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Faculty of Health Sciences, Department of Pharmacy

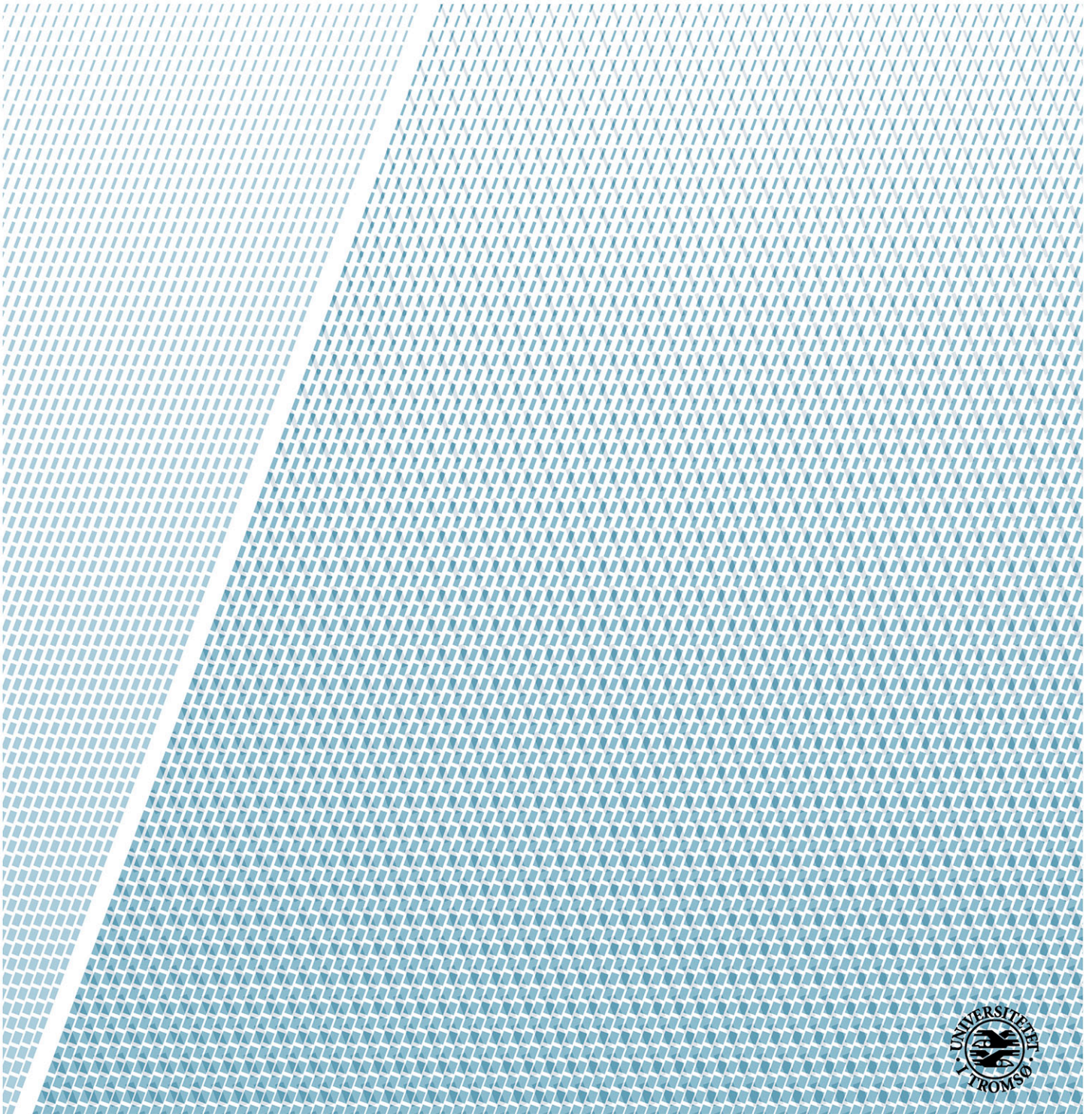
Molecular Cancer Research Group

A study of Fez1 and Fez2

Establishment of Fez1 and Fez2 knock out cells lines

Localization of Fez1 and Fez2

Jenny Thuy Tien Nguyen



Acknowledgments

This master thesis was carried out at the Molecular Cancer Research Group, Institute of Medical Biology, University of Tromsø – The artic University of Norway from August 2016 to May 2017.

It has been a period of intense learning for me, not only in the scientific area, but also on a personal level. Therefore I would like to express my deepest thanks to the people who have supported and helped me throughout this period.

I would first like to give my deepest thanks to my main supervisor associate-professor Eva Sjøttem, who has spent time to read my master thesis and has given me constructive feedback, and my lab-supervisor, Hanne Britt Brenne for always finding time to help when it was needed. They were both very generous with their time and knowledge and assisted me during the work with my thesis. Thank you both for your motivation, patience and encouragement. In addition, I would like to thank everyone who has given me some of their time and helped me during this master thesis.

Last but not least, I must express my very profound gratitude to my family and friends. Thanks all for the encouragements and supports during my studies. This accomplishment would not have been possible without all of you. And special thanks to my mom, for providing me with unfailing support and continuous encouragement throughout my years of study, I love you mom!

Jenny Thuy Tien Nguyen

Tromsø, May 2017

Abstract

Autophagy is an essential cellular process that is important to maintain homeostasis by degrading proteins, lipids and organelles during critical times like cellular or environmental stress conditions. Autophagy plays a critical role in human pathologies, since defective autophagy can lead to diseases like cancer, neurodegenerative- and inflammatory diseases. Fez1 (Fasciculation and elongation protein zeta 1) is a protein that has been reported to inhibit autophagy. In the first part of this study an attempt to establish stable knock out cell lines of Fez1, its homologue Fez2 and double Fez1-Fez2 by using CRISPR/Cas9 technology was performed. The knock out strategy was applied on two different human cell lines, Hek293 Flp-In and the neuronal cell line SH-SY5Y, and more than 200 putative clones were screened. Unfortunately, none of them displayed complete knock out of Fez1 or Fez2. This may indicate that Fez1 and Fez2 are essential for cell survival or cell growth in these cell lines.

In the second part of this study, confocal imaging was applied to study the localization of Fez1 and Fez2, and if they colocalized with proteins involved in autophagy and intracellular transport. Interestingly, upon over-expression both Fez1 and Fez2 colocalized with proteins involved in autophagosome initiation and maturation like WIPI2 and the Atg8 proteins. However, they did not colocalize with syntaxin17, which is involved in the fusion step between autophagosomes and lysosomes. Both proteins also colocalized with tubulin and the transport protein KIF5B. This may suggest a role for Fez1 and Fez2 in the transport of early autophagosomes. The colocalization with tubulin, specifically γ tubulin, was verified at the endogenous level by immunostaining.

Abbreviations and glossary

AMP	Ampicillin
APS	Ammonium persulphate
ATP	adenosine triphosphate
Atg	Autophagy-related
BSA	Bovine Serum Albumin
Cas9	CRISPR-associated protein 9
CMA	Chaperone mediated autophagy
CIP	Calf intestine phosphate
CRISPR	Clustered regularly interspaced short palindromic repeats
DSB	double stranded break
ddNTPs	dideoxynucleotide triphosphates
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
EGFP	Enhanced green fluorescent protein
ESCRT	endosomal sorting complexes required for transport
FBS	Fetal Bovine Serum
Fez	Fasciculation and elongation protein zeta
GABARAP	γ-aminobutyric type A ($GABA_A$)-receptor associated protein
HBSS	Hank's Balanced Salt solution
HDR	homology-directed repair
HRP	Horseradish peroxidase
Hsc70	Shock-cognate protein of 70kDa
HOPS	homotypic fusion and vacuole protein sorting
JIP1	c-Jun N-terminal kinase-interacting protein 1
KHC	kinesin heavy chain
LAMP-2A	lysosome-associated membrane protein type 2A
LB	Lurica-Bertani
LC3	light chain 3
LIR	Light chain interaction region
mTORC1	mechanistic Target of rapamycin kinase complex 1
NHEJ	Non-Homologous End Joining
PAS	phagophore assembly site
PAM	protospacer adjacent motif
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	paraformaldehyde
PE	Phosphatidylenolamine
PKCζ	protein kinase C-zeta
PI3P	phosphatidylinositol triphosphate

PtdIns3K	phosphatidylinositol 3-kinase
RT	Room temperature
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophore
SCOC	short coiled-coil protein
SOC	Super optimal broth with Catabolite repression medium
SNARE	Soluble N-ethylamide-sensitive factor attachment protein receptor
Stx17	Syntaxin-17
TALENs	Transcription activator effector nuclease
TBS-T	Tris buffered saline with tween 20
TEMED	Tetramethylethylenediamine
ULK	unc-51 like autophagy activating kinase
UPS	Ubiquitin-Proteasome system
UVRAG	UV irradiation resistance gene
ZFN	Zinc Finger nuclease
WIPI	WD repeats domain phosphoinositide-interacting protein 2
WT	Wild type

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Introduction

Cellular homeostasis

The ubiquitin-Proteasome system (UPS) and autophagy are two major systems that maintain cellular homeostasis, an important process for cell survival that maintains a well-controlled balance between protein synthesis and degradation (protein turnover). Disturbance of this balance may lead to accumulation of dysfunctional organelles and misfolded proteins within the cells, a hallmark for many diseases such as neurodegenerative diseases and cancer (Ariosa & Klionsky, 2016). Thus, to avoid the consequences of faulty or damaged proteins and organelles, it is important to ensure that incorrectly folded proteins or injured cellular compartments are removed, hence why protein degradation is an important process for intracellular renovation (Cooper & Hausman, 2004).

The ubiquitin-proteasome system (UPS)

This system target proteins for degradation via a selective pathway, by marking them with ubiquitins, a 76 amino acid polypeptide that is present in all eukaryotic cells. The ubiquitins attach to the amino group of a lysine residue, thereby resulting in a multiubiquitin chain. This chain is then recognized and the protein is degraded by the proteasome, a multisubunit protease complex (Cooper & Hausman, 2004).

Three distinct classes of enzymes are involved in the ubiquitination of targeted proteins. As shown in Figure 1, the ubiquitin-activating enzyme (E1) activates the carboxyl-terminal in an ATP-dependent manner by forming a thiolester bond. Activated ubiquitin is then transferred to ubiquitin-conjugated enzyme (E2). Through an additional thiolester bond, ubiquitin ligase (E3) links ubiquitin from E2 enzyme to a lysine of the target protein. Apparently there is only one common E1, but dozen of E2s and a large number of E3 enzymes. Different E3s recognize different substrate proteins, and the specificity of these enzymes provides selectivity to the process (Chen & Dou, 2010; Cooper & Hausman, 2004).

After ubiquitination, the polybiquitination protein is recognized and degraded by the proteasome. The 26S proteasome consist of several subunits, a 20S catalytic core and two 19S regulatory caps on both end of the 20S core. The 20S core proteasome contains of 28 subunits that are assembled into four rings that forms a barrel-shaped molecule. The two non-catalytic outer rings consist of α-subunits and are called α-rings, while the two inner catalytic rings consist of β-subunits and are called β-rings (Chen & Dou, 2010).

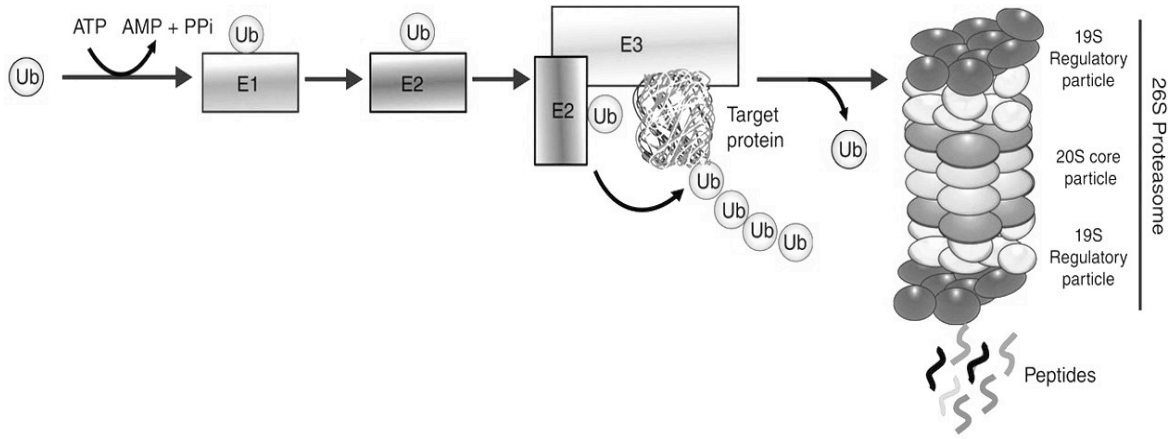


Figure 1. Mechanism of the ubiquitin-proteasome system.

Autophagy

Autophagy is an self-degenerative process involved in maintaining cellular homeostasis by forming an autophagosome, a double-membrane that can selectively or non-selectively engulfs intracellular-cargo (Glick, Barth, & Macleod, 2010). The cargo autophagosome sequester the cargo away from the cytosol and degrade it by fusion with lysosomes. The cargo can be cytosolic material like abnormal protein aggregates, and excess or damaged organelles, or intracellular pathogens (Johansen & Lamark, 2014). Autophagy is activated by nutrient starvation and promotes cell survival until nutrients becomes available again. The formation of autophagosomes is a complex process involving autophagy-related (Atg) protein (Svenning & Johansen, 2013). Autophagy can currently be divided into three main types: macroautophagy, microautophagy and chaperon-mediated autophagy (Figure 2) (Glick et al., 2010).

Microautophagy

Microautophagy is a process involving direct engulfment of sequestered portions of cytoplasmic cargo by autophagic tubes, which mediate invagination and vesicle scission of the vacuole membrane for degradation (Uttenweiler & Mayer, 2008). It can be divided into nonselective and selective. Nonselective autophagy is used for the turnover of bulk cytoplasm under starvation conditions, whereas selective autophagy targets specific organelles like mitochondria (mitophagy) for degradation (Mijaljica, Prescott, & Devenish, 2014).

Chaperone mediated autophagy (CMA)

CMA is a process where proteins containing a specific motif related to the pentapeptide KFERQ in their amino acid sequence (Kaushik & Cuervo, 2012) get removed without disturbing neighboring proteins (Cuervo & Wong, 2014). These targeted proteins are identified in the cytosol by a chaperone protein called shock-cognate protein of 70kDa (hsc70) (Wang & Mao, 2014), that binds to the target motif in the target protein and translocate it across the lysosome membrane, where it interacts with lysosome-associated membrane protein type 2A (LAMP-2A), resulting in their unfolding and degradation (Glick et al., 2010).

Macroautophagy

Macroautophagy is a process characterized by forming a double-membrane, referred to autophagosome (Glick et al., 2010), that envelopes cytoplasmic components and thereafter fuses it with lysosome or endosome for degradation (Svenning & Johansen, 2013).

Micro- and macroautophagy are both mechanisms that are able to engulf cytoplasmic cargo to degrade it, in contrast to CMA that depends on a signature sequence and interaction with chaperone proteins to import proteins into the lysosomes (Figure 2). In this study, the focus will be on macroautophagy, hereafter called autophagy, and the molecular mechanism is described below.

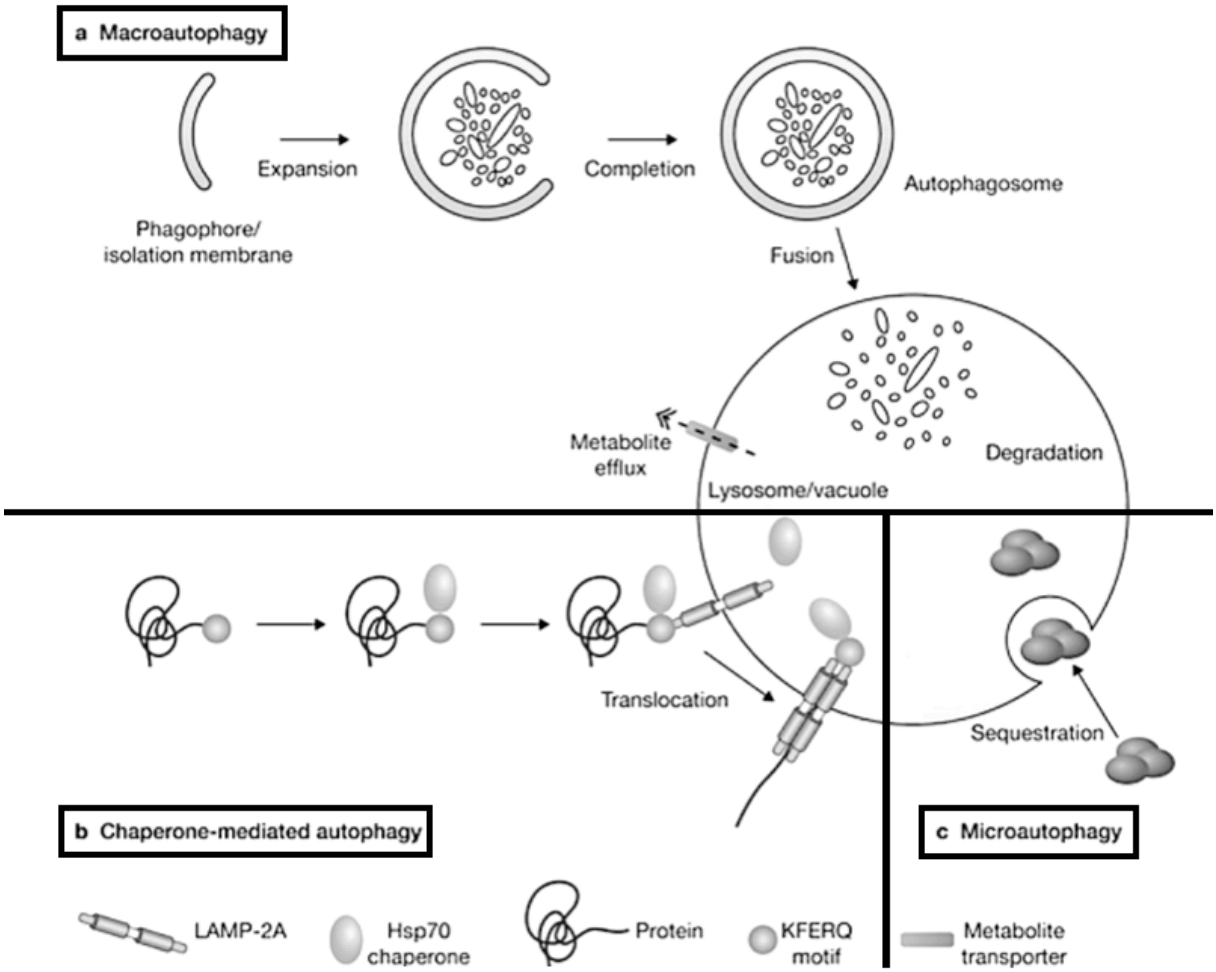


Figure 2. Mechanisms of the three main types of autophagy. *a) Macroautophagy b) Chaperone-mediated autophagy and c) Microautophagy (available from: Boya, Reggiori, and Codogno (2013))*

Selective autophagy

Autophagosome induction

Research in general suggest that nutrient-sensing pathways like target of rapamycin kinase complex 1 (TORC1) in yeast, called mechanistic TORC1 (mTORC1) in mammals, induce autophagy in cells (Ariosa & Klionsky, 2016). In the lack of nutrition, TORC1 is inactivated leading to activation of autophagy by dephosphorylating Atg13 (Figure 3). Dephosphorylated Atg13 has reduced affinity for Atg1 (Papinski & Kraft, 2016), thus forming a complex called Atg1 complex (Atg1-Atg17-Atg29-Atg-31). This complex recruits other Atg proteins to the phagophore assembly site (PAS) to initiate autophagosome formation (Fujioka et al., 2014). In mammals, unc-51 like autophagy activating kinase 1 (ULK1) and ULK2 corresponds to

Atg1 in yeast (Ariosa & Klionsky, 2016). Whereas during nutrition rich conditions, mTORC1 inhibit autophagy by phosphorylating Atg13, thereby inhibiting a complex formation with Atg1 resulting in decreased Atg1 kinase activity.

Autophagosome nucleation

The next step is the autophagosome nucleation of the initial phagophore membrane, a process regulated by class III phosphatidylinositol 3-kinase (PtdIns3K) complex, which consist of the lipid kinase Vps34, Beclin-1 (mammalian homolog of Atg6), Atg14 and Vps15 (Yin, Pascual, & Klionsky, 2016). This complex generate phosphatidylinositol triphosphate (PI3P) (Glick et al., 2010), a molecule that recruits Atg proteins that recognize and preferentially bind to PtdIns3P, like Atg18 and Atg21 (WIPI1 and WIPI2 in mammals) to the nucleation site (Ariosa & Klionsky, 2016; Fujioka et al., 2014).

WD repeats domain phosphoinositide-interacting protein 2 (WIPI2) links to PtdIns3P and recruits Atg12-Atg5-Atg16L1 complex by binding directly to Atg16L1 upon starvation. Thus, enable LC3 lipidation, which is important for autophagosomal formation (Dooley, Wilson, & Tooze, 2015).

Vesicle expansion

Vesicle expansion requires two ubiquitin-like systems, Atg5-Atg12 conjugation and Atg8-phosphatidylenolamine (Atg8-PE). Atg7 and Atg10 serve as E1 activating and E2 conjugating enzymes respectively, and allow the Atg5-Atg12 conjugate to form a complex with Atg16 that serves as the E3 ligase and thereby facilitate the lipidation of Atg8, forming Atg8-PE. The Atg4 protease must be able to cleave the Atg8's terminal arginine residue before PE molecule can be attached to Atg8.

In mammals, several isoforms of the Atg8 homolog, including microtubule-associated protein light chain 3 (LC3) and γ-aminobutyric type A ($GABA_A$)-receptor associated protein (GABARAP) undergo a similar conjugation process to that in yeast. The synthesis and processing of LC3-isoform, LC3B is presents in early stages of phagophore assembly and is used to detect autophagy in cells, since LC3B increases during autophagy. Whereas GABARAP acts in later stages in the autophagic pathway (Ariosa & Klionsky, 2016) and colocalize with LC3 at autophagosomes (Glick et al., 2010).

In addition to their roles in vesicle expansion, Atg8/LC3 can recruit adaptor proteins called autophagy receptors like ubiquitin-binding protein p62 (also known as sequestosome 1) to the autophagosome through a short LC3-interacting region (LIR) motif, and thereby mediate selective autophagy (Rubinsztein, Codogno, & Levine, 2012).

Fusion

Glick et al. suggest that the autophagosomes will fuse with the lysosomal compartment to form an autolysosome once they arrived at the destination (Glick et al., 2010). Tubulin is an acetylated cytosolic protein that is essential for fusion of autophagosome to lysosomes (Banreti, Sass, & Graba, 2013). Lysine acetylation enhances kinesin-1 and c-Jun N-terminal kinase-interacting protein 1 (JIP1) recruitment on microtubules, thereby allowing phosphorylation and activation of JNK, which may initiate autophagosome formation by triggering release of Beclin1 from Bcl-2-Beclin1 complex (Banreti et al., 2013; Geeraert et al., 2010).

The fusion allows the release of the inner autophagosome vesicle into the lysosomal body, but the exact process for autophagosome fusion is still unknown at present. However Yin et al. (2016) and Ariosa and Klionsky (2016) among others suggest that components such as homotypic fusion and vacuole protein sorting (HOPS) and endosomal sorting complexes required for transport (ESCRT) proteins and syntaxin-17 a soluble N-ethylamide-sensitive factor attachment protein receptor (SNARE) proteins, that is important for fusion with endosome or lysosome. It is localized to the outer membrane of completed autophagosomes, and depletion of Stx17 causes accumulation of autophagosomes without degradation, are involving in the fusion process (Ariosa & Klionsky, 2016; Ikuta et al., 2007; Yin et al., 2016).

Degradation and efflux

After fusion, the autophagosomes and its contents are degraded by various hydrolases, and some of the autophagosome contents like amino acids are recycled for further use (Ariosa & Klionsky, 2016).

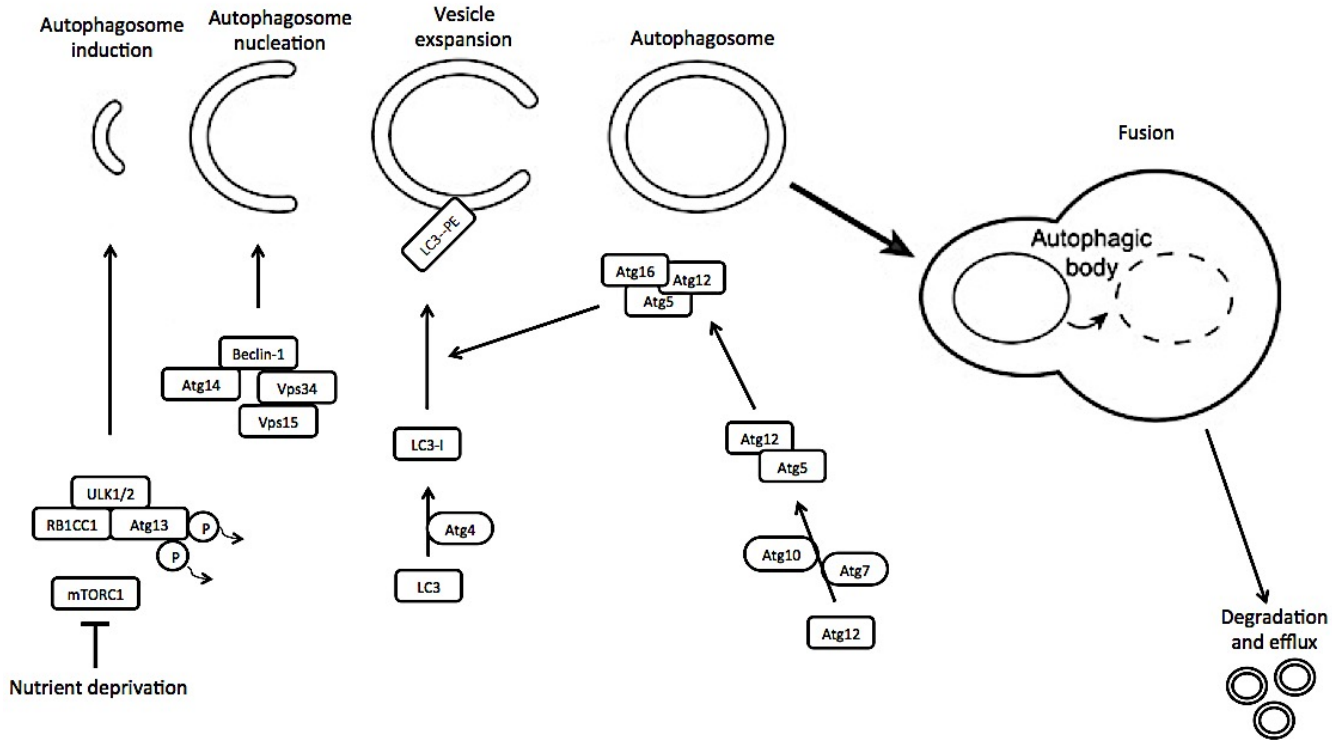


Figure 3. The mechanism of autophagy

P62/ SQSTM1

Polyubiquitin-binding protein p62, also known as sequestosome-1 (SQSTM1), was the first identified autophagy receptor. Research has revealed that it is an ubiquitin-binding scaffold protein that colocalizes with ubiquitinated protein aggregates in many neurodegenerative diseases including Alzheimer, dementia with Lewis body and Parkinson (Birgisdottir, Lamark, & Johansen, 2013; Bjørkøy et al., 2009; Pankiv et al., 2007).

Several studies addressed that p62/SQSTM1 protein is degraded by autophagy. p62 consists of a N-terminal Phox-BEM1 (PB1) domain that is important for polymerization, and a C-terminal UBA domain that interacts with ubiquitinated proteins. p62/SQSTM1 possess a short LC3 interaction region (LIR) (Birgisdottir et al., 2013; Pankiv et al., 2007) and it was reported to facilitate direct interaction to LC3 and GABARAP family proteins via a specific sequence motif. Thus it may link ubiquitinated proteins to the autophagic machinery to enable their degradation in the lysosome (Bjørkøy et al., 2009; Pankiv et al., 2007).

p62 is used as a marker for autophagic flux, since inhibiting of autophagy cause accumulation of p62, whereas induced autophagy leads to decreased autophagy levels (Bjørkøy et al., 2009).

Autophagy-related (Atg) proteins and their roles in autophagy

Atg proteins participate in various stages of the autophagic process, and as of 2016 it has been identified more than 40 Atg proteins in yeast, where the majority participates in the regulation of autophagosome formation (Liu & Klionsky, 2016). The Atg8-family plays a critical role for the maturation of autophagosome, in addition to function as an adaptor protein for selective autophagy (Y. K. Lee & Lee, 2016).

While there are two subfamilies, LC3 and GABARAP within the Atg8 family in mammals (Nguyen et al., 2016; Weidberg et al., 2010), there is only one Atg8 gene in yeast. The Atg8 family undergoes a unique ubiquitin-like conjugation to phosphatidylethanolamine (PE) on the autophagic membrane, a process essential for autophagosomes formation (Shpilka, Weidberg, Pietrokovski, & Elazar, 2011).

GABARAP

The GABARAP subfamily consists of 3 proteins, GABARAP, GABARAP-L1 and GABARAP-L2. The GABARAP proteins are involved in later stages of maturation of autophagosomes.

LC3B

LC3 has three subfamilies; LC3A, LC3B and LC3C. Lipidated LC3 level increases during autophagy, hence it is used as an autophagy marker. During the autophagy process, it is involved in elongation of the phagophore membrane, in addition to selectively recruit autophagy receptors such as p62.

The Fez family – Fez1 and Fez2

Biological functions of Fez1 and its role in neuronal diseases

Fasciculation and elongation protein zeta 1 (Fez1) and Fez2 are the mammalian orthologs of the Caenorhabditis elegans uncoordinated (UNC)-76 protein, and they all contain a coiled-coil region in their C-terminal (Alborghetti et al., 2013; Bloom & Horvitz, 1997; Toshitsugu Fujita et al., 2004). UNC-76 is a protein necessary for normal axonal bundling and elongation of axons, and cause locomotory defect if mutated (Maturana, Fujita, & Kuroda, 2010). Hence, mutation of Fez1 may result in axonal transport defects (Toshitsugu Fujita et al., 2004; Gindhart et al., 2003).

The Fez1 gene is located on chromosome 11 and possesses four regions of coiled-coil domains, in contrast to Fez2, who only have two. Coiled-coils are structural motif consisting of two to five α-helices (Mason & Arndt, 2004), that forms a left-handed supercoil by wrapping around each other (Burkhard, Stetefeld, & Strelkov, 2001; Mason & Arndt, 2004; Truebestein & Leonard, 2016) and mediate protein-protein interactions (Strauss & Keller, 2008) as well as subunit oligomerization of a large number of proteins (Burkhard et al., 2001). The coiled-coil domains of Fez1 can be utilized for intra- and intermolecular interactions (Maturana et al., 2010), and can be classified as a hub protein, since it can interact with a large number of proteins (Alborghetti, Furlan, & Kobarg, 2011).

The coiled-coil domain of Fez1, interacts with the short coiled-coil protein SCOC (Maturana et al., 2010), which is involved in Golgi-transport by interacting with ARL1. SCOC depletion inhibits starvation-induced autophagy monitored as decreasing LC3 lipidation. Thus, SCOC is a positive regulator of autophagy and overexpression of SCOC was reported to increase LC3 lipidation (Joachim, Wirth, McKnight, & Tooze, 2012).

Furthermore, it was observed that Fez1 overexpression in contrast, was found to inhibit autophagy. Increased amount of SCOC lead to reduction of Fez1 binding to ULK1. Therefore, Joachim et al. (2012) suggested that formation of Fez1-ULK1 complexes may be regulated by SCOC. In addition, it was suggested that Fez1 stabilizes the interaction between and SCOC and UV irradiation resistance gene (UVRAG), a tumor suppressor and a stoichiometric component of class III PtdIns3K complex that is essential for autophagosome formation (Joachim et al., 2012).

The biological functions of Fez1 are not yet fully understood, but it is currently believed that Fez1 negatively regulates autophagy (Joachim et al., 2012). Several studies suggested that Fez1 is brain specific (Haedicke, Brown, & Naghavi, 2009; Ikuta et al., 2007; Maturana et al., 2010), while Fez2 is reported to be expressed in most tissues (Toshitsugu Fujita et al., 2004). Hence, Maturana et al. (2010) suggested that Fez1 is involved in the development and function of the central nervous system and has therefore multiple roles when it comes to neuronal development, neuropathologies, neuronal resistance and viral infections (Maturana et al., 2010). Early work by Haedicke et al. (2009) reported that high levels of Fez1 in neurons contribute to HIV-1 resistance, although the mechanism is still unknown. Additional work by Malikov et al. (2015) seems to prove this theory by reporting that HIV-1 virus require kinesin-1 to travel to the nucleus where it fuse with the cell's DNA (Haedicke et al., 2009; Le Douce, Herbein, Rohr, & Schwartz, 2010; Malikov et al., 2015). Therefore by disrupting Fez1 functions in neurons, it might affect neuron's HIV-1 resistance, intracellular transport of synaptic proteins and mitochondria (Chua, Jahn, & Klopfenstein, 2013; Haedicke et al., 2009; Malikov et al., 2015).

Regulation of axonal transport by interaction with kinesin superfamily

Kinesin-1 is essential for regulating axonal transport, and several studies have suggested that Fez1 interacts with the human ortholog of kinesin-1, KIF (Toshitsugu Fujita et al., 2004; Gindhart et al., 2003; Maturana et al., 2010) and tubulin (T. Fujita et al., 2007). In order to activate the kinesin motor, Fez1 need to bind directly to the tail of kinesin heavy chain (KHC) while JIP1 binds to kinesin light chains (KLCs) simultaneously (Blasius, Cai, Jih, Toret, & Verhey, 2007).

Kinesins (KIFs) are one of the three superfamilies that are involved in intracellular transport, and the two remaining are called dyneins and myosins. KIF5B is a subgroup of KIF5 within the Kinesin superfamily, and is expressed ubiquitously in contrast to other KIF5 subgroups; KIF5A and KIF5C, that are expressed specific in neurons. KIF5B are molecular motor proteins that are responsible for transporting molecular cargo along microtubule tracks from the plus end of the microtubule, and use the chemical energy of adenosine triphosphate (ATP) hydrolysis to drive conformational changes that generate motil force. (Hirokawa, Noda, Tanaka, & Niwa, 2009). Fujita et al. (2007) reported that within PC12 cells, Fez1 not only interact with kinesin and motor protein KIF5B but also with tubulin. They showed that the

interaction of the FEZ1/kinesin complex with tubulin is actually involved in the anterograde transport of mitochondria. In addition, when Fez1 were silenced by siRNA, the anterograde movements of mitochondria within the neuritis of NGF-treated PC12 cells were also strongly inhibited, the same result was also reported by Maturana et al. (2010). Furthermore, knockdown of Fez1 resulted in vesicle accumulation and mitochondria localization in the cell body, thus indicating transport failure of organelles (Alborghetti et al., 2013).

The Fez1 mutants Fez1 S58A and Fez1 S58E

Fez1's phosphorylation ability is important for intracellular transport. Fez1 S58A is a phosphorylated defective protein of Fez1, where the amino acid nr.58, a serine, is mutated and can't be phosphorylated. This leads to it being unable to mediate axonal transport of the presynaptic SNARE protein syntaxin-1 (Chua et al., 2012).

While in Fez1 S58E the acidic amino acid glutamate has been inserted to the Fez1 protein instead of serine 58, thus resulting in a phosphor-mimicking protein. The phosphorylated form of Fez1 is essential for activation of phosphorylating dependent axonal transport of the presynaptic SNARE protein syntaxin-1.

Fez2

Fez2 is a homolog of Fez1, but compared to latter, little is known about it, so additional studies is needed. However it has been reported that Fez2 interact with protein kinase C-zeta (PKCζ), and may have an important role when it comes to morphological changes of various cells. Therefore, the biological roles of Fez2 are though to be similar to those of Fez1 (Maturana et al., 2010).

CRISPR/Cas9 knockout

Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) protein act as sequence-specific adaptive immune system in bacteria. It is used to protect themselves against viruses or other foreign genetics elements. When bacteria encounter an invading source of DNA, the Cas nuclease will turn the foreign DNA into small DNA fragments and then incorporate it into the CRISPR locus genome as the protospacers. Thus, providing a heritable record of past infections. CRISPR are short identical segments of DNA that are identical and interspaced by protospacer. Each protospacer is associated with a

protospacer adjacent motif (PAM), which is present in the target DNA, but not the crRNA that targets it (Ran et al., 2013; Rath, Amlinger, Rath, & Lundgren, 2015).

The CRISPR/Cas system can be classified into three major types: I, II and III, of which type II is the most studied and is the basis for the current genome engineering technology available. What separate these three major types are the differences of repertoires of Cas genes, the organization of Cas operons and the structure of repeats in the CRISPR arrays (Chylinski, Makarova, Charpentier, & Koonin, 2014). In addition, type II differs from the other two CRISPR systems as it only require one Cas protein (Cas9) to cleave foreign DNA. Cas9 contains a HNH nuclease domain and a RuvC-like nuclease domain that will generate a double stranded break (DSB) at the target site (Ran et al., 2013; Zhang, Wen, & Guo, 2014). DSBs can either be repaired by cellular Non-Homologous End Joining (NHEJ) or by homology-directed repair (HDR) pathway. The NHEJ pathway leads to insertions and/or deletions (indels), which will disrupt the targeted locus. While the HDR pathway will allow precise replacement mutations if a donor template with homology is supplied to the targeted locus (Ran et al., 2013; Zhang et al., 2014).

The CRISPR/Cas9 system (Figure 4) consists of a guide RNA (gRNA) that will guide the Cas9 nuclease to target site. The gRNA consist of two components: a target-specific CRISPR RNA (crRNA) and an auxiliary trans-activating crRNA (tracrRNA). The genomic target is defined by a 20-nucleotide (nt) sequence. Thus, by altering the 20-nt sequence of the gRNA, the CRISPR/Cas9 system can be guided to target any genomic region that is complementary to that sequence (Ran et al., 2013).

CRISPR/Cas9 depends on RNA for sequence-specific cleavage in contrast to other genome editing technologies like Zinc Finger nuclease (ZFNs) and Transcription activator effector nuclease (TALENs), which are depending on protein guided DNA cleavage. Since only programmable RNA is required to generate sequence specificity in CRISPR/Cas9, it is more specific, efficient and easier to customize as well as more suited for gene editing than ZFNs and TALENs according to (Ran et al., 2013).

According to Zhang et al. a major concern with CRISPR/Cas9 method is higher risk for off-target cleavage in human cells compared to ZFNs and TALENs, since it has been observed that CRISPR/Cas9 have tolerance for base pair mismatches between gRNA and its complementary target sequence. And large genomes often contain multiple DNA sequences that are identical or highly homologous to target DNA sequences. Therefore, will CRISPR/Cas9 may cleave highly homologous DNA sequences, which can lead to mutations at undesired sites.

CRISPR/Cas9 was used in this study in an attempt to generate knock out of Fez1 and Fez2 in Hek293 Flp-In cells.

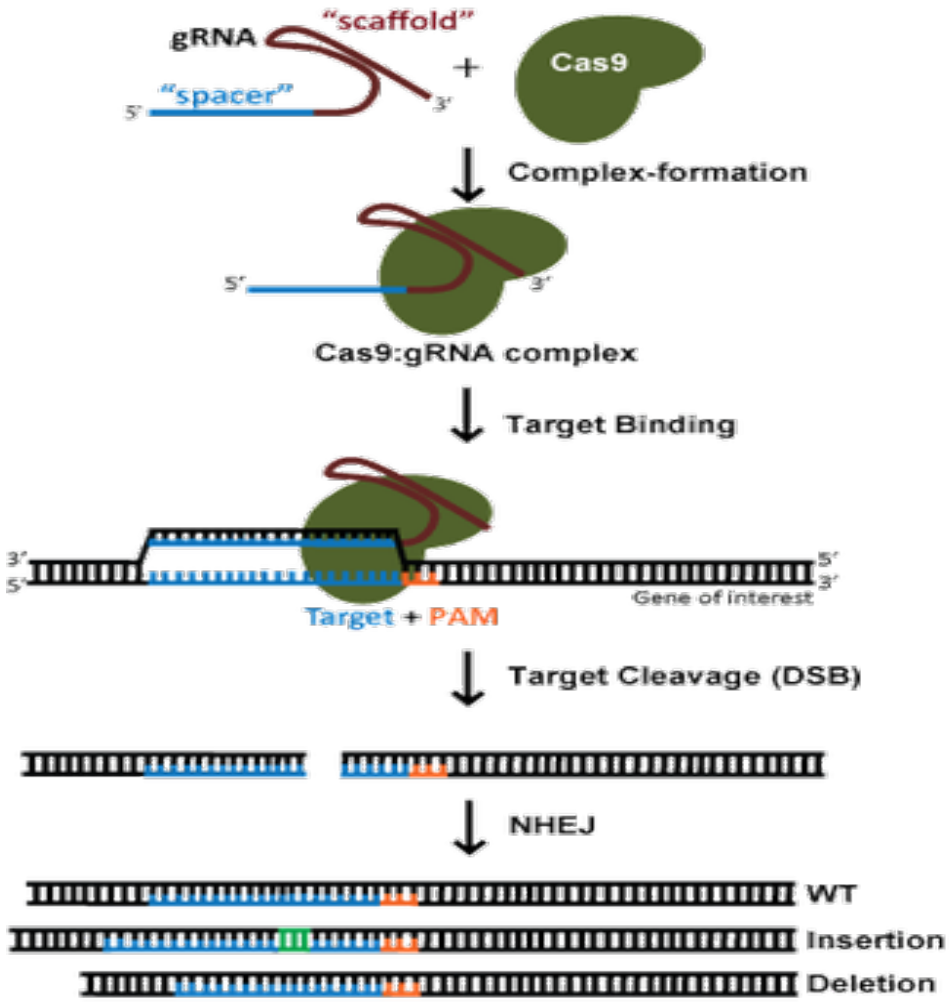


Figure 4. Mechanism of the CRISPR/Cas9 technology (available from:(Addgene, n.d)

Aims of this study

Autophagy is an essential process for maintaining cellular homeostasis. Fez1 have been reported to negatively regulate this process. Fez1 is involved in autophagy by interacting with kinesin1 and autophagy regulator SCOC via its coiled-coil domain. In contrast to Fez1, SCOC has been reported to be a positive regulator of autophagy

The use of knock out cell lines and imaging technology are important tools in functional studies of proteins. The aim of this study was:

- To establish knock out Fez1 and Fez2 cell lines by using CRISPR/Cas9 technology, for further functional studies.

- To study the colocalizations of over-expressed Fez1 or Fez2, with various autophagy and transport proteins in HeLa cell lines, using fluorescence confocal imaging.

- To study the localizations in of various EGFP-Fez1 and EGFP-Fez2 proteins inducible expressed in Hek293 Flp-In cells, with endogenous autophagy and transport proteins by immunostaining and fluorescence confocal imaging.

Materials and methods

Materials

Bacteria

Table 1. Bacteria used in this study.

Bacteria
Competent E.coli DH5α

Plasmids

Table 2. Plasmids used in this study.

Plasmid	Reference
pX458 (pSpCas9(BB)-2A-GFP)	Ran et al, 2013 / addgene
GFP-kf5B (337-963)	H. Olsvik, 2012
GFP-Stx17	Hakura et al, 2012
GFP-Tubulin	Clontech
GFP-WIPI2b	Dooley et al, 2014
pDEST-EGFP	Clontech
pDEST-EGFP-Fez1	E. Alemu, unpublished
pDEST-EGFP-Fez1 3xLIRm	E. Alemu, unpublished
pDEST-EGFP-Fez1 S58A	E. Alemu, unpublished
pDEST-EGFP-Fez1 S58E	E. Alemu, unpublished
pDEST-EGFP-Fez2	E. Alemu, unpublished
pDEST-EGFP-GABARAP	E. Alemu, unpublished
pDEST-EGFP-LC3B	Bjørkøy et al, 2005
pDEST-mCherry-Fez1	E. Alemu, unpublished
pDEST-Myc-Fez1	E. Alemu, unpublished
pDEST-Myc-Fez2	E. Alemu, unpublished
pGFP-p62	Bjørkøy et al, 2005

Mammalian cells

Table 3. Mammalian cells used in this study.

Mammalian cell types	
HeLa	ATCC CCL2
Hek293 Flp-In	ATCC CRL-1573
SH-SY5Y	ATCC CRL-2266

Growth media

Table 4. Growth media that were used for culturing various bacterial cultures.

Growth medium for bacterial cultures	**Contents**
Lurica-Bertani (LB) medium	10g Bacto Trypton 5g Bacto yeast extract 10g NaCl dH_2O to 1L pH adjusted to 7,5 with NaOH Antibiotic: Ampicillin (100ug/mL)
LB agar plate	10g Bacto Trypton 5g Bacto yeast extract 10g NaCl 15g agar dH_2O to 1L pH adjusted to 7,5 with NaOH Antibiotic: Ampicillin (100ug/mL)
Super optimal broth with Catabolite repression (SOC) medium	20g Bacto Trypton 5g Bacto yeast extract 10mL 250mM KCl 5g $MgCl_2$ 20mM glucose dH_20 to 1L pH adjusted to 7,5 with NaOH

*Media are bought from "mediekjøkken" at UNN SUMP

Table 5. Growth media that were used for culturing mammalian cells.

Mammalian cell types	**Growth medium contents**	**Supplier**
Human embryonic kidney cells (HEK293) Flp-ln T-rex Source: ATCC CRL-1573	500ml Dulbecco's Modified Eagle's Medium – low glucose 10% (vol/vol) standardized fetal calf serum 1% Penicillin/Streptomycin 100ug/ml Hygromycin	Sigma Aldrich (SA) Biochrome AG SA Thermo Fisher Scientific (TFS)
HeLa WT cells	500ml Dulbecco's Modified Eagle's Medium – low glucose 10% (vol/vol) standardized fetal calf serum 1% Penicillin/Streptomycin	SA Biochrome AG
SH-SY5Y		TFS

List of various solutions

Table 6. Buffers, enzymes, chemicals and their contents or CAS-number for purchased items that were used in various methods in this study.

Method	Solutions	Contents/CAT-number	Supplier
BCA protein assay kit/ Cell harvesting	2xSDS gel loading buffer	100mM Tris-HCl, pH 6,8 200nM DTT 4% SDS (w/v) 0,2% Bromophenol Blue (w/v) 20% glycerol (w/v)	-
	1XSDS	2xSDS diluted with dH_2O	-
Cell culturing	Dublerco's modified eagle's medium (DMEM) – low glucose	#D6046	SA
	Fetal Bovine Serum (FBS) Superior	#S0615	Biochrom GmbH
	Hygromycin B	50mg/ml in PBS	Invitrogen
	Penicillin-Streptomycin	#P0781	SA
	Trypsin	#T4049	SA
CRISPR/Cas9	10xCutSmart buffer	#B7204S	NEB
	10xT4 Ligation buffer	-	New England Biolabs (NEB)
	10xligation buffer	-	NEB
	10xNEB1 buffer	#B7001	NEB
	Alkaline Phosphatase, Calf Intestinal (CIP)	#M0290	NEB
	BbsI	#R0539	NEB

	T4 Polynucleotide kinase	#M0201	NEB
	T4 DNA Ligase	#M0202	NEB
	T4 DNA ligase Buffer	-	NEB
Cryopersevation of E. Coli/ mammalian cells/ bacteria cultures	50% Glycerol	-	SA
	Dimethyl sulfoxide (DMSO)	#D5879	SA
Immunostaining	4',6-diamidino-2-phenylindole (DAPI)	#62248 1:5000 in PBS	TFS
	4% Paraformaldehyde	-	VWR*
	5% Bovine Serum Albumin (BSA)	#A3983 Diluted in PBS	SA
	Fibronectin from bovine plasma	#F1141 Diluted 1:100 in PBS	SA
	Hank's Balanced salt solution (HBSS)	#H8264	SA
	Methanol >99,8%	-	SA
	Mowiol	2,4g Mowiol 4-88 6g Glycerol 12ml 0,2M Tris, pH 8,5	SA SA SA
	Immersol 518F	-	Zeiss
Precipitation of DNA	3M NaOAc, pH 5,2	-	-
	70% ethanol	Diluted from 96% ethanol with dH_2O	SA
	96% Ethanol	-	SA
	TE buffer	10nM Tris-HCl, pH 8,0 1mM EDTA	

PCR-Genomic DNA	1M Tris, pH 7,4	-	SA
	50mM NaOH	Diluted with dH2O	SA
	Deoxynucleotides mix, 10mM	#D7295	SA
	Taq DNA Polymerase PCR Buffer (10X)	#18067017	TFS
Gel-electrophoresis	20xMinigel buffer	193,75g Tris-Cl 27,22g NaOAc 14,9g EDTA dH_2O to 2L	-
	6xT gel loading buffer	0,25% Bromophenol blue 60mM EDTA, pH 8,0 0,6% SDS 40% (w/v) sucrose sterile filtered	-
	1 kb DNA ladder	1,0ul 1kb ladder stock (1,03 ug/ul) 24,0ul TE buffer, pH 8,0 5,0ul 6xT gel loading buffer	-
	100 bp DNA ladder		
	1% agarose gel	1g agarose 100mL 1xminigel buffer	SeaKem LE
	1,5% agarose gel	1,5g agarose 100mL 1xminigel buffer	SeaKem LE
	2% Metafor Agarose gel	1g metaphor agarose 50ml 1xminigel buffer	
	Gel Loading Dye, Purple (6X)	#B7024	NEB
	PAGE GelRed Nucleic Acid Gel Stain (10,000X)	#41008	Biotium

SDS-page gel	2xSDS gel loading buffer with Bromophenol Blue (BFB)	100mM Tris-HCl, pH 6,8 200mM DTT 4% SDS (w/v) 0,2% Bromophenol Blue (w/v) 20% glycerol (w/v)	-
	4xConcentrating gel buffer	60,55g Trizima-base 4g SDS dH_2O to 1L	-
	4xSeparating gel buffer	181,62g Trizima-base 4g SDS dH_2O to 1L pH 6,8 adjusted with HCl	-
	4% Concentrating gel	1mL 40% acrylamide mix 2,5mL Concentration gel buffer, pH 6,8 6,4mL dH2O 100ul 10% Ammonium persulphate (APS) 10ul Tetramethylethylenediamine (TEMED)	-
	8% Separating gel	2mL 40% acrylamide 2,5mL separating gel buffer 5,4mL dH2O 100ul 10% APS 10ul TEMED	-
	10% Separating gel	2,5mL 40% acrylamide 2,5mL separating gel buffer 4,9mL dH2O 100ul 10% APS 10ul TEMED	-

	16% Seperating gel	4mL 40% acrylamide 2,5mL separating gel buffer 3,4mL dH2O 100ul 10% APS 10ul TEMED	-
Restriction enzyme digestion	NEB 1.1 buffer	#B7201	NEB
Sanger sequencing	BigDye 3.1	-	Applied Biosystems
	5xSequencing buffer	-	Our in house sequencing lab
Transfection/ Revers transfection	TransIT-LT 1	#MIR2300	Mirus Bio LCC
	Metafectene pro	#T040	Biontex laboratories
	Lipofectamine RNAiMAX reagent	#13778150	Invitrogen
	Tetracycline	5mg/ml	-
Western blot	Biotinylated protein ladder	#7727	Cell signaling
	ProSieve® QuadColor™ Protein Markers, 4.6-300kDa	#00193837	Lonza
	SuperSignal molecular weight protein ladder	#84785	TFS

	1xTBS-T buffer (Tris buffered saline with tween 20)	75ml 2M NaCl 10mL 1M Tris-HCl, pH8,0 1mL Tween 20 914mL dH_2O	-
	0,2M NaOH	-	-
	5% milk solution	2,5g dried milk (Magermilch powder) 50mL TBS-T	-
	5xTransfer buffer	195nM Glycine 240nM Tris 0,15% SDS dH_2O to 1L	-
	Transfer buffer	200ml 5xTransfer buffer 150mL 96% Ethanol dH_2O to 1L	-
	Ponceau S dye	-	SA
General	Phosphate buffered saline (PBS)	10XPBS diluted with dH_20	TFS

*We Enable Science

Antibodies

Table 7. Displays a list of antibodies, both primary and secondary, that were used in different methods.

Methods		Antigen	Animal	Dilution	Supplier
Western blot	Primary	Fez1	Rabbit	1:1000	Chua, J et al (2012)
		Fez1	Rabbit	1:1000	Cell signaling
		Fez1	Mouse	1:1000	ABNova
		Fez2	Rabbit	1:1000	SA
		αGFP (EGFP-TAG)	Rabbit	1:5000	Abcam
		Actin	Rabbit	1:1000	Sigma
		LC3B	Rabbit	1:1000	Sigma
	Secondary	HRP anti-rabbit	-	1:1000	SA
		HRP anti-mouse	-	1:1000	SA
		Anti-biotin HRP linked	-	1:1000	Cell signaling
Immunostaining	Primary	GABARAP	Rabbit	1:300	Abgent
		γ-tubulin	Mouse	1:200	SA
		p62/SQSTM1	Guinea pig	1:2000	Progen
		Golgi 58K	Mouse	1:200	SA
	Secondary	Anti-rabbit Alexa Fluor-555	Goat	1:1000	Invitrogen
		Anti-mouse Alexa Fluor-555	Goat	1:1000	Invitrogen
		Anti-mouse Alexa Fluor-647	Goat	1:1000	Invitrogen
		Anti-guinea pig Alexa Fluor-647	Goat	1:1000	Invitrogen

siRNA

Table 8. siRNA that were used under transfection.

SiRNA	Concentration (uM)	Supplier
Control	50	-
Fez1 -01	20	-
Fez2	20	TFS, #16708A

Primers

Table 9. Primers that were used in this study.

Method	Primer	Sequences	Supplier
CRISPR/Cas9	U6	5'-GGG CAG GAA GAG GGC CTA T-3'	Invitrogen
PCR - Genomic DNA	Fez1-PCR-forward	AGCCTGGTCTAAACTCACATTACGC	Invitrogen
	Fez1-PCR-reverse	TTCTTCACGGGAGCTAGGTTCTCGG	Invitrogen
	Fez2-PCR-forward	GACGGAGTCTCACTCTGTTGCCCAG	Invitrogen
	Fez1-PCR-reverse	TAAACAGCCAGGCCCTGAAACTTAG	Invitrogen

DNA oligos

Table 10. Various guide sequences for pX458 (pSpCas9(BB)-2A-GFP) Fez1 and Fez2 used as CRISPR/Cas9 oligos. All these oligos were from Invitrogen.

Guide sequences	
Fez1-T1A-fw	CACCGTTCATCCAGACTCACCAGTG
Fez1-T1A-rev	AAACCACTGGTGAGTCTGGATGAAC
Fez1-T2A-fw	CACCGCTCCTCCGGGTCCTCCGAGC
Fez1-T2A-rev	AAACGCTCGGAGGACCCGGAGGAGC
Fez1-T1B-fw	CACCGCCTTCGACCCTCCTGCTCGG
Fez1-T1B-rev	AAACCCGAGCAGGAGGGTCGAAGGC
Fez1-T2B-fw	CACCGTTCATCTCCCCACCATCTCG
Fez1-T2B-rev	AAACCGAGATGGTGGGGAGATGAAC
Fez2-T1A-fw	CACCGTACAATACCAGGATGTACAC
Fez2-T1A-rev	AAACGTGTACATCCTGGTATTGTAC
Fez2-T2A-fw	CACCGAAAGGTTCTCCAGTATTCAC
Fez2-T2A-rev	AAACGTGAATACTGGAGAACCTTTC
Fez2-T1B-fw	CACCGAAGTGTGAGCACGAGTGGT
Fez2-T1B-rev	AAACACCACTCGTGCTCACACTTC
Fez2-T2B-fw	CACCGTGGCTTTTAAAATACGGTCT
Fez2-T2B-rev	AAACAGACCGTATTTTAAAAGCCAC

Blotting paper & membrane

Table 11. Blotting papers and membrane, used in western blot.

Blotting papers/ membrane	**Supplier**
Blotting papers, grade GB005 (1.5mm)	GEHealthcare life sciences
Blotting papers, grade GB003 (0.8mm)	GEHealthcare life sciences
Amersham Protran 0.45 NC (Nitrocellulose blotting membrane 0,45µm)	GEHealthcare life sciences

List of various kit

Table 12. Kits used in this study.

Kit	Supplier
Chemiluminescent Peroxidase Substrate-3	Sigma Aldrich
GenElute™ Mammalian Genomic DNA Miniprep Kit	Sigma Aldrich
GenElute™ Plasmid miniprep Kit	Sigma Aldrich
GenElute™ PCR clean-up Kit	Sigma Aldrich
Pierce® BCA protein Assay Kit	Thermo Fisher Scientific

Instruments

Table 13. Instruments used in different methods.

Method	Instrument	Brand
Cell culturing	LAF cabinet	ScanLaF
	Leitz DM IL microscope	Leica Microsystems
	Cell Counting Chamber	Bürker
Checking induced cells	Leitz DM IRB microscope	Leica Microsystems
Concentration measuring	NanoDrop 2000	TFS
Gel electrophoresis	Mini-Sub Cell GT systems	BioRad
	MultiDoc-It Digital Imaging System, transilluminator	UVP
Immunostaining	Confocal laser scanning microscope 780	Zeiss
Protein measuring	VersaMax ELISA Microplate Reader	Molecular Devices
Western blot	Curix 60 film Processor	AGFA
	Hoefer Mighty Small II Mini Vertical Electrophoresis Systems	TFS
	ImageQuant LAS4000	GE Healthcare Life Sciences
	Trans-Blot Turbo transfer system	BioRad

Methods

CRISPR/Cas9 knockout

This method was used in an attempt to make Fez1 and Fez2 knock out cell lines by editing parts of the genome. Cas9 act as "molecular scissors" and cut the two strands of DNA at a specific location in the genome. While a piece of pre-designed RNA sequence "guide" Cas9 to the right part of the genome. Oligos containing DNA guide sequences for Fez1 and Fez2 were first phosphorylated and annealed before they were ligated into linearized pX458 plasmid. The ligation mix was thereafter transformed into E.coli DH5α cells (Table 1) for amplification. Finally, insertion of the oligos into the pX458 was validated by DNA sequencing of purified plasmids.

Procedure:

Cloning of oligo into pX458

The guide sequence was selected by using the online CRISPR Design tool (*http://tools.genome-engineering.org*) that identifies and ranks suitable sites in addition to predicts off-target sites for inputted sequences within the plasmid. Oligos containing the target sequence in the Fez1 and Fez2 genes were cloned into the CRISPR vector pSpCas9(BB)-2A-GFP (pX458) as shown below (Figure 5) (available from: *https://www.addgene.org/48138/*).

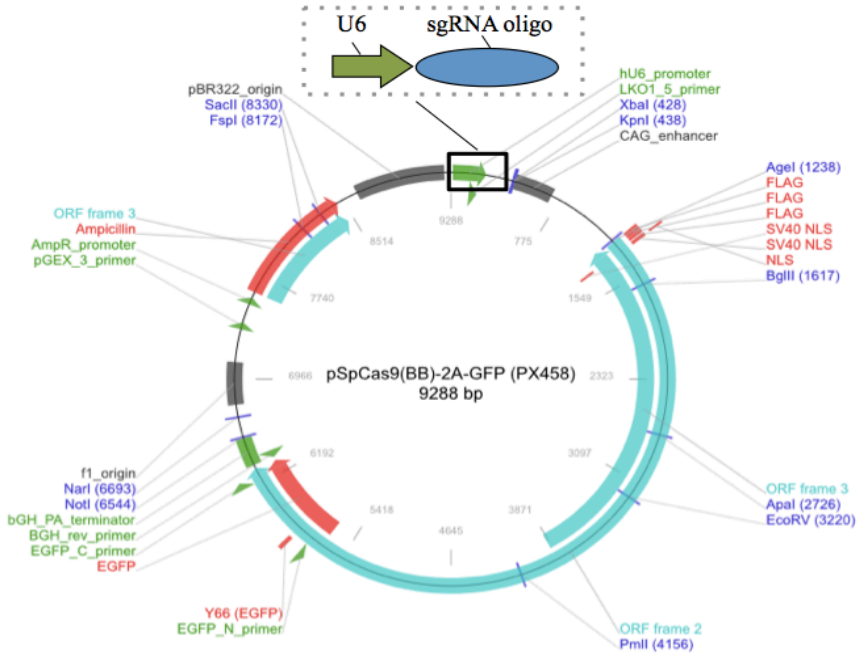


***Figure 5**. pX458 vector showing where oiligo with guide sequences were inserted.*

Growing E.coli from a freezer stock

Freezer stock of E.coli containing the pX458 plasmid was cultured in a sterile 15ml tube, filled 4ml LM + ampicillin (AMP) medium. A sterile pipette tip was first dipped in the freezer stock and then dropped in the tube with medium. In addition, the freezer stock was streaked on the surface of an agar plate. Both the agar plate (Table 4) and the tubes were cultured over night at 37°C, the tubes was shaken at 230 round per minutes (rpm).

Plasmid purification using GenElute^{TM} miniprep kit

This method was applied to isolate and purify plasmid DNA from proteins, RNA and chromosomal DNA within bacterial cells. It is based on silicia-binding and alkaline-sodium dodecyl sulfate (SDS) lysis procedures. The cells were subjected to a lysis solution and the chromosomal DNA was denatured under this alkaline condition. Upon neutralization, the chromosomal DNA aggregates and form insoluble clots, while plasmid DNA remains in the supernatant. The plasmid DNA was afterwards purified from the supernatant by binding to a column. Thereafter, washed to remove contaminants and finally the DNA was eluted with elution solution.

Procedure:

All steps were performed at room temperature (RT) and centrifuged at 12 000 x g if not stated otherwise. 1,5 – 3ml of the overnight cultures were transferred to eppendorf tubes and centrifuged for 1min. The supernatants were then removed and 200ul resuspension solution with RNAse was added to resuspend the pellet in the tubes. When the solutions were homogeneous, 200ul lysis solution was added. The tubes were then gently inverted for a few times before they were set on hold. After 4min, 350ul neutralization/ binding solution was added, inverted a few times and centrifuged for 10min. Collection tubes with column were then assembled, and 500ul column preparation solution was added. The tubes were centrifuged for 1min, and the flow-through was discarded. The cleared lysate from the eppendorf tubes were then transferred to the collection tubes with column and centrifuged for 1min. The flow-through liquid was discarded. Thereafter, 750ul wash solution diluted with ethanol was added. The tubes were then centrifuged for 1min and the flow-through was discarded. The tubes were then further centrifuged for 2min to dry the column. After the columns were changed to new collection tubes, 100ul elution solution were added and

centrifuged for 1min. The concentrations of the samples were then measured with NanoDrop 2000.

DNA precipitation

According to Sigma-Aldrich, the manufacture of GenElute^{TM} miniprep, the eluted DNA is clean enough to be used in digestion, ligation, sequencing, PCR and transfection. However to ensure that the DNA is really purified for transfection of mammalian cells, it was precipitated.

Procedure:

In a 90ul plasmid sample, 9ul 3M NaOAc (pH 5,2) was first added followed by 200ul 96% cold ethanol (-20 ºC). The samples were then vortexed for 10sec and thereafter left untouched for 20min, before they are centrifuged for 30min at 13 000 x g. The supernatant were then carefully removed. 200ul 70% ethanol was then added to the tube and centrifuged for another 10min, before discarding the supernatant. The eppendorf tubes were then left for drying without the cap for 30min at room temperature or 10min under vacuum. The dry pellet was dissolved in 40ul TE, before the concentrations were measured with Nanodrop 2000.

Phosphorylation and annealing of Oligos

The guide oligos (Table 10) were diluted in dH_2O to a final concentration of 100uM. To phosphorylate and anneal the oligos, the following solution was mixed in a 0,5ml microsentrifuge tube:

1ul	Oligo forward (100uM)
1ul	Oligo reverse (100uM)
1ul	10xT4 Ligation buffer
6ul	dH_2O
1ul	T4 Polynucleotide kinase

The solution was then incubated in a water bath at 37°C for 30min (phosphorylation) and then put into a PCR machine with the following program: 95°C for 5min and ramped down to 25°C at 5°C per minute (annealing).

Restriction enzyme digestion

Restriction enzymes can recognize and cut DNA at specific nucleotide sequences, called restriction sites (Berg JM, Tymoczko JL, & L., 2002; JM, JL, & L., 2002), and is used to insert genes or other pieces of DNA into plasmids.

The following mixture was incubated at 37°C for 1-1,5 hours to linearize the pX458 vector.

2ul 10xNEB1 buffer
1ug pX458 vector
0,5ul BbsI
dH_2O to 20ul

After 1-1,5 hours, 0,5ul calf intestinal phosphatase (CIP) was added to the solution before it was further incubated for 15min. CIP removes the phosphate from the plasmid ends, thus avoiding self-ligation during the cloning step. The BbsI digested plasmid (linearized pX458) was then purified with a GenElute™ PCR clean-up kit. Thereafter was 100ng of linearized pX458 run on a 1% agarose gel to verify if the plasmid was linearized.

Purification of digested vector/ GenElute™ PCR clean-up

Digested plasmids and PCR amplified product need to be purified from remaining primers, nucleotides, enzymes, oil, and salts. This is because these components can interfere with processes such as DNA sequencing. GenElute PCR clean-up combines silicia binding with a microspin format. Within the spin column, DNA gets attached to the silica membrane, while contaminants pass through. The bound DNA was then washed, and the clean DNA was eluted in a suitable buffer.

Procedure:

All centrifugation was conducted at 12 000 x g if not stated otherwise. 500ul column preparation solution was added to each collection tube assembled with column. The tubes were then centrifuged for 1min and the flow-trough were discarded. A mixture of 100ul biding solution and 2ul reaction mix were added to each tube, and then centrifuged for 1min before discarding the flow-through. Thereafter, 500ul wash solution was added to each tube and centrifuged for another 1min before discarding the flow-through. The collection tubes with column were then further centrifuged for 2min. The columns were then transferred to a

new collection tubes. 50ul elution solution were added and incubated for 1min at room temperature (RT), before being centrifuged for 1min.

Agarose gel electrophoresis

Agarose gel electrophoresis is a method used to separate and analyze nucleic acids that differ in size, charge, or conformation in an electric field. Smaller fragments migrate more easily through the pores of the gel and will therefore migrate faster than larger fragments. In general, the higher the concentration of agarose, the smaller the pore size, because increased concentration reduce the migration speed and thus making it possible to separate smaller DNA molecules. DNA is negatively charged, and will migrate through the agarose gel matrix towards the anode when an electric field is applied across the gel. The migration of nucleic acids is also affected by electrical parameters like current and voltage. Buffer compositions and temperature, low ionic strength of electrophoresis buffer leads to low electrical conductance, resulting in very slow migration rate of DNA. While in buffers with high ionic strength, electrical conductance is very efficient and thus leading DNA to migrate through the gel faster. But it will also generate significant amounts of heat that may cause melting of the gel and DNA denaturation. (Isbir, Kirac, Demircan, & Dalan, 2013; P. Y. Lee, Costumbrado, Hsu, & Kim, 2012)

Procedures:

1g agarose powder and 100ml 1xminigel buffer were mixed to make a 1% agarose gel. When making a 1,5% agarose gel, the solution contains 1,5g agarose powder and 100ml 1xminigel buffer. The solution was warmed in a microwave oven until a clear, transparent solution is achieved. Under this process the solution was taken out and stirred regularly. The solution was then casted in a gel caster assembled with a sample comb. The gel must polymerase for at least 20min, before the samples were loaded.

The wells were either loaded with molecular weight ladder or test samples mixed with gel loading dye purple. The gel was first run at 60V and then 90V after all the samples have exited the wells and then run until the dye line is approximately 80% of the way down the gel. It was then put into a container with a solution that's contains 2ul GelRed for at least 20min, for staining. GelRed stains the DNA by intercalating between DNA bases (Biotium, 2013). Thereafter, the gel was visualized with an UV transilluminator. The length of the DNA

fragments can be determined by comparing with a DNA ladder, a collection of DNA fragments of known lengths.

Ligation of oligo into pX458 vector

The phosphorylated and annealed oligos were diluted 1:200 in dH_20 before ligated to the linearized and purified pX458 plasmid. The following solution was incubated at 25°C for 2 hours:

1ul	10xligation buffer
50ng	linearized pX458 vector
1ul	diluted phosphorylated and annealed oligo
1ul	T4 DNA ligase
dH_2O to	10 ul

Transformation of E.coli DH5∝

The ligation mixture was transformed into competent E.coli, which are cells that have been treated to transform efficiently, since not all bacteria are capable of taking up exogenous DNA (Sigma, 2016). 50ul competent E.coli and 5ul ligation product was mixed and incubated for 20min on ice before heat shocked for 2min in a water bath at 37°C. The mixture was incubated on ice for another 2min before adding 200ul prewarmed to RT, SOC. The tubes were then incubated for 1 hour at 37°C with gentle shaking at 150rpm. 200ul of each tube was thereafter plated out on various RT LB plates with ampicillin and incubated over night at 37°C.

Validation of correct insertion

To check if the oligo was inserted correct, 4 different colonies of each LB plate where picked with a sterile pipette tip and put into a 3ml tube LB medium with 100ug/ml ampicillin. The tubes were incubated overnight at 37°C under shaking. Miniprep was then used to isolate and purify the plasmid DNA from the cultures.

The concentrations of all isolated and purified plasmid DNA were then measured with NanoDrop 2000. The isolated and purified plasmid DNA was then validated with restriction enzyme digestion by incubating the following mixture at 37°C for 1 hour:

1ul	10xCutSmart buffer
3ul	purified pX458 plasmid
0,25ul	BbsI
dH2O to 10ul	

This was then run on a 1% agarose gel with 2ul 6xT loading buffer added in each sample.

Sanger sequencing

Sanger sequencing is a chain terminating sequencing method. During sequencing, DNA polymerase copy single-stranded DNA templates and incorporate dideoxynucleotide triphosphates (ddNTPs) into a chain of nucleotides, thus terminating the strand elongation. ddNTPs lacks a 3' hydroxyl group of deoxynucleotide triphosphates (dNTPs), which is required to form a link with the next nucleotide, since it cannot form a bond with the 5'phosphate. This results in varying lengths of short DNA strands that are ordered by size and can reveal the original DNA by reading the end letters from the shortest to the longest piece (Heather & Chain, 2016).

Plasmids with the correct restriction pattern were verified by sequencing from the U6 promoter using pX330-seq-U6 primer (Table 9). The following sequencing mixture was made:

1ul	BigDye
1ul	5xSequencing buffer
150ng	plasmid
1ul	U6 primer (10uM)
dH_2O to 10ul	

Thereafter, the mixture was put into a PCR machine with the following program: 96°C for 20sec, 51°C for 15sec, and 60°C for 4min. This cycle was repeated 34 times and then holds at 4°C until the samples were sent to the DNA sequencing core facility. The sequencing results were analyzed by Chromas and BLAST.

Cell culturing

Cell culturing involves dispersal of cells in an artificial environment, with suitable nutrient solutions, temperature, humidity and gaseous conditions. To avoid apoptosis and senescence, the cells were split regularly, and then incubated in an incubator at 37°C with 5% CO_2 and ideal conditions of O_2 and humidity. All types of cells were split every 2-4 days, or when the cell confluence reached approximately 80%.

Procedure:

The medium was removed from cell culture flasks (Table 14) by an aspirator, and then washed with 1xPBS before they were trypsinated. After a certain time, the cells were checked with a microscope to ensure that the cells have detached. To help the cells detach, the cell culture flasks were hit gently. When the cells were detached, new medium was added either in the same cell culture flask or a new one. Since the same cell culture flask can be used to split cells up to 3 times.

Table 14. *Solutions used during cell culturing and splitting.*

Cell culture flasks sizes (mL)	PBS (mL)	Trypsin (mL)	Medium (mL)
T25	4	0,8	At least 4
T75	5	1	At least 9
T175	5	1,5	At least 16

Cryopreservation of mammalian cells and E.coli DH5α:

Cryopreservation is a process where cells get preserved by cooling to very low temperatures. This is to enable stocks of cells for long-term storage and to minimize genetic changes and contamination. With this method the cells can maintain their viability, until they are defrosted when they are needed (European Collection of Cell Cultures & Sigma, 2010). Formation of ice crystals during freezing may cause damage to the cells by rupturing the cell membrane, thus cryoprotectants like glycerol or dimethyl sulfoxide (DMSO) was added. DMSO is an intracellular agent, which can penetrate inside cells and reduce the formation of ice crystals that could lead to membrane rupture and damage the cells. While glycerol work by replacing part of the extracellular and intracellular water so that the amount ice formed is lower while

the unfrozen fraction remains larger. The degree of shrinkage of the cell will therefore be limited.

The cells were first frozen at -70°C before being transferred to a liquid nitrogen tank. This is because at some point during freezing, crystalline water will be formed. While the volume of the unfrozen fraction decrease, the concentration of cells and solutes in the unfrozen fraction will increase. The increase in osmotic strength causes an efflux of water from the cells, and minimizes the chance of intracellular ice formation. As cooling continues, the viscosity of the unfrozen fraction becomes too high for any further crystallization (FAO, 2012).

Procedure:

Freeze stock of mammalian cell lines

Cells were detached by trypsination and the medium with detached cell cultures was then centrifuged for approximately 10-15min at 1000 rpm. The medium was then removed and a mixture of Fetal Bovine Serum (FBS) with 10% DMSO was added to each tube with pellets, and mixed by pipetting. 1ml was then transfer to each cryotube, they were afterwards frozen at -70°C, then transferred to a liquid nitrogen tank after a week.

Freeze stock of E.coli DH5α

1,2ml of the over night culture and 300ul of 50% glycerol were mixed in a cryotube. The tubes were then marked and frozen at -70°C.

Mycoplasma testing

Mycoplasma is bacteria that lack a cell wall around their cell membrane. The lack of a rigid cell wall makes mycoplasmas resistance to most common antibiotics, since most of them target cell wall synthesis. Mycoplasma contamination is difficult to detect because unlike bacterial and fungus contamination, it will not cause visible changes to the growth media or be detected by a light microscope. Mycoplasma competes with cells for nutrients in the growth media and can therefore induce changes to cell cultures, such as reduced cellular proliferation, morphological changes, cell aggregation and/or poor transfection efficiencies. Thus, the reliability, validity and reproducibility of the experimental results can be affected (Finley, 2013; Lonza, 2017a, 2017b; Young, Sung, Stacey, & Masters, 2010).

Procedure:

Samples from various cell cultures were cultured in T25 cell culture flasks. They were then leaved to grow until approximately 80% confluent. When the cell cultures reached the desired confluent level, the medium was removed and replaced with medium that doesn't contain antibiotics. After 2-3 days, 100ul of the medium was taken out and boiled for 10 min. Thereafter centrifuged at 10 000 rpm for 10 min. The supernatants were then transferred to new eppendorf tubes, and frozen at -20 ºC until they were analyzed by PCR by a technician in the group.

Transfection of mammalian cells

Transfection is a method used to introduce foreign genetic materials (DNAs and RNAs) into cells to produce genetically modified cells. The transfection can either be stable or transient. Stable transfection integrate genetic materials into the host cell genome and will sustain transgene expression even after the host cell replicate. In contrast, transiently transfected genes are not integrated into the host cell genome and will only be expressed for a limited period. Hence, transiently transfected genetic materials will be lost after some cell division (Kim & Eberwine, 2010).

Both transient and stable transfections were performed in this study. The medium used under transfection was without antibiotics, while the medium used after transfections had both antibiotics and FBS. The reason why antibiotics were not added under transfections was because it may reduce the transfection efficiency.

The main purpose of this method is to introduce the plasmid DNA and siRNA into Hek293 and HeLa cells (Table. 3), for genomic engineering (CRISPR/Cas9), for establishing of stable cell lines and for localization studies using a confocal microscope.

Procedure:

6-well plates were used for both forward and reverse transfection. Approximately 300 000 cells were cultured in each well if not stated otherwise. The difference between forward and reverse transfection are that in forward transfections, cells were seeded 1-2 days before transfection reagent were added. In contrast, cells were seeded in the wells at the same time as the transfection reagent in reverse transfection.

Forward transfection:
Cells were seeded in 2ml medium with 10% FBS in a 6-well dish, and thereafter incubated for 24 hours before they were transfected.

Both Lipofectamine and Lipofectamine RNAiMAX are cationic-lipid based transfection reagents that are made up of liposomes, a positively charged head group and a hydrophobic tail group. The positively charged head group interacts with DNA, which is negatively charged and the transfection complex gets transferred into the cell by endocytosis (Thermo Fisher Scientific, n.d).

- **Transfection of plasmid DNA**:
  As shown in Table 15, pX458 plasmids with two different guide sequences were added together with 100ul medium in 6 different tubes. 2ul metafectene and 100ul medium with 10% FBS were added to 6 other eppendorf tubes. The tubes containing metafectene were thereafter added to each tube with plasmids, and the mixtures were incubated at RT for 15-20min. During this time, complexes with DNA lipid carries are formed.

  Each eppendorf tube with plasmid DNA was added dropwise into each tube with transfection reagent. Mixed once with a pipette and then incubated for 15-20min at RT. The solutions were then added dropwise to the wells of the cell-cultured plates and incubated for 24 hours. After incubation, the cells were checked if they were successfully transfected using a fluorescence microscope. Old medium were then removed and the cells were trypsinated and transferred to cell strainer tubes for cell sorting. A FACSAria cell sorter sorted cells expressing GFP in 96-well culturing plates containing 100ul medium with 10%FBS, one green cell in each well. The cells were incubated around 3 weeks, with change of medium once a week. When the cells had grown into a visible colony, they were transfected into a 12-well dish, and further to 6-well dishes for western blot, isolation of genomic DNA and freezing. Transfection using Plasmid DNA was also used to test different antibodies

***Table 15**. Plasmids containing CRISPR/Cas9 guided sequences and the amount that were used to transfect into Hek293 Flp-In cells by the transfection reagent, Metafectene. Each eppendorf tube contains two different plasmids, 500ng of each.*

Tube	Plasmids	Concentrations (ng)	Total concentration per tube (ng)
1	Fez1 – T1A	500	100
	Fez1 – T1B	500	
2	Fez1 – T2A	500	100
	Fez1 – T2B	500	
3	Fez2 – T1A	500	100
	Fez2 – T1B	500	
4	Fez2 – T2A	500	100
	Fez2 – T2B	500	
5	Fez1 – T1A	500	100
	Fez2 – T1A	500	
6	Fez1 – T2A	500	100
	Fez2 – T2A	500	

- **Transfection of siRNA:**

  Small interfering RNA (siRNA) is duplexes that target complementary mRNA substrates for degradation. It was used to validate antibodies that were used to detect Fez1 and Fez2 in this study. The tubes marked as A contains; 150ul medium with 10% FBS, siRNA (Table 16) and 2,5ul Lipofectamine RNAiMAX. While the tubes marked as B contains 100ul with 10% FBS and 2,5ul Lipofectamine RNAiMAX. The tubes marked with A were added dropwise to the tubes marked as B.

***Table 16**. Contents of the tubes marked as A. The tubes contain various siRNA that were to transfect into Hek293 Flp-In EGFP, EGFP-Fez1 and EGFP-Fez2 cells by using transfection reagent, RNAiMAX.*

SiRNA	Control siRNA	Fez1 siRNA (20uM)	Fez2 siRNA (20uM)
Quantity (ul)	1	2,4	2,4

The tubes were incubated for 20min at RT and the contents were then added dropwise to each well and incubated 48 hours before harvesting for western blot.

Pierce Bicinchoninic Acid (BCA) Protein Assay

A method used to measure protein concentrations of various cell lysates for further experiments. This technique is based on BCA for colorimetric detection of proteins and exhibits strong absorbance at 562nm, that is almost linear within 20-2000ug/ml working range. The unknown samples were then compared alongside a series of dilutions of known concentrations of BSA (Table 12).

Procedure:

Each well of the micro-plate were loaded with either different BSA standards or a mixture of 10ul sample and 190ul working reagent. The working reagent was premixed with 50 parts A and 1 part B. The plates were incubated for 30min at RT and then colorimetric analyzed in a VersaMax ELISA microplate reader. The results were thereafter analyzed by using excel to compare the samples with unknown concentrations with a standard curve, made of BSA dilutions.

Western blot

Sodium Dodecyl Sulfate **(**SDS) is an anionic detergent that is present in the SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE) buffer and gel loading buffer. This detergent disrupts the tertiary structure of proteins to linearize and binds to proteins to make them negatively charged, thereby when exposing to current, the proteins will migrate towards the positive electrode in the gel (Mahmood & Yang, 2012).

Western blot was used to identify proteins that first were separated by SDS-PAGE based on molecular weights. All proteins that were originally on the gel were transferred over to a nitrocellulose membrane (Table 11). Further, the membrane was incubated with specific primary antibodies that bind to proteins of interest, while unbound antibodies were washed away. The membrane was then treated with horseradish peroxidase (HRP)-conjugated secondary antibodies, that will recognizes and bind to the primary antibodies. The secondary antibody is linked to a reporter enzyme that produces color or light, which allows it to be easily detected and imaged.

In this study, western blot were used to investigate if various EGFP Fez1, Fez2 and their mutants were expressed in the Flp-In cells and whether CRISPR/Cas9 has successfully knocked out Fez1 and Fez2.

Procedure:

Preparation of the cell lysate

Around 300 000 cells were seeded out in 6-wells cell cultures plates and incubated in a cell incubator over night. The cells were then harvested; growth medium removed and washed with PBS once, before 60ul 2xSDS loading buffer with 200mM DTT was added to each well. The cell lysate were then transferred to 1,5ml eppendorf tubes and boiled at 100°C for 10min or until when the lysate were no longer viscous.

SDS-page preparation

Components of a separating gel (Table 6) were mixed and poured between the plates of an assembled dual gel caster until 2/3 filled. Water was then added on top of the separating gel to keep it straight and from drying out. The gel was left to polymerize for at least 20min. The water was then discarded and a gel comb was added before a 4% concentrating gel solution (Table 6) was added on top of the separating gel. The gel was left to polymerize for at least another 20min.

The glass and metal plate along with the casted gel and spacer were then detached from the dual gel caster and put into a BioRad power chamber and fastened with a clip on each side. SDS-running gel buffer were then added to the vertical and bottom chamber. A mixture of 5ul ProSieve Quad Color Protein marker and 5ul Biotinylated protein ladder were loaded into a well, while the samples were added into the other wells. The gel was then run at 90V with water cooling until the migration front reach the concentrating gel, then it was run at 160V for 1 hour or until the migration front reached the bottom.

Western blot

A “sandwich” was assembled in a cassette with three Whatman paper at the bottom, a nitrocellulose membrane, a SDS-page gel over and then another three Whatman paper on top. All cut into 6,5 x 8,5 cm pieces and soaked in transfer buffer. The cassette was then closed with a lid and placed in a blotting chamber of the Trans-Blot Turbo transfer system, and run for 0,1A per gel for 1 hour or 0,2A if 2 gels in were the same chamber.

After 1 hour, the nitrocellulose membrane was detached from the sandwich, and proteins on the membrane were stained with Ponceau S for 3min. A picture was then taken of the membrane after washing it with dH_2O. Ponceau S was then washed away completely by soaking the membrane in TBS-T under shaking for 2-3min and then rinsed 2-3times with TBS-T. The membrane was then blocked with 5% dried milk in TBS-T under shaking for 20min to 1hour. Afterwards was the membrane incubated with primary antibody diluted in 2,5ml 5% milk in TBS-T (Table 7). This was incubated over night at 4°C on a rotator.

The membrane was taken out of the tube and washed with TBS-T 4 times, 5min between every round. Secondary antibodies; HRP anti-rabbit and anti-biotin HRP linked were diluted according to Table.7 in 2,5% milk in TBS-T to detect the proteins and ladder, respectively. Thereafter was the mixture added to a new 50ml tube before the membrane was added, and incubated for 1 hour under rotation at RT.

Imaging of Chemiluminescent

The membrane was washed with TBS-T 6 times before 1ml per membrane of Chemilumiscent peroxidase substrate-3 mixture (0,5ml of solution A and solution B) was added. It was then incubated in the dark for 5min. The signal was detected using ImageQuant LAS 4000.

Isolating genomic DNA from cells

A quick protocol and a genomic DNA miniprep kit are two methods used to isolate genomic DNA from cells to be used as template in PCR reactions.

Quick protocol

Process:

300ul 50mM NaOH were added to the wells of a 24-well cell culture plate, that contained approximately 100 000 cells. The lysate were transferred to eppendorf tubes and incubated at 95°C for 20min. They were then cooled on ice before 60ul 1M Tris, pH 7,4 was added to each tube and centrifuged for 10min at 12 000 x g. Further, 2ul of the lysate were used as template in PCR reactions, while the remainder were stored at -20°C.

GenElute™: Mammalian genomic DNA miniprep

Procedure:

Cells were grown to confluency in a 6-well dish and then loosened by trypsination before being pelleted by centrifugation at 1000 rpm for 5min. The pellet inside the tubes was resuspended with 200ul of resuspension solution. 20ul of protein kinase K solution was added followed by 200ul of lysis solution. The samples were vortexed for about 15sec and then incubated at 70°C for 10min. 500ul column preparation solution was then added to each binding column and centrifuged for 1min at 12 000 x g. The flow-through was then discarded. Thereafter, 200ul 96% ethanol was added to the lysate and the tubes spun for about 5-10sec. The lysate was then transferred to the binding column and centrifuged for 1min at 12 000 x g. The column was transferred to a new collection tube. 500ul wash solution was then added and they were centrifuged for 3min. The flow-through was discarded and the tubes were centrifuged once more to ensure that all ethanol was removed. The column was then again transferred to new collection tubes. 200ul elution solution was added and incubated for 5min, before they were centrifuged for 1min to elute the genomic DNA.

Polymerase chain reaction (PCR) for genomic DNA

PCR is a method used to amplify a DNA template to produce specific DNA fragments in vitro. The aim is to make enough of the target DNA region so that it can be analyzed or used in further experiments like sequencing, gel electrophoresis, or cloned into a plasmid. The PCR product is then exposed to different temperatures for a certain time period. The series of temperature and time are referred as one cycle of amplification. Theoretically, each PCR cycle doubles the amount of target sequence in the reaction. Each cycle of PCR includes template denaturation, primer annealing and primer extension.

Process:

The following solution was mixed in a PCR tube for each genomic DNA sample:

5ul 10xPCRbuffer
1 ul DMSO
4ul dNTP
1ul DNA polymerase
1ul Forward primer (Table 9)
1ul Reverse primer (Table 9)
2ul genomic DNA
dH2O to 50ul

The PCR mixtures were then transferred to a PCR machine with the program described under Figure 6.

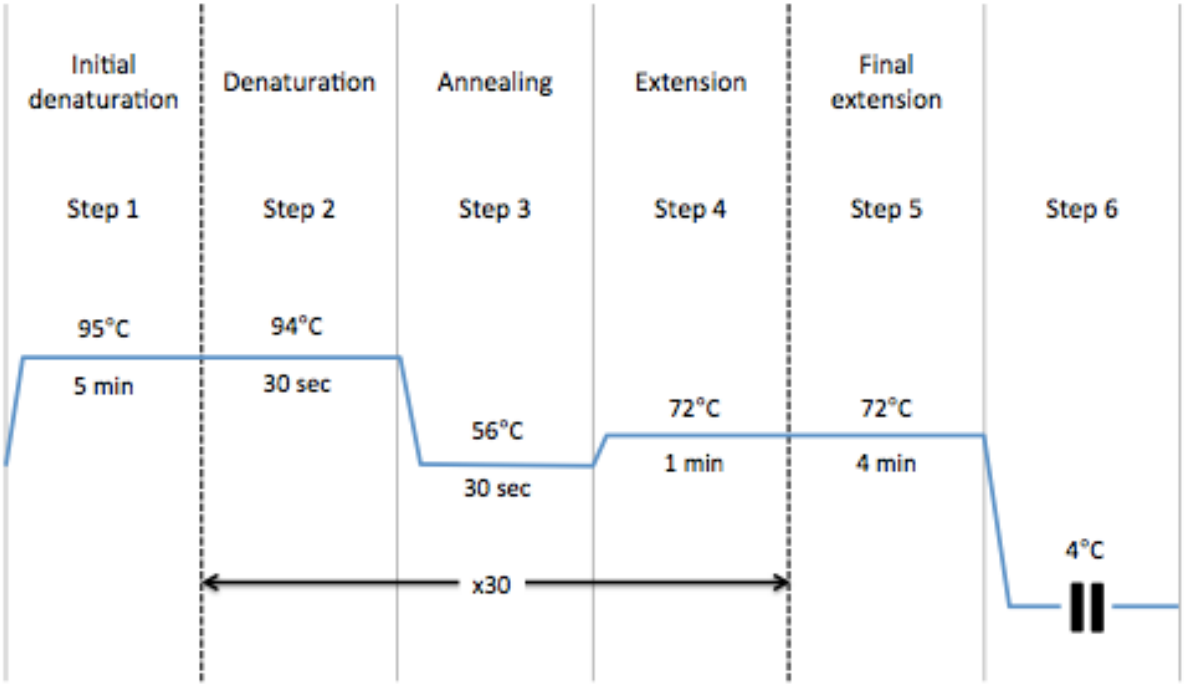


Figure 6. *The PCR program used in this study to amplify genomic sequence with temperatures and time. Step1-2: denaturing of target DNA. Step 3: Primer annealing. Step 4: Primer extension. Step 2-4 was run for 30 cycles. The next cycle starts at step 2. Finally step 6: the PCR products were kept at this temperature until it was further used/freeze.*

MetaPhor® Agarose gel electrophoresis

Like regular agarose, MetaPhor agarose is a method used to separate and analyze nucleic acids as described earlier for regular agarose gel. But what separate these two agarose types is that MetaPhor is a high-resolution agarose. It can differentiate DNA fragments that differ in size by 2% and have twice the resolution capabilities of the finest-sieving agarose products according to the producer, Lonza (Lonza, 2007).

Procedure:

1g MetaPhor agarose gel powder and 50ml 1xminigel buffer were used to make a 2% MetaPhor agarose gel. ¼ of the buffer were first added to an Erlenmeyer flask under stirring. The agarose powder was then added to the flask, and the remaining buffer was used to wash of any powder residue from the Weighing boat. The flask was then warmed in a microwave for 2min at medium temperature and 2min at high temperature. The solution was taken out every minute for stirring to ensure that all powder has dissolved and thereafter cooled to room temperature.

It was afterwards casted in a gel caster that was assembled with a well-making comb for 20min. To obtain optimal resolution and gel handling characteristics the solidified gel was incubated at 4°C for at least another 20min. All sample preparations had to be done before the solidified gel was taken out to room temperature. Since it is porous and can easily break if it's taken out at room temperature for a long period.

After removing the comb, the wells were either loaded with 6ul 100kb DNA protein ladder or 6ul samples. Each sample contains 5ul PCR product and 1ul gel loading dye purple 6x. The gel was first run at 60V and then 90V after all the samples had exited the wells, and run until the dye line is approximately 80% of the way down the gel. It was afterwards put in a container with 3ul GelRed for 1 hour before DNA fragments were visualized by UV light.

Immunostaining

Immunostaining is a method used to study protein expression, localization and distribution within individual cells or tissues. Specific antibodies were used to detect specific proteins in fixed cells. The cells were first exposed to primary antibodies directed against the protein of interest. Bound antibodies were then detected by secondary antibodies that are directed against a part of the invariant portion of the primary antibody. The technique used in this thesis is called immunofluorescence, and it use fluorphore-conjugated antibodies that will become visible when they fluorescence. Fluorphores are proteins that absorb light at one wavelength and emit light at another wavelength. These antigen-antibody complexes were then detected and visualized by a confocal microscopy. Detections of antigen in cultured cells were then conducted by using a confocal microscopy.

Procedure:

All the incubations were conducted at room temperature (RT) if not stated otherwise. The coverslips were first put into each well of 24-well cell culture plates, before they were completely covered in ethanol for sterilization for 5-10min. After the coverslips were sterilized, 100ul Fribronectin diluted in 1xPBS was added (1:100) for at least 15min before they were washed with 1xPBS twice. Each well was then cultured with approximately 50 000 cells in 500ul DMEM + 10%FBS. There plates were incubated in a CO_2-incubator for 24 hours.

Here, Hek293 cells stably expressing GFP-tagged Fez1 or Fez2 constructs, and transiently co-transfected HeLa cells, were used for localization studies of Fez1 and Fez2.

A. **Hek293 Flp-In cells**: Expression of Flp-In EGFP-protein construct was induced with tetracycline the same or next day. Inducing solution: 500ul DMEM with 10% FBS and 1ul/ml tetracycline.

B. **HeLa wild type (WT) cells**: HeLa cells were seeded one day before transfection. The cells were transfected with *Trans*IT-LT1 Reagent that was pre-warmed to RT and gently vortexed before use. A sterile eppendorf tube were first added with 50ul DMEM, and then 200ng plasmid DNA was added. Finally the 1ul TransIT-LT1 diluted in 50ul DMEM (Table 17) was added to the DNA mix, and mixed gently.

Table 17*. Plasmid combinations and the amount used to transfect into HeLa cells by TransIT-LT1 transfection reagent.*

Tube	Plasmid 1 (100ng)	Plasmid 2 (100ng)
1 + 2	pDEST-mCherry-Fez1	pDEST-EGFP
3 + 4	pDEST-mCherry-Fez1	pDEST-EGFP-Fez1 S58A
5 + 6	pDEST-mCherry-Fez1	pDEST-EGFP-Fez1 S58E
7 + 8	pDEST-mCherry-Fez1	pDEST-EGFP-Fez1 3xLIRm
9 + 10	pDEST-mCherry-Fez1	pDEST-EGFP-Fez2
11 + 12	pDEST-mCherry-Fez1	pDEST-EGFP-GABARAP
13 + 14	pDEST-mCherry-Fez1	pDEST-EGFP-LC3B
15 + 16	pDEST-mCherry-Fez1	pGFP-p62
17 + 18	pDEST-mCherry-Fez1	GFP-WIPI2b
19 + 20	pDEST-mCherry-Fez1	GFP-Kf5B (337-963)
21 + 22	pDEST-mCherry-Fez1	GFP-Stx17
23 + 24	pDEST-mCherry-Fez1	GFP-Tubulin

1ul room tempered *Trans*IT-LT1 Reagent was then added directly into each solution without coming in contact with the sides of the plastic tube. After the contents are mixed completely. The tubes were incubated for 15-30min, before the contents were added drop-wise into different areas of each well. The cell culture plates were then gently rocked back and forth and from side to side for even distribution, and incubated for 24 hours in a CO_2-incubator.

Two coverslips for each plasmid combination (Figure 7), where one of them were starved the day after transfection by replacing DMEM with Hank's Balanced Salt Solution (HBSS), and incubated for 1-2 hours. DMEM and HBSS were then removed and the coverslips were fixated with 500ul 4% paraformaldehyde (PFA) for 15-20min. Then washed with PBS once, before they were permeabilized with 500ul methanol for 5min. They were then washed with PBS twice before blocked with 5% fresh made BSA in PBS for 20min.

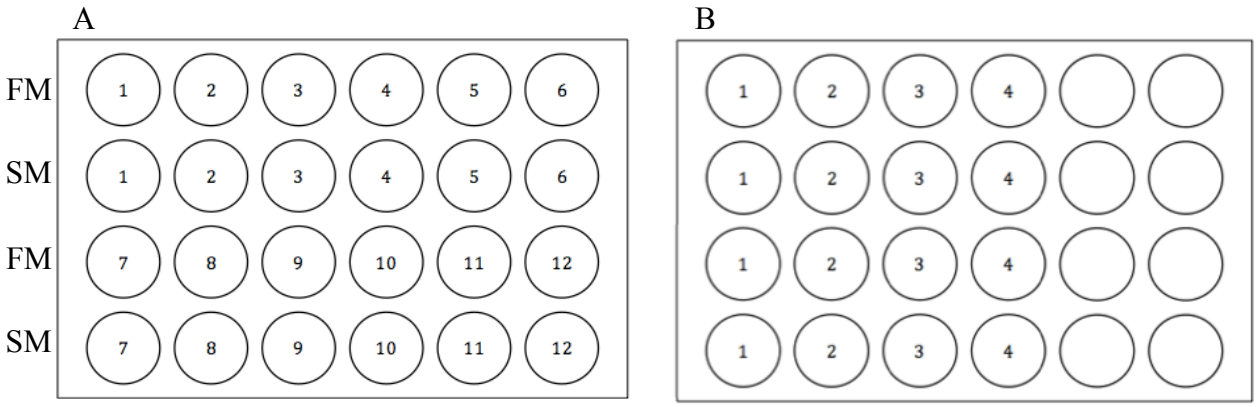


Figure 7. *24-wells cell culture plates with* ***A.*** *Transfected HeLa WT cells with 2 wells of each plasmid combinations. The plasmid combinations in the wells are shown in table 17.* ***B.*** *Hek293 Flp-In cells with 4 wells of each cell type. 1: Hek293 Flp-In EGFP-Fez1, 2: Hek293 Flp-In EGFP-Fez1 S58A, 3: Hek293 Flp-In EGFP-Fez1 S58E and 4: Hek293 Flp-In EGFP-Fez2. FM= DMEM+10%FBS, SM= HBSS*

Primary antibodies (Table 7) were diluted in fresh 1% BSA in PBS, and 25ul were spotted on a parafilm that was attached to a flat surface with dH_2O. Further the coverslips were picked up from the wells with a needle and a tweezer. The coverslips were then placed on top of the 25ul spotted antibody solution diluted as shown in Table 7, with the cells facing the solution and then incubated for 1 hour in RT.

After 1 hour, each coverslip was washed in a 1% BSA solution by dipping them in the solution 5-6 times. They were then placed on a new parafilm that were spotted with 25ul of secondary antibodies diluted in 1% BSA in PBS and incubated for another 1 hour.

Each coverslip was thereafter washed 5-6 times in 1% BSA solution and then 5-6 times in dH_2O. Excess wash solutions on the coverslips were removed by touching the edge of a filter paper. The coverslips were then mounted on an object glass. Each coverslips were placed on a 10ul Mowiol solution with the cell side facing the solution. They were then dried over night at RT or for 2 hours at 37°C, before they were analyzed by a Zeiss 780 inverted confocal microscope and the Zen program.

Results

Establishment of potential knock out cell lines with CRISPR/Cas9 technology

A way to study the functions of a gene is to shut it down, knock it out or overexpress it in cells. Thereafter, compare the manipulated cells with control cells (WT) to check if there are any differences. Here we used the CRISPR/Cas9 technology in an attempt to establish stable knock out cell lines of Fez1, Fez2 and double Fez1-Fez2.

Cloning of guide sequences into CRISPR/Cas9 vector pX458

Guide sequences for targeting the Cas9 endonuclease to exon1 in Fez1 and Fez2 were obtained by bioinformatic analysis as described in Ran et al., 2014. Four guide sequences with low off-target effects were selected for each gene. Oligos encoding the selected guide sequences were phosphorylated and annealed, before they were ligated into linearized pX458 plasmid. Ligated plasmids were transformed into E. coli for amplification. Purified plasmids were digested with BbsI to verify if the insertion were successful. A successful insertion will result in result in an undigested plasmid, while no insertion will linearize the plasmid and give a band of around 8000 bp in an agarose gel. The agarose gel indicated that all except two of the purified plasmids (lanes 9 and 12) was successfully cloned (Figure 8). The insertion was further verified by DNA sequencing, which showed that plasmids containing guide sequence T1 and T2 for Fez1 and T1 and T2 for Fez2 was successfully cloned into the pX458 vector (Table 10).

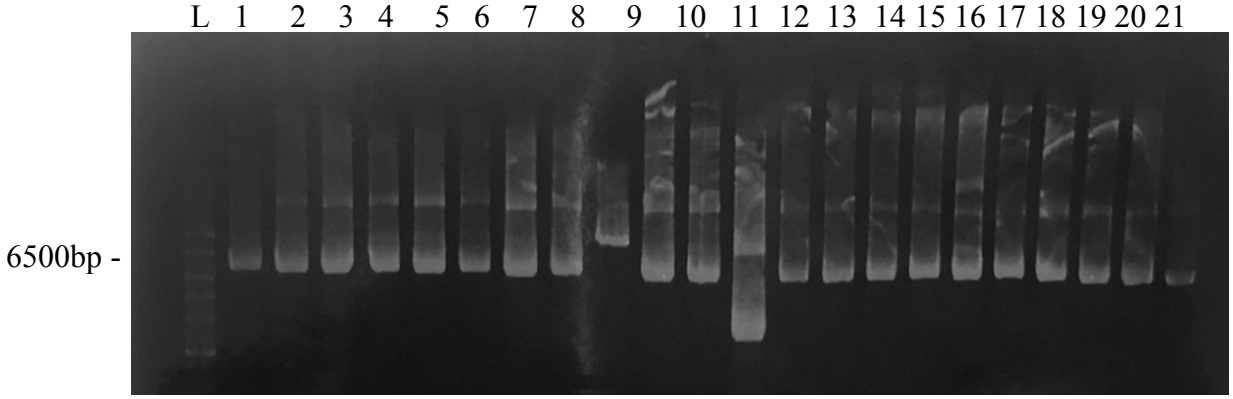


Figure 8. Agarose gel of BbsI digested pX459 plasmids ligated with Fez1 and Fez2 guide sequences. Lanes 1-5: Fez1 T1 A and B in pX458, lanes 6-10: Fez1 T2 A and B in PX459, lanes 11-15: Fez2 T1 A and B in pX459, lanes 16-21: Fez2 T2 A and B in pX459.

Verification of Fez1 and Fez2 antibodies

Two protocols is used to verify if knock out clones is established by the CRISPR/Cas9 technology, i) Western Blotting to identify cells with no expression of the targeted protein, and ii) Sequencing of the genomic region targeted by Cas9 to verify mutation of the targeted genes. Therefore we wanted to establish a WB protocol with antibodies specifically detecting Fez1 and Fez2. For this purpose, Hek293FlpIn cell lines stably expressing EGFP-Fez1 or EGFP-Fez2 were cultured in 6-well dishes, transfected with siRNA, and induced with tetracycline before harvesting the cells for western blot. siRNA targets complementary mRNA for cleavage and degradation, so that the expression of Fez1 or Fez2 protein is reduced. Hence by comparing them with cells not transfected with Fez1 or Fez2 siRNA, it is possible to detect Fez1 and Fez2 band, since these bands will be weakened upon siRNA transfection.

Four different antibodies were tested (Figure 9). The result indicates that all tested antibodies were able to specifically detect Fez1, Fez2 or both proteins except from the antibody from ABNova. The antibody from Chua et al. (Chua et al, 2012).was the most suitable antibody to use since it could detect both Fez1 and Fez2, while the remaining antibodies detected either Fez1 or Fez2.

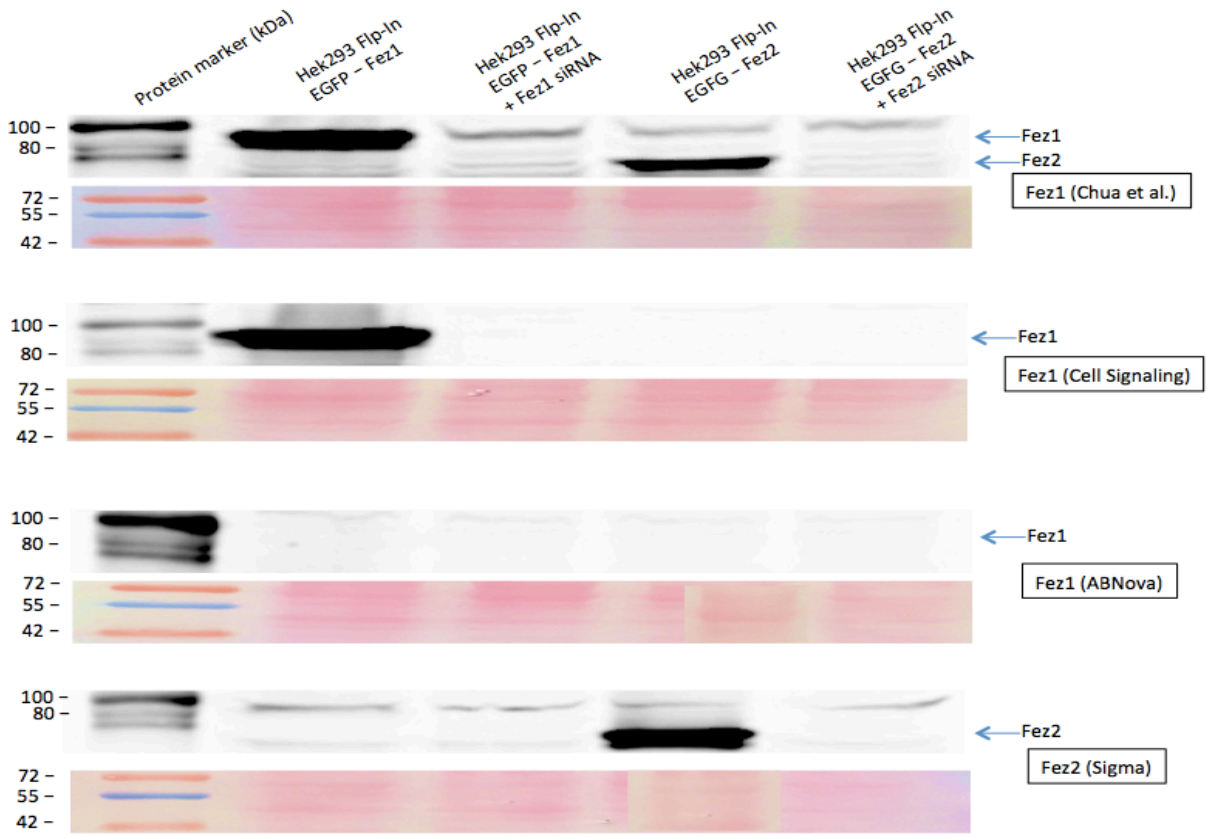


Figure 9. Three of four antibodies tested were specific for Fez1 or Fez2. *Western Blot testing Fez1 and Fez2 antibodies from different suppliers. Extracts from Hek293 Flp-In cell lines expressing EGFP-Fez1 (lanes 2,3) or EGFP-Fez2 (lanes 3,4) and transfected by siRNA (lanes 3, 5) were separated by SDS-Page, blotted to nitrocellulose membrane probed with the indicated antibodies. Ponceau staining was used as a loading control.*

No Fez1 or Fez2 knock out clones were established in Hek293 Flp-In cells

To establish knock out clones, the CRISPR/Cas9 plasmids encoding Fez1 or Fez2 guide sequences were transfected into two different cell lines; Hek293 Flp-In cell line and SH-SY5Y cell line. The Flp-In cell line is easy to reconstitute, while the SH-SY5Y cell line is a nerve cell line with high expression of Fez1. The cells were seeded in 6-well dishes, transfected with PX459 plasmids, and sorted into 96-well dishes one day post transfection. The sorting was based on GFP expression, since the pX458 vector contains the EGFP gene under a mammalian promoter. Two guide sequences were transfected together into each well, to obtain a genomic deletion in between the targeted regions. One cell were seeded in each well in the 96 well dishes. The cells were left to grow for around three weeks. Cells that had grown to visible colonies in the wells were picked and grown further for testing by western blotting and genomic PCR.

To verify if Fez1 or Fez2 were successfully knocked out, around 9-40 clones were picked from each 96-well dish, and analyzed by Western blotting (Figure 10-16). Fez1 has a molecular weight of approximately 60kDa, while Fez2 run in a gel as a 50kDa protein. As shown in Figure 10 to 16, all clones displayed two main bands of around 50 and 60kDa, indicating presence of Fez1 and Fez2. One band or the other were weakened in some clones, which may indicate partial knock-out. However, complete knock out were not seen in any clones.

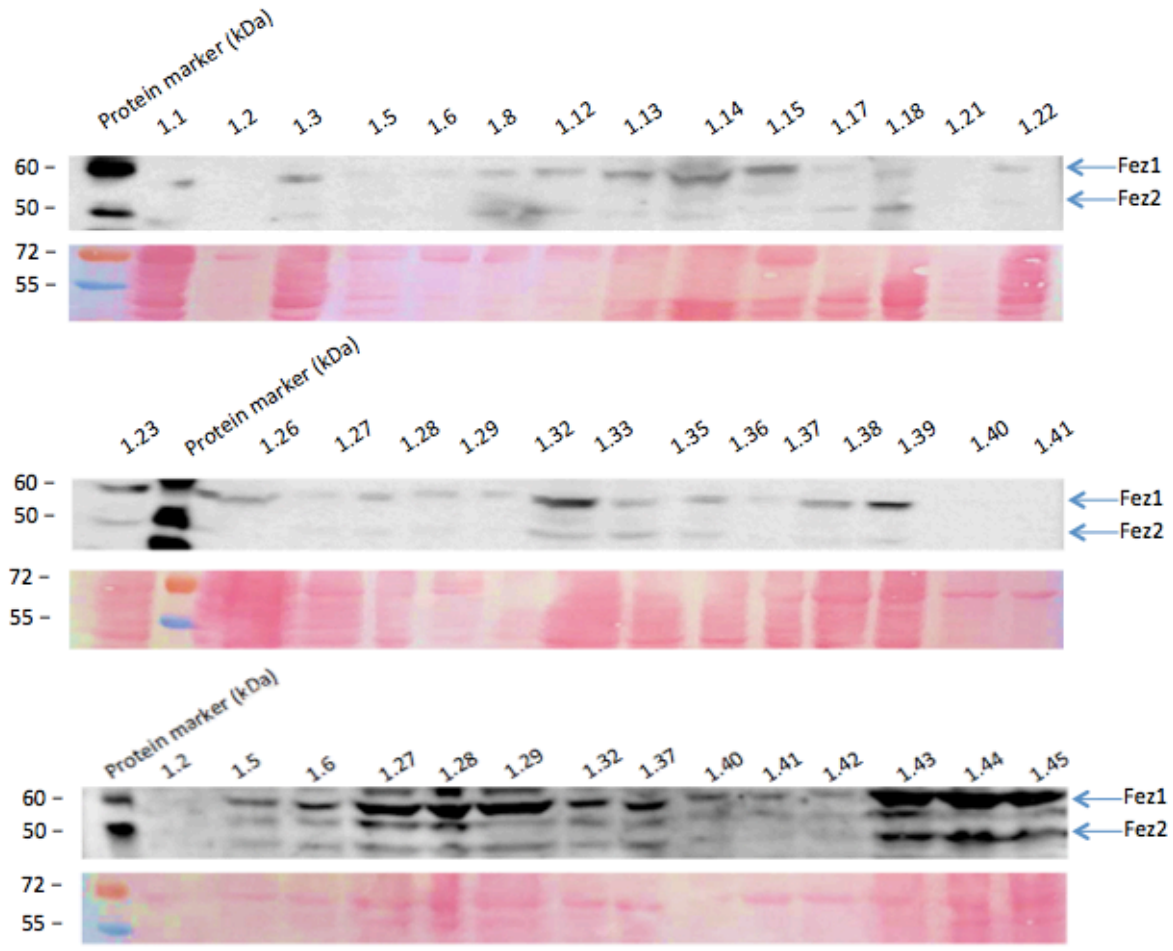


Figure 10. Western Blot of cell extracts from Hek293 Flp-In CRISPR/Cas9 clones targeting guide sequence Fez1 T1A and T1B. *Extracts from the clones were separated by SDS-Page, blotted to nitrocellulose membrane probed with the Fez1 and Fez2 antibody from Chua et al. The numbers above each lane indicate clone name. Ponceau staining was used as a loading control.*

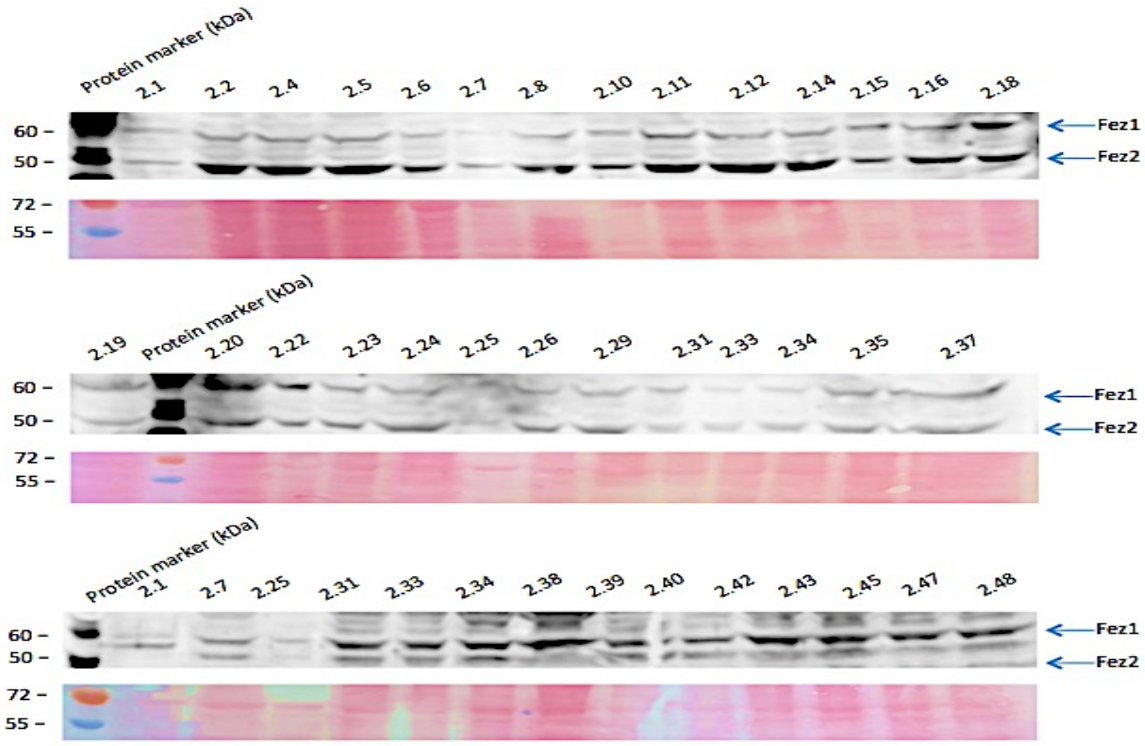


Figure 11. Western Blot of cell extracts from Hek293 Flp-In CRISPR/Cas9 clones targeting guide sequence Fez1 T2A and T2B. *Extracts from the clones were separated by SDS-Page, blotted to nitrocellulose membrane probed with the Fez1 and Fez2 antibody from Chua et al. The numbers above each lane indicate clone name. Ponceau staining was used as a loading control.*

Figure 12. Western Blot of cell extracts from Hek293 Flp-In CRISPR/Cas9 clones targeting guide sequence Fez2 T1A and T1B. *Extracts from the clones were separated by SDS-Page, blotted to nitrocellulose membrane probed with the Fez1 and Fez2 antibody from Chua et al. The numbers above each lane indicate clone name. Ponceau staining was used as a loading control.*

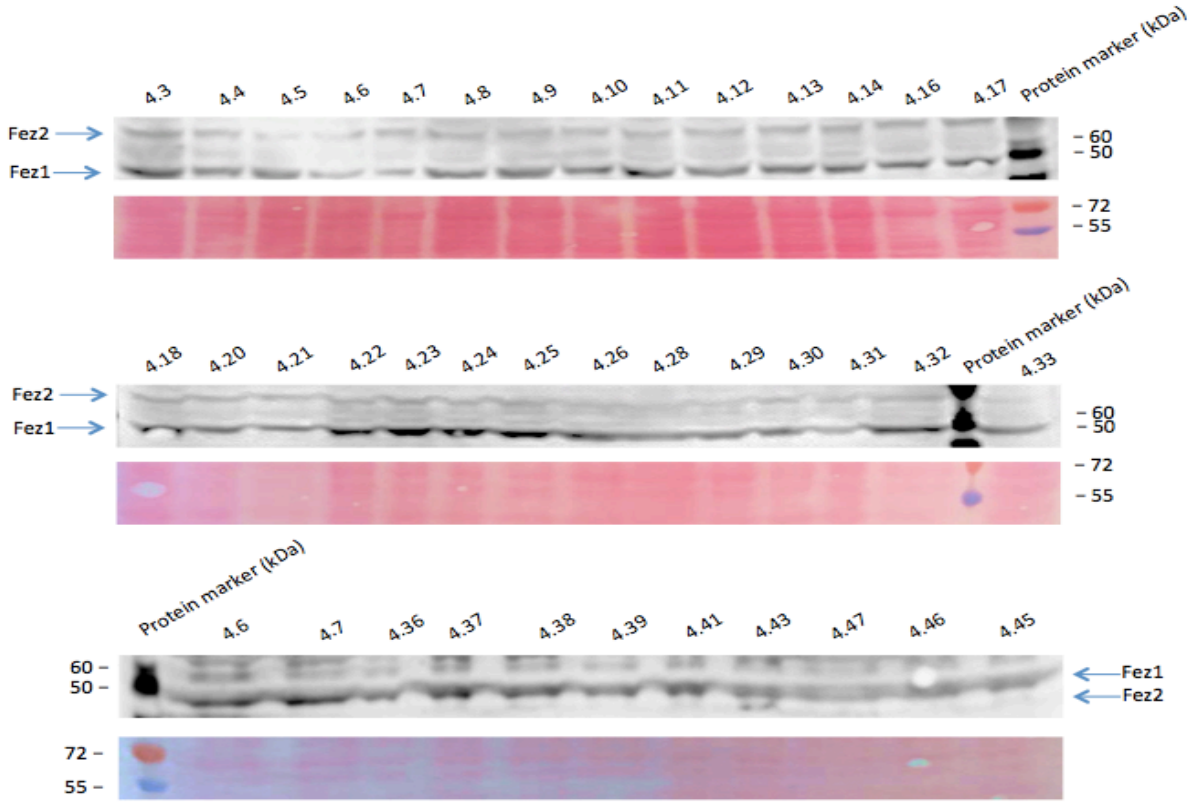


Figure 13. Western Blot of cell extracts from Hek293 Flp-In CRISPR/Cas9 clones targeting guide sequence Fez2 T2A and T2B. *Extracts from the clones were separated by SDS-Page, blotted to nitrocellulose membrane probed with the Fez1 and Fez2 antibody from Chua et al. The numbers above each lane indicate clone name. Ponceau staining was used as a loading control.*

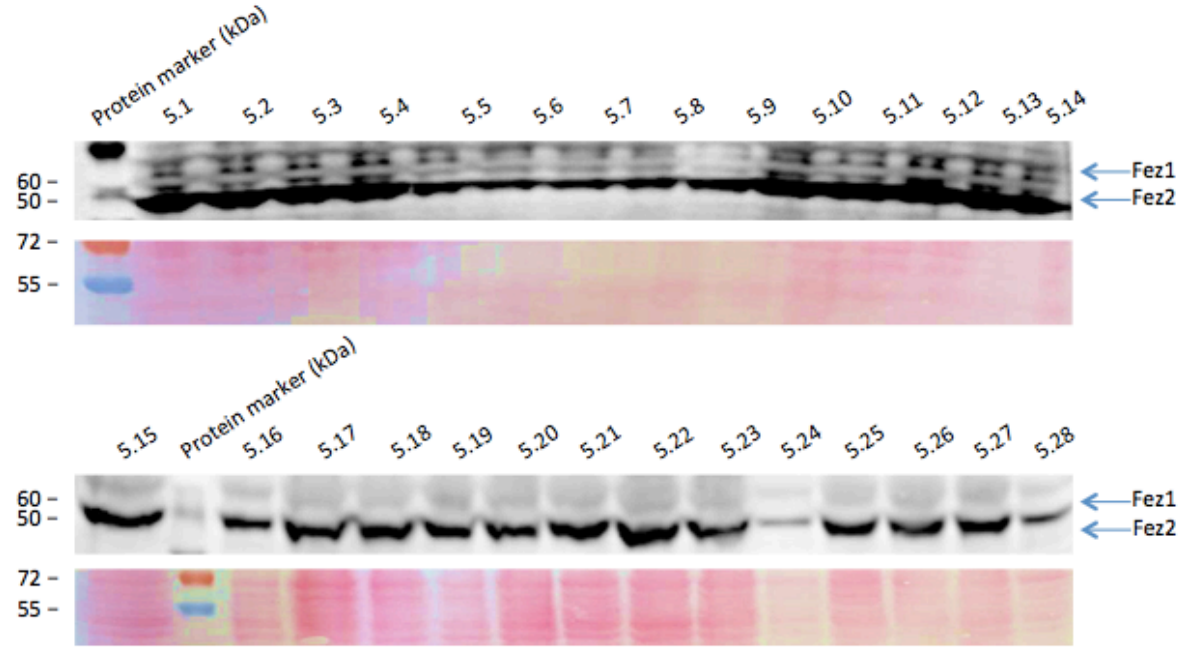


Figure 14. Western Blot of cell extracts from Hek293 Flp-In CRISPR/Cas9 clones targeting guide sequence Fez1 T1A and Fez2 T1A. *Extracts from the clones were separated by SDS-Page, blotted to nitrocellulose membrane probed with the Fez1 and Fez2 antibody from Chua et al. The numbers above each lane indicate clone name. Ponceau staining was used as a loading control.*

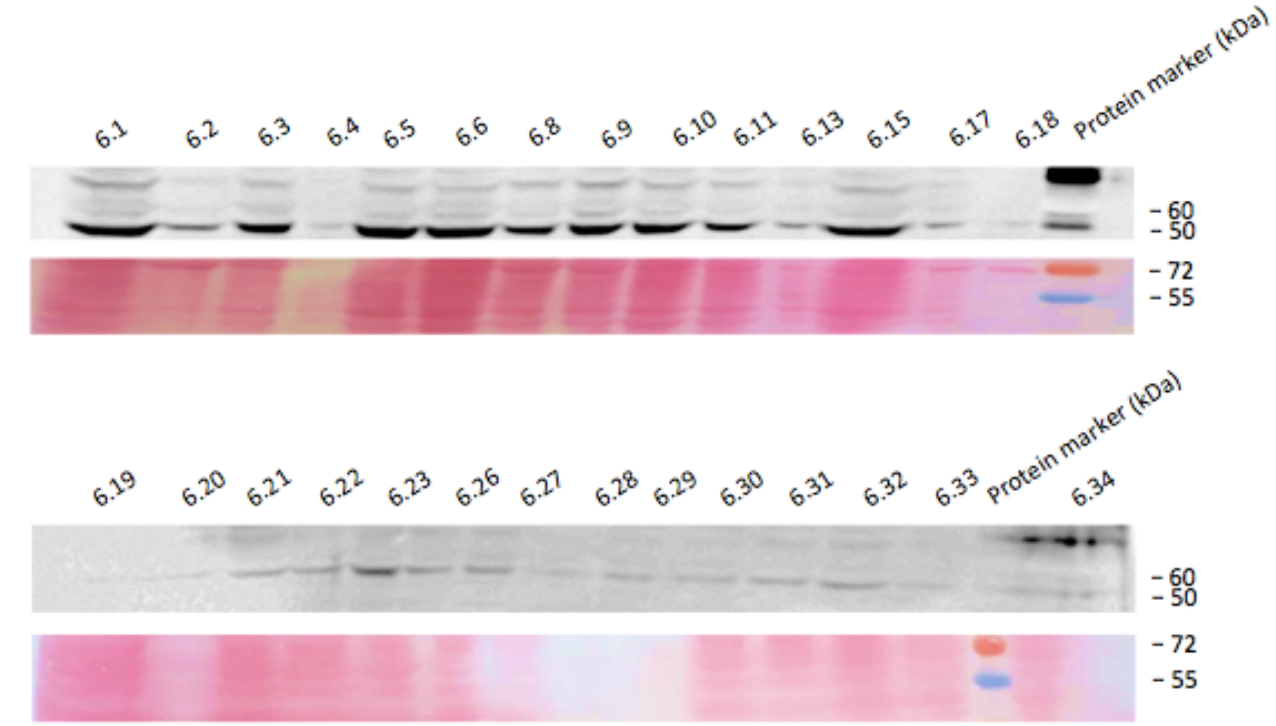


Figure 16. Western Blot of cell extracts from Hek293 Flp-In CRISPR/Cas9 clones targeting guide sequence Fez1 T2A and Fez2 T2A. *Extracts from the clones were separated by SDS-Page, blotted to nitrocellulose membrane probed with the Fez1 and Fez2 antibody from Chua et al. The numbers above each lane indicate clone name. Ponceau staining was used as a loading control.*

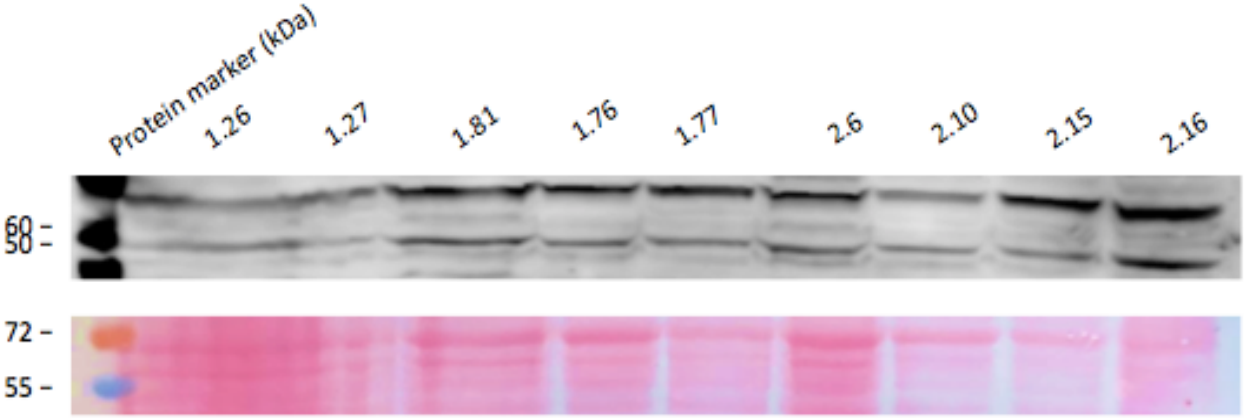


Figure 15. Western Blot of cell extracts from Hek293 Flp-In CRISPR/Cas9 clones targeting guide sequence Fez1 T1A+T1B (samples 1.26-1.77) and Fez1 T2A+T2B (samples 2.10-2.16). *Extracts from the clones were separated by SDS-Page, blotted to nitrocellulose membrane probed with the Fez1 and Fez2 antibody from Chua et al. The numbers above each lane indicate clone name. Ponceau staining was used as a loading control.*

No Fez1 or Fez2 knock out clones were established in SH-SY5Y cells

As stated above, knock out was also performed using the nerve cell line SH-SY5Y, since it has been shown that nerve cells express high levels of Fez1. Only a few clones were expanded and investigated by Western blotting (Figure 17). Unfortunately, no complete knock out clone was obtained in the SH-SY5Y cells.

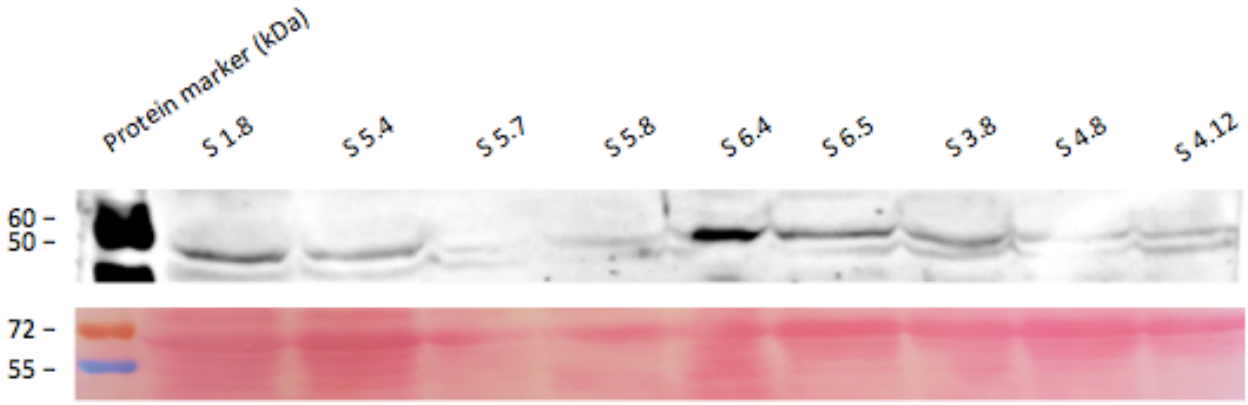


Figure 17. Western Blot of cell extracts from SH-SYS5 CRISPR/Cas9 clones targeting various guide sequences. *Extracts from the clones were separated by SDS-Page, blotted to nitrocellulose membrane probed with the Fez1 and Fez2 antibody from Chua et al. The numbers above each lane indicate clone name. Fez1 T1A+T1B (sample S1.8), Fez2 T1A+T1B (sample S3.8), Fez2 T2A+T2B (sample 4.18-4.12), Fez1 T1A+Fez2 T1A (sample S5.4-S5.8) and Fez1 T2A+Fez2 T2A (sample S6.4). Ponceau staining was used as a loading control.*

Analyzing putative Fez1 knock out clones by PCR over genomic region

As mentioned above, some of the clones analyzed by Western blotting displayed weakened Fez1 bands, which may indicate knock down. To verify these clones further, the genomic region targeted by CRISPR/Cas9 was amplified by PCR and run on agarose gel beside PCR product from non-treated cells. If the genomic locus were cut by Cas9 and repaired – a PCR product with different size than the WT locus would be expected.

Genomic DNA was isolated from the clones of interest, and used as template in a PCR reaction with PCR primers located on each side of the region targeted by the guided Cas9. The PCR products were analyzed by electrophoresis with metaphor gel (Figure 18). Except for clone 1.45 (black ring), all PCR products were similar as PCR products from WT cells, indicating no change of the genomic locus. Clone 1.45 gives 2 PCR products. One similar to WT and one shorter, suggesting that the Fez1 allele on one of the of the chromosomes were successfully knocked out.

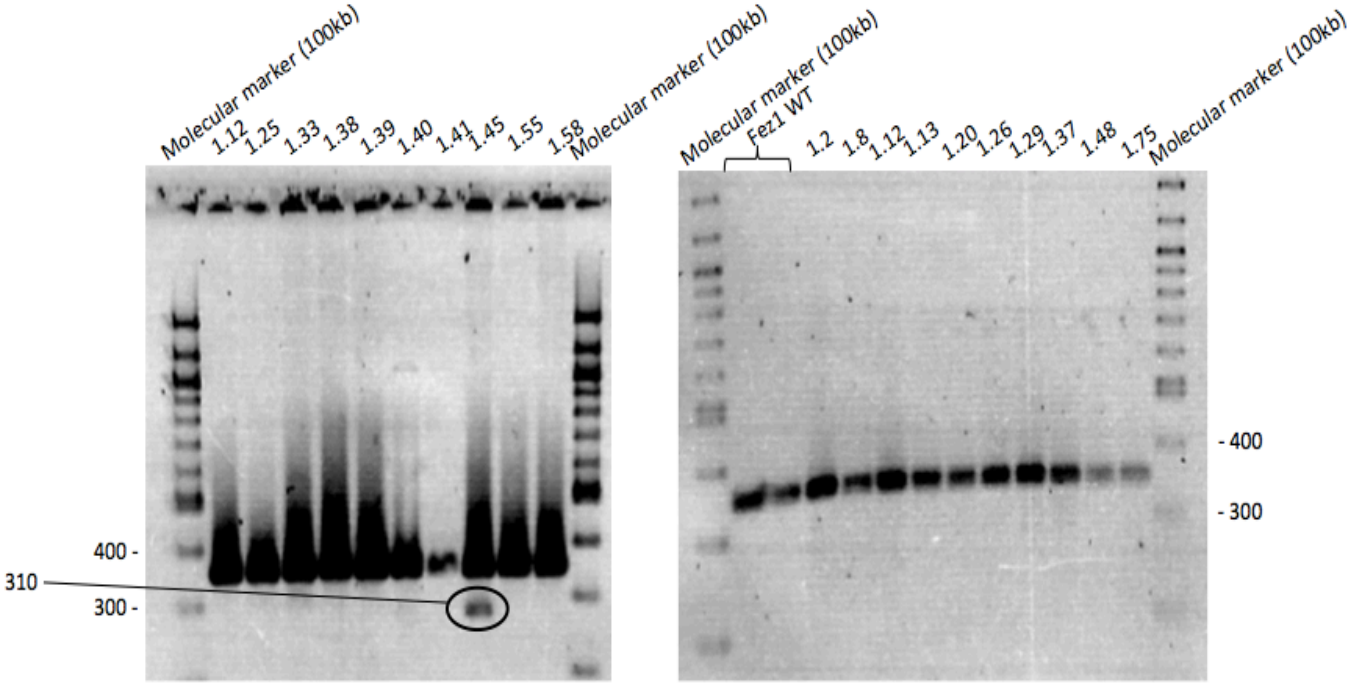


Figure 18. Agarose gel of PCR products amplified from the Fez1 genomic region of some putative knock out clones.

Fez1 colocalizes with KIF5B and tubulin

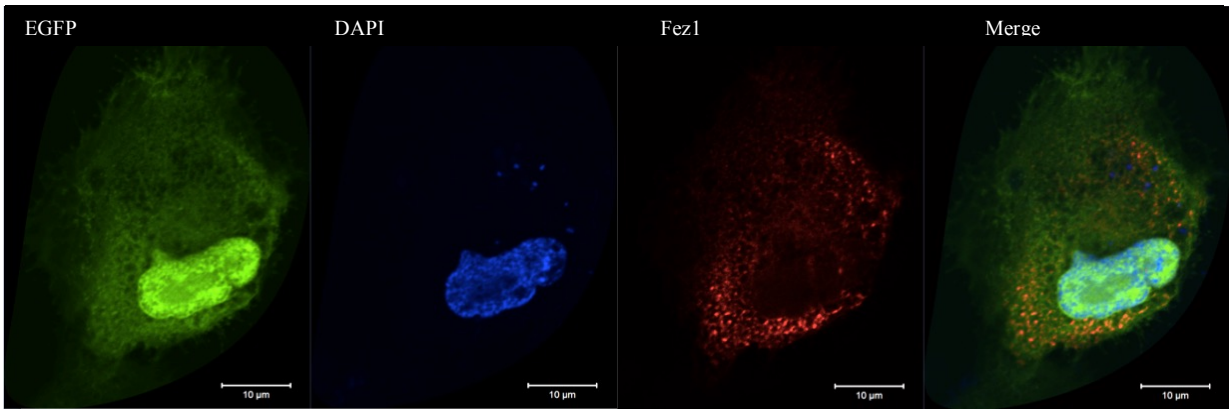
To study the localization of a protein in the cell, and identify which protein it co-localizes with, is one way to understand the function of a protein. Fez1 is reported to be involved in intracellular transport, and this is regulated by phosphorylation of serine 58 in Fez1. When S58 is phosphorylated, Fez1 binds to the transport protein KIF5B, which mediates transport along microtubules in cells. Here we used red (mCherry) fluorescence tag on Fez1 and green fluorescent tag (EGFP) on various Fez1 mutants and transport proteins, to study their localization and co-localization in cells by fluorescent confocal microscopy.

HeLa WT cells were seeded in 24-well dishes and co-transfected with vectors expressing different GFP-tagged proteins and mCherry-Fez1, while DNA were stained with DAPI.

First, the localization of mCherry-Fez1 in HeLa cells that were transfected with EGFP-control vector and mCherry-Fez1 expression vector was investigated. The transfected cells showed EGFP localization throughout the cell under normal and starved conditions (Figure 19). Whereas, Fez1 showed cytoplasmic localization under normal conditions, but seems to accumulate in the nucleus under starvation in some cases as shown in red in Figure 19B.

Next, we analyzed the localization of KIF5B and Fez1. Both proteins were cytoplasmic localized and they were strongly co-localized under both normal and starved conditions, as visualized in Figure 20.

A)



B)

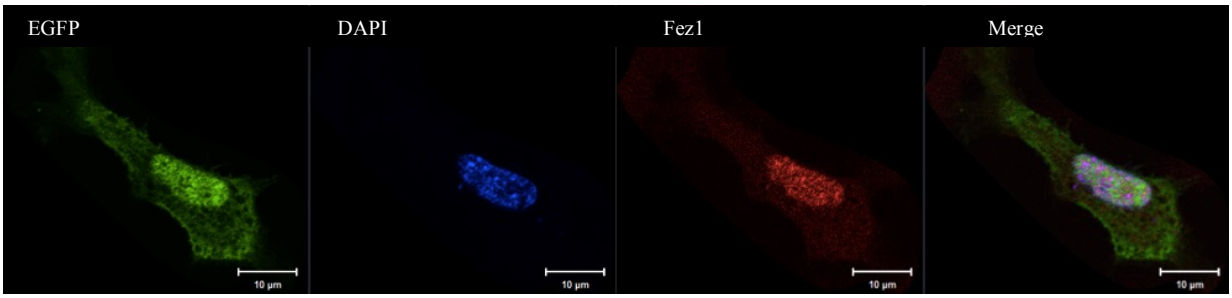
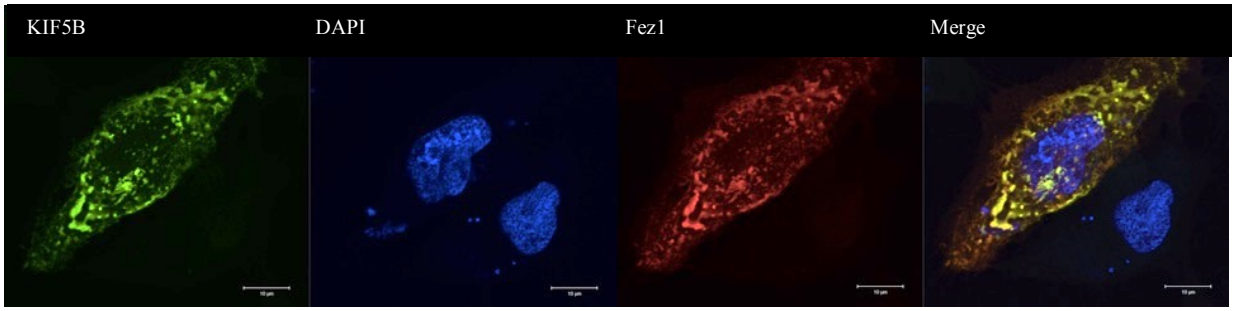


Figure 19. Localization of Fez1 and EGFP-control in HeLa WT cells*. The cells were transfected with pDEST-EGFP (green), DAPI (blue) and pDEST-EGFP-Fez1 (red) under A) normal and B) starved conditions.*

KIF5B mediates transport on microtubule filaments, and we therefore asked if Fez1 might colocalize with tubulin. Figure 21A shows that tubulin and Fez1 are cytoplasmic colocalized under normal conditions. However, it seems like both proteins were co-accumulated in dots under starved conditions (Figure 21B).

A)



B)

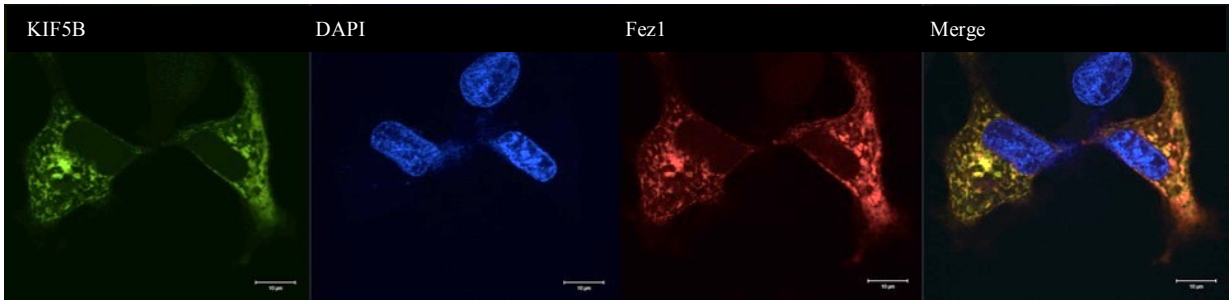
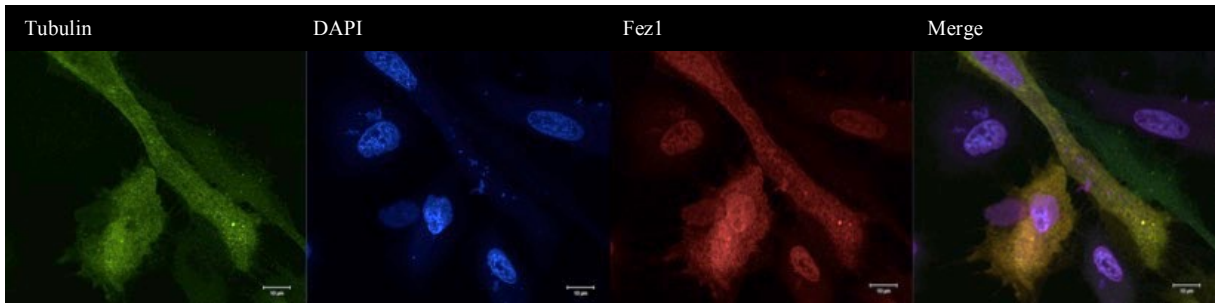


Figure 20. KIF5B is localized similarly as WT Fez1. *HeLa WT cells transfected with GFP-KIF5B (337-963) (green), DAPI (blue) and pDEST-EGFP-Fez1 (red) under A) normal and B) starved conditions.*

A)



B)

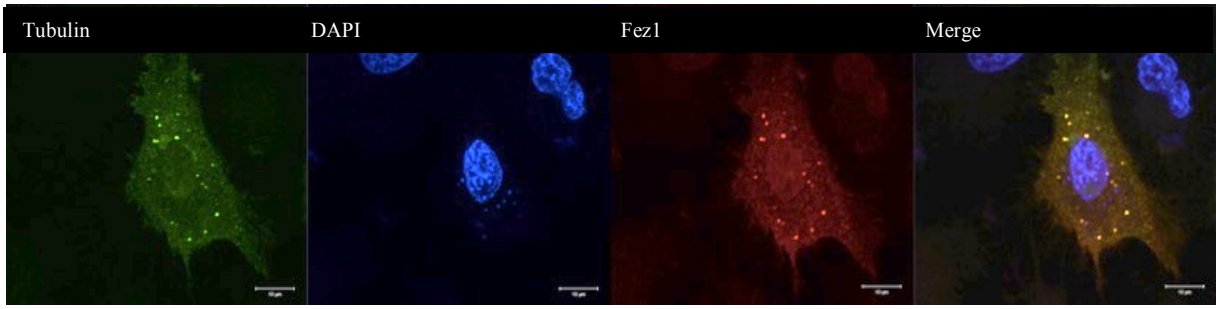
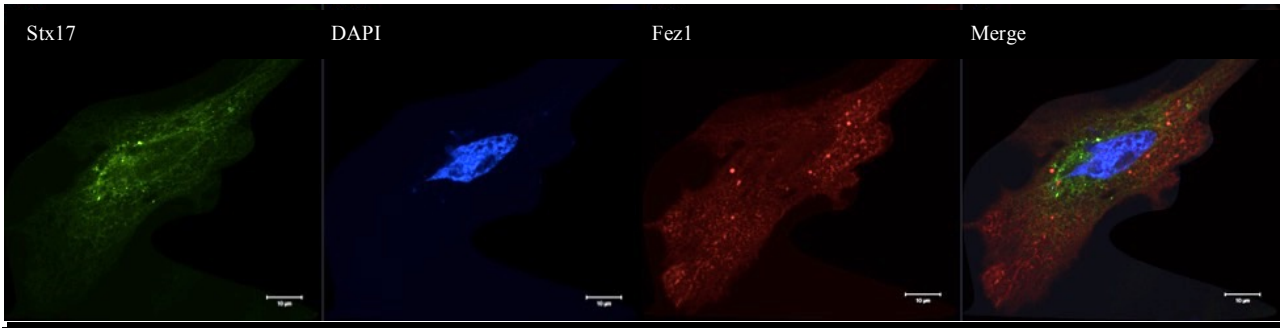


Figure 21. Tubulin is localized similarly as WT Fez1. *HeLa WT cells transfected with GFP-Tubulin (green), DAPI (blue) and pDEST-EGFP-Fez1 (red) under A) normal and B) starved conditions.*

Syntaxin 17 (Stx17) is a membrane bound protein that is associated with intracellular vesicles like autophagosomes. Co-expression of Stx17 with Fez1 shows that Stx17 and Fez1 do not co-localize under either normal or starved conditions (Figure 22)

A)



B)

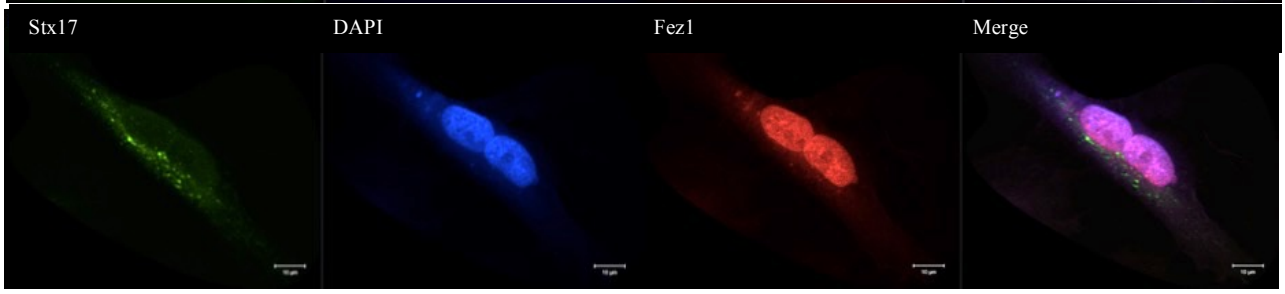


Figure 22. Syntaxin-17 were not colocalized with WT Fez1. *HeLa WT cells transfected with GFP-Stx17 (green), DAPI (blue) and pDEST-EGFP-Fez1 (red) under A) normal and B) starved conditions.*

Since phosphorylation of S58 in Fez1 is reported to regulate the Fez1-KIF5B interaction, we next tested if the S58 mutants S58A, which can not be phosphorylated, and S58E, which mimic phosphorylation, display similar localization as WT Fez1 in cells.

Fez1 and the mutant Fez1 S58A, showed to complete overlap under both normal and starved conditions (Figure 23). They are localized throughout the cytoplasm, and enriched in the same dots or cellular structures.

Similar results were obtained for Fez1 and Fez1 S58E. Interestingly, they both accumulate in a perinuclear dot both under normal (Figure 24A) and starved (Figure 24B) conditions. Furthermore, both proteins were more accumulated under starved conditions as indicated by the appearance of more dots under starved than normal condition.

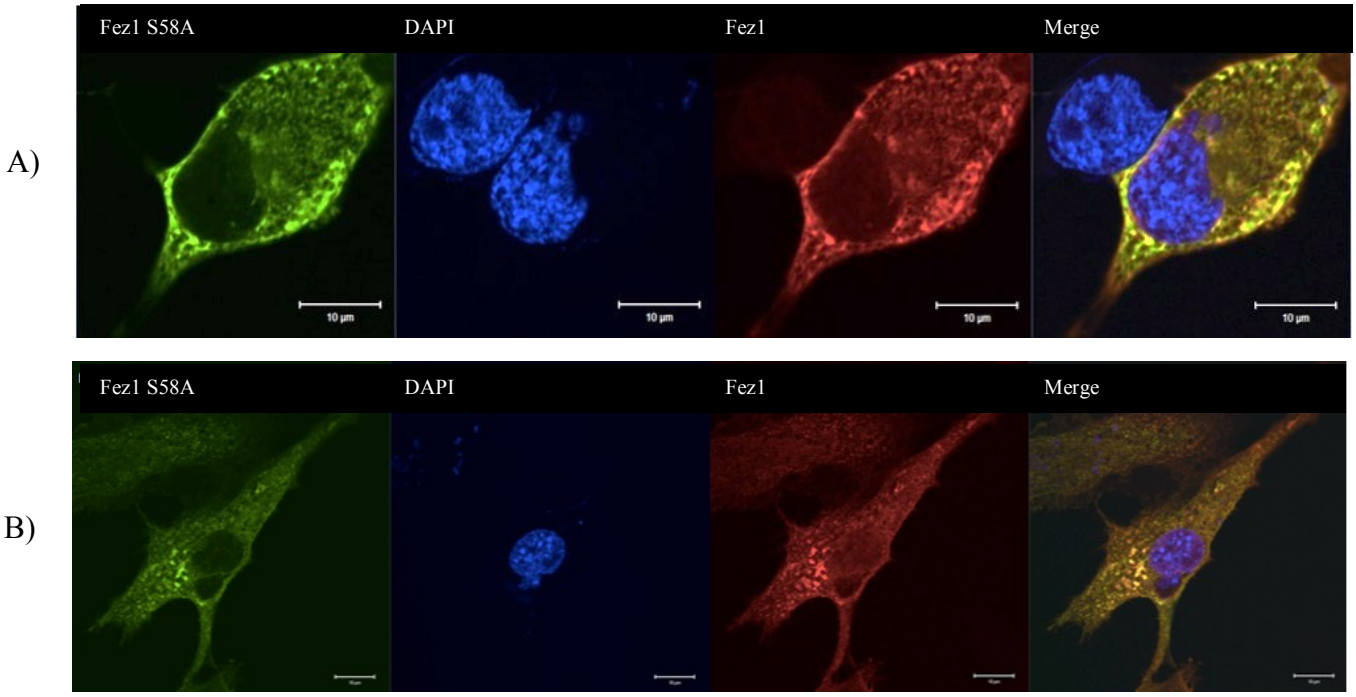


***Figure 23**. **The Fez1 S58A mutant is localized similarly as WT Fez1.** HeLa WT cells transfected with pDEST-Fez1-S58A (green), DAPI (blue) and pDEST-EGFP-Fez1 (red) under A) normal and B) starved conditions.*

A)

B)

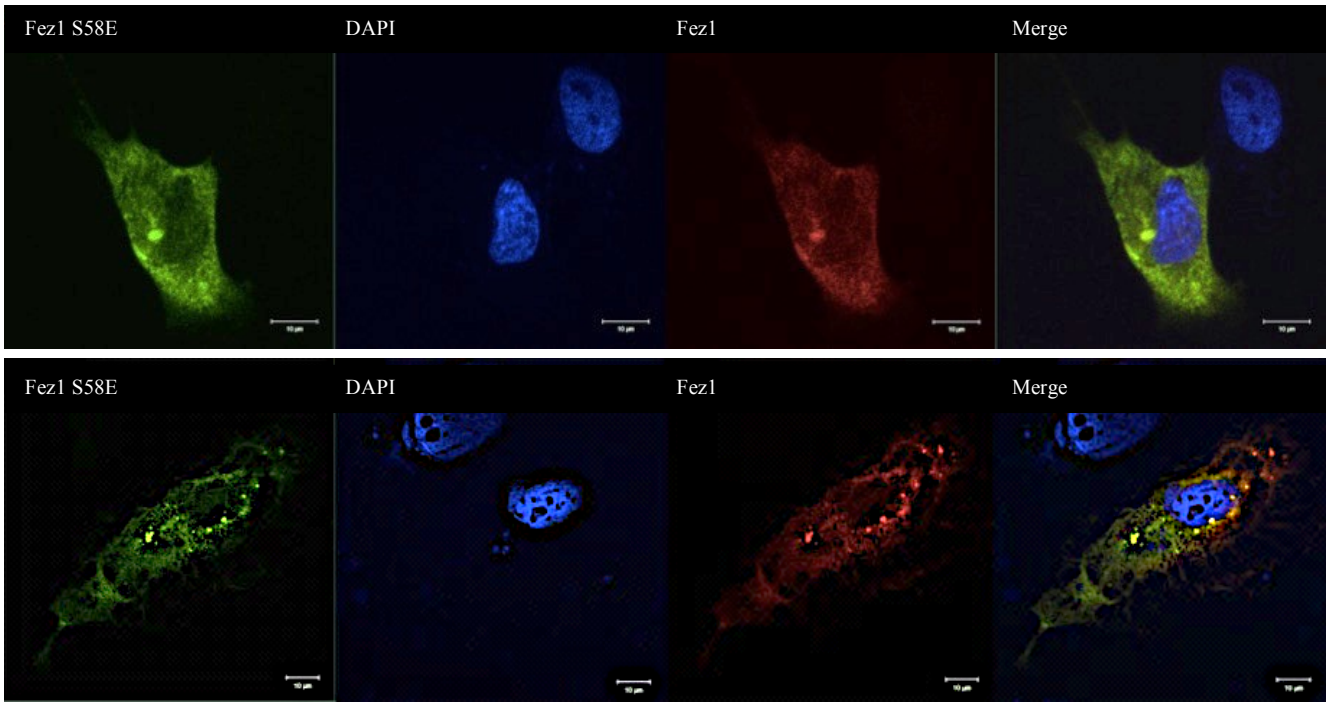


Figure 24. TheFez1 S58E mutant is localized similarly as WT Fez1. *HeLa WT cells transfected with pDEST-EGFP-Fez1 S58E (green), DAPI (blue) and pDEST-EGFP-Fez1 (red) under A) normal and B) starved conditions.*

Fez1 and Fez2 colocalize in cells

Fez2 is a homologue of Fez1, but is much less studied. In order to study if Fez1 and Fez2 are similarly localized in cells, HeLa cells were transfected with mCherry-Fez1 and EGFP-Fez2 expressing plasmids. As shown in Figure 25, Fez1 and Fez2 display a very similar localization pattern. Both proteins show cytoplasmic localizations under normal and starved conditions and they are accumulated in the same dots.

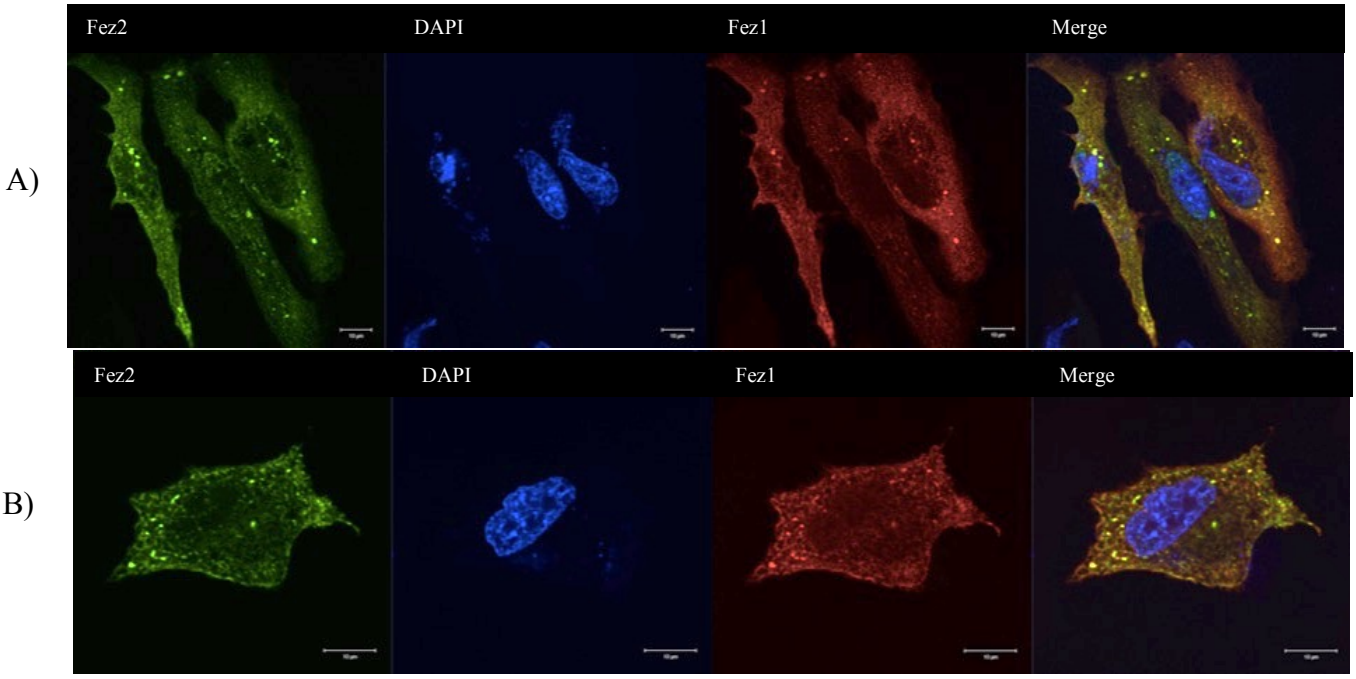


***Figure 25**. **Fez2 is localized similarly to WT Fez1.** HeLa WT cells transfected with pDEST-EGFP-Fez2 (green), DAPI (blue) and pDEST-EGFP-Fez1 (red) under A) normal and B) starved conditions.*

Fez1 colocalizes with the autophagy proteins p62, GABARAP, LC3B, and WIPI2b

Fez1 is reported to be a regulator of autophagy, inhibiting the autophagy process in cells. Here we wanted to investigate if Fez1 colocalize with proteins involved in the autophagy process. First, we co-expressed mCherry-Fez1 and the autophagy receptor protein p62 fused to EGFP in the HeLa cells. p62 forms round dots in the cytoplasm. Interestingly, Fez1 seems to be recruited to these dots (Figure 26). Furthermore, both proteins seem to accumulate under starved conditions compared to under normal conditions. Hence, Fez1 co-localizes very well with the autophagy receptor p62.

A)

B)

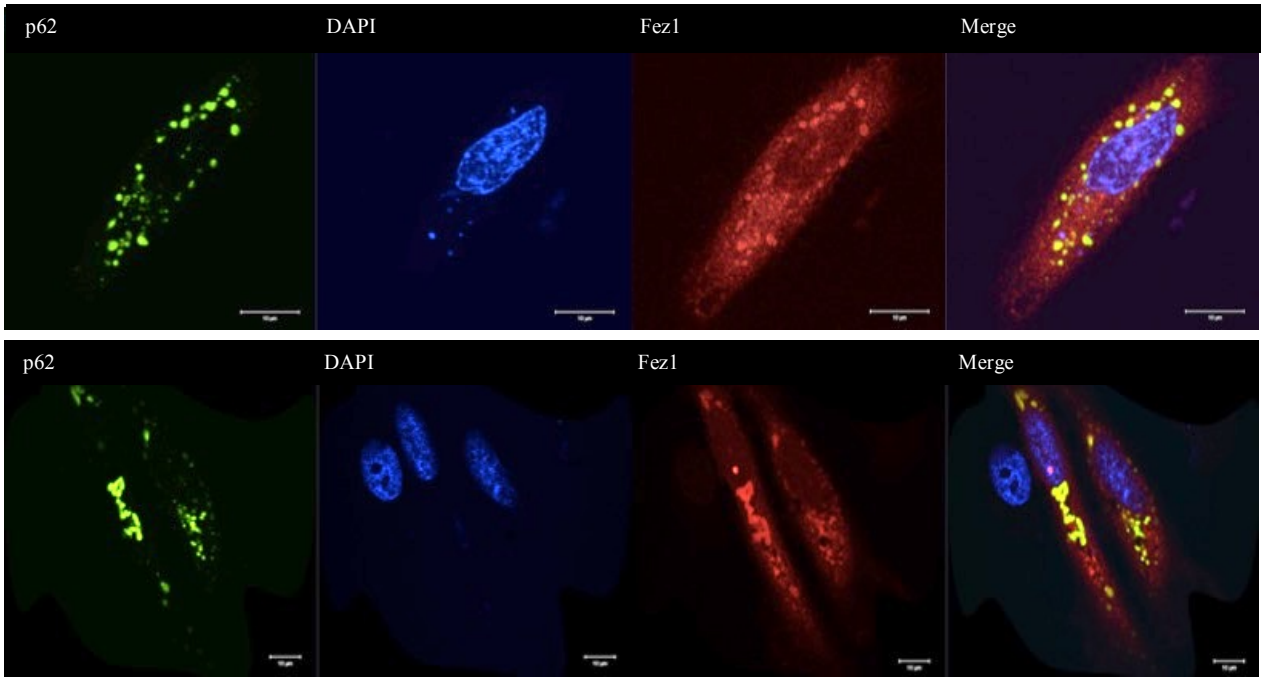
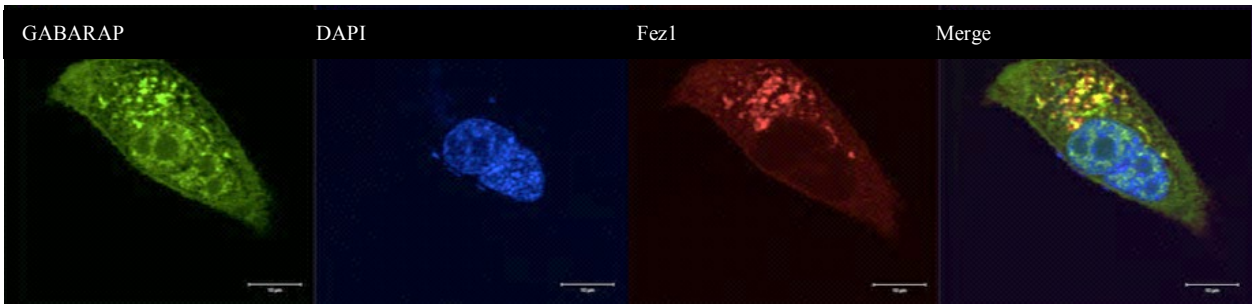


Figure 26. p62 is localized similarly as WT Fez1. *HeLa WT cells transfected with pGFP-p62 (green), DAPI (blue) and pDEST-EGFP-Fez1 (red) under A) normal and B) starved conditions.*

GABARAP and LC3B are members of the ATG8 family of autophagy proteins. Upon induction of autophagy, they are lipidated and associate with the autophagosome membrane. Autophagy receptors bind to the ATG8s in the autophagosome membrane, and thereby bring the cargoes that are going to be degraded to the autophagosome. Since Fez1 colocalized with the autophagy receptor p62, we next questioned if Fez1 colocalizes with the Atg8 proteins GABARAP and LC3B. HeLa cells were transfected with expression plasmids for mCherry-Fez1 and EGFP-Gabarap or EGFP-LC3B. Interestingly, Fez1 colocalized with both Gabarap (Figure 27) and LC3B (Figure 28) in certain dots in the cytoplasm. It was also observed that LC3B is upregulated under starved conditions.

A)



B)

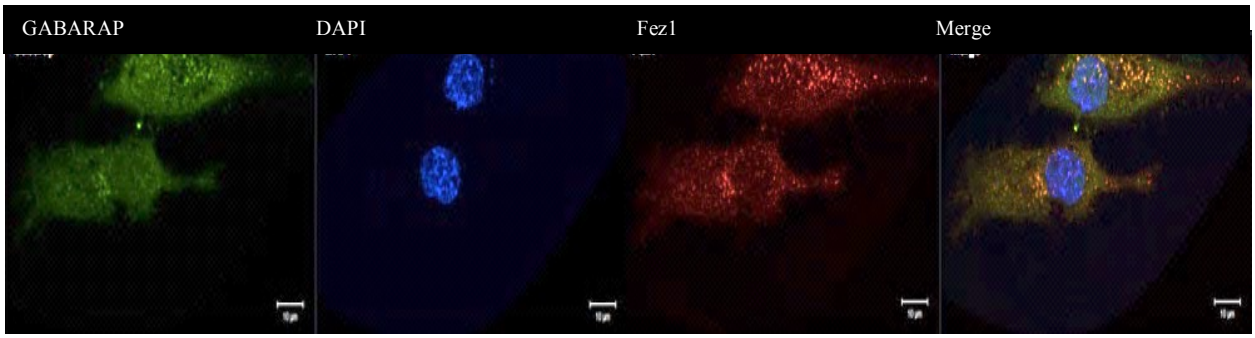
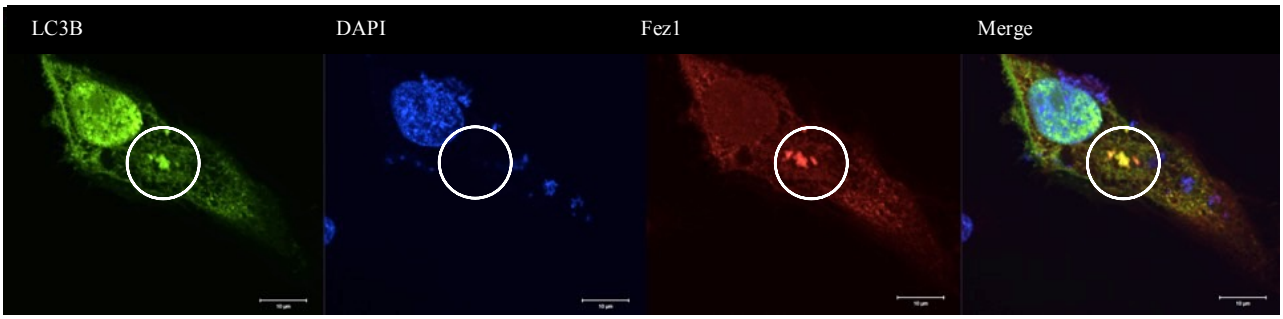


Figure 27. GABARAP is localized similarly as WT Fez1. *HeLa WT cells transfected with pDEST-EGFP-Gabarap (green), DAPI (blue) and pDEST-EGFP-Fez1 (red) under A) normal and B) starved conditions.*

A)



B)

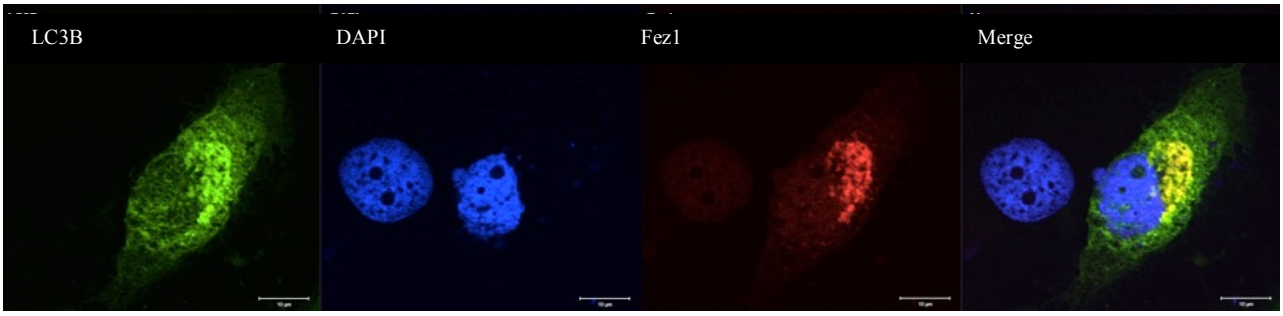


Figure 28. LC3B is localized similarly as WT Fez1. *HeLa WT cells transfected with pDEST-EGFP-LC3B (green), DAPI (blue) and pDEST-EGFP-Fez1 (red) under A) normal and B) starved conditions.*

The autophagy receptors and the Atg8 proteins are mainly involved in the late processes of autophagy. To investigate if Fez1 could be implicated in regulation of the early processes of autophagy, localization of mCherry-Fez1 and EGFP-WIPI2 was investigated. WIPI2 binds to phosphorylated lipids and is involved in recruiting the proteins involved in the early steps of autophagosome formation. Notably, Fez1 is enriched in the WIPI2 dots, and the two proteins seem to be colocalized both under normal and starved conditions. Furthermore, it seems like they both were upregulated under starved conditions in relations to normal conditions (Figure 29).

A)

B)

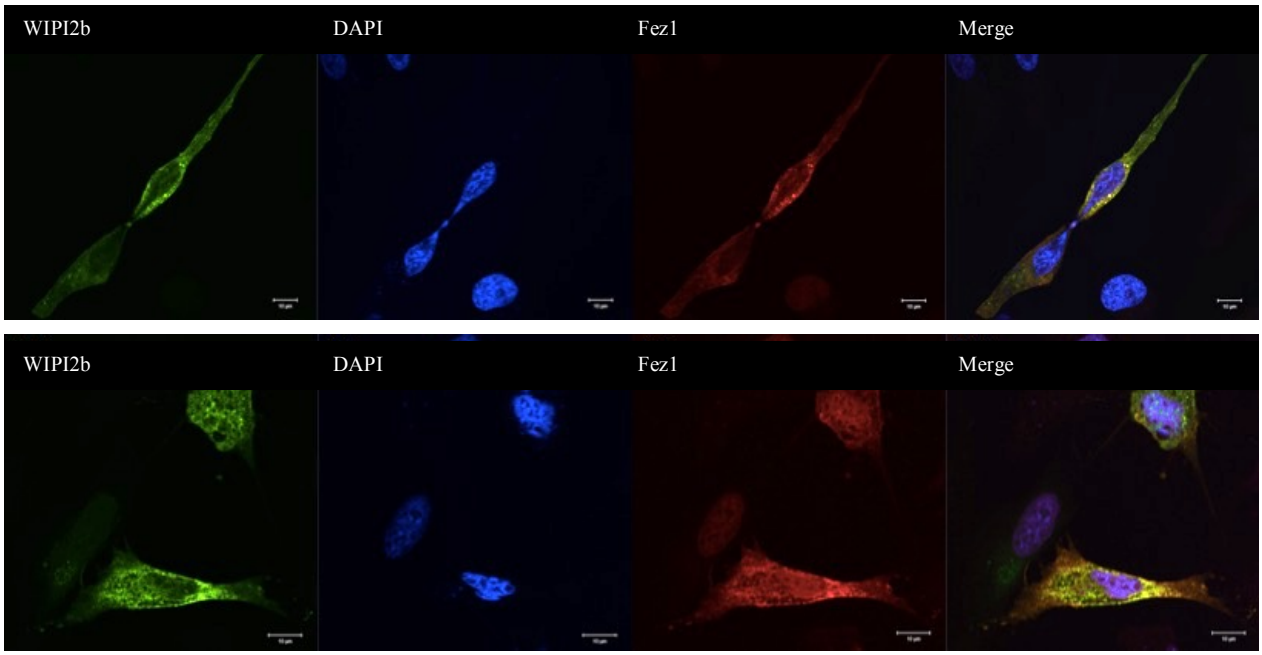


***Figure 29**. **WIPI2b is localized similarly as WT Fez1.** HeLa WT cells transfected with GFP-WIPI2b (green), DAPI (blue) and pDEST-EGFP-Fez1 (red) under A) normal and B) starved conditions.*

All together, these over-expression studies indicate that Fez1 may interact with proteins involved both in the early and in the late stages of autophagy. Furthermore, Fez1 also colocalize with KIF5B and tubulin, which both may be involved in transport of autophagy vesicles or proteins involved in the autophagy process.

Over-expression of Fez1 or Fez2 does not impair LC3B lipidation

The colocalization of over-expressed Fez1 with several autophagy proteins raised the question whether Fez1 over-expression has an effect on autophagy flux. LC3B is used as an indicator for autophagy flux and displays two bands on an SDS-PAGE gel, where the upper and lower band represents LC3B-I and LC3B-II respectively. LC3B-II is the lipidated form that is associated with the autophagosome membrane. Change in the LC3B-II form relative to the LC3B-I form indicates change in the autophagic activity. To investigate the effect of Fez1 and Fez2 on LC3B, the Hek293 Flp-In EGFP-Fez1 and EGFