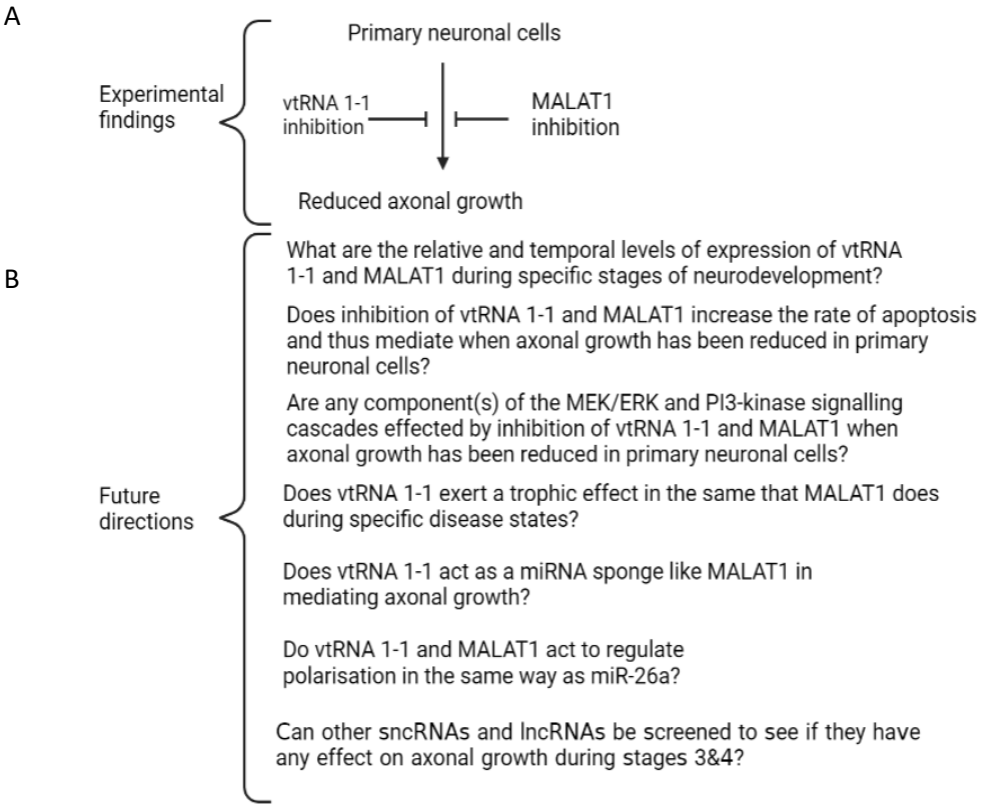


Figure 18-Summary of experimental findings and now to further investigate the role of vtRNA 1-1 and MALAT1 in axonal growth. Inhibition of vtRNA 1-1 and MALAT1 reduced axonal growth rates (A) and potential future lines of investigation to see how vtRNA 1-1 and MALAT1 specifically regulates axonal growth (B).

Websites

[1]Coding and Non-Coding RNA | Bio-Rad

[2] Non-Coding RNAs (ncRNAs) | Abcam

[3] Antisense LNA GapmeRs (qiagen.com)

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Appendix

First automated attempt to quantify P62 puncta

The find maxima function did reveal an expected increase in P62 levels with increasing concentrations of vtRNA 1-1 (Figure 1B) (Horos *et al.*, 2019a) but the resultant data was too widely spread as demonstrated in (Figure 1B) and the number of regions of interest (ROIs) analysed in each field of view was not of a significantly large number. The find maxima function used in ImageJ only considers pixels of the local maxima and a limitation of the find maxima function is that potential P62 puncta that are not as strongly expressed are missed by the find maxima function as demonstrated in (Figure 1A).

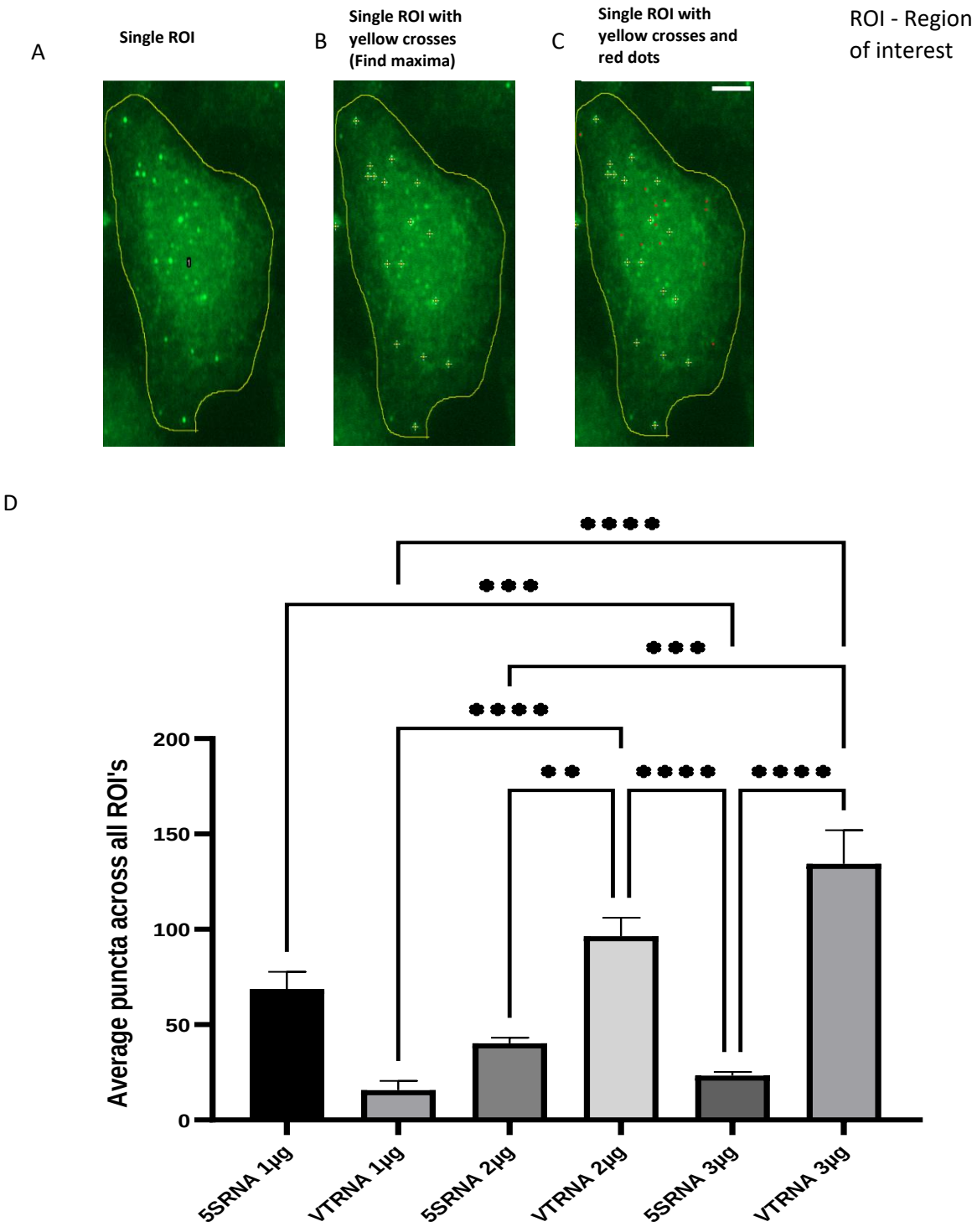

Figure 1-Find maxima function used to quantify P62 puncta in transfected HeLa cells A-Single ROI using with the freehand function in ImageJ, Single ROI with find maxima function utilised (yellow crosses indicate areas of pixels with the local maximum intensity and potential P62 puncta). Same ROI with red dots to show potential P62 missed by the find maxima function (C). (Scale bar= 6 µm). Quantification of P62 puncta in (D) with the bar graph presented as mean +/- the standard error of the mean. We employ a Kruskal-Wallis Tukey's multiple comparisons test of 20 ROIs across each experimental condition, (****p<0.0001) (***p<0.0010) (**p<0.0085).

Second automated attempt to quantify P62 puncta

A further automated attempt was made to quantify the puncta using ImageJ and Cell Profiler (Figure 2). To attempt to isolate P62 puncta the background was subtracted by 10 pixels (Figure 2A) in ImageJ. Images were then transferred to Cell Profiler and further visualisation of the P62 puncta was made in Cell Profilier using the enhance and supress function (Figure 2B). An 8-bit binary image was created in ImageJ and quantified by the particle analyser function in ImageJ (Figure2C). However background noise remained an issue and P62 puncta is likely to have been lost through using this automated pipeline and there were also problems when transfering the images from ImageJ to Cell Profilier as the sizes of the images changed as did the resultant scale bar (Figure 2B). The second automated attempt to quantify the P62 puncta did not demonstrate a dose dependent effect of vtRNA 1-1 on increasing P62 levels (Figure 2D) and manual counting of P62 puncta was therefore undertaken as shown in the main body of the thesis (Figure 13B).

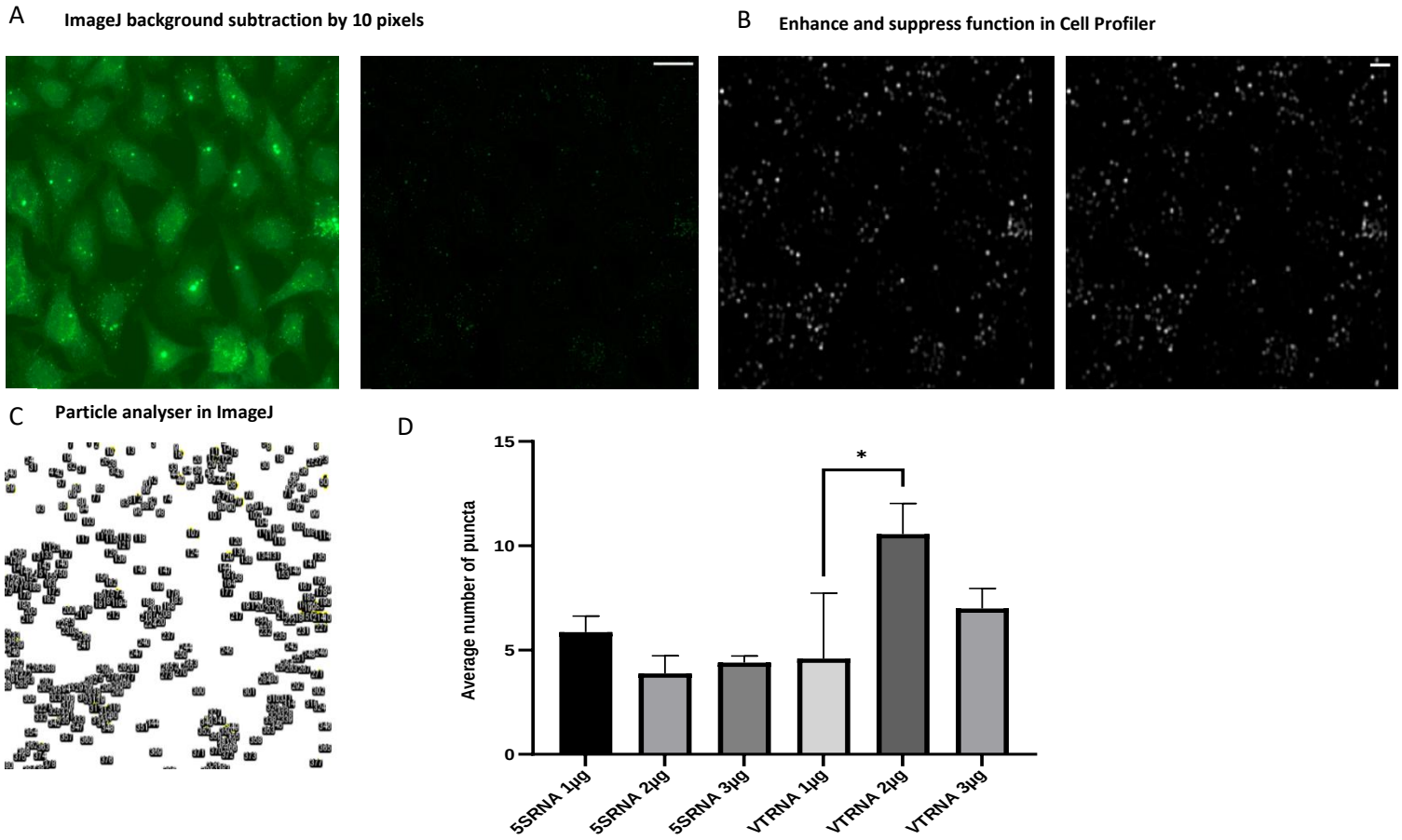

Figure 2-Second automated attempt to quantify P62 puncta -Transfected HeLa cell (A) with its background subtracted by 10 pixels using ImageJ (Scale bar in A&B=25 μm) (B) Use of the Enhance and Supress function to better visualise potential P62 puncta used in Cell Profiler, 8-bit binary image created in ImageJ and particle analyser in ImageJ to quantify P62 puncta (C). Bar graphs are presented as mean +/- the standard error of the mean (D). Kruskal-Wallis Dunn's multiple comparisons test of second automated attempt to quantify P62 puncta (*p< 0.0133). Number of Dapi positive cells 5SRNA=494 and vtRNA=355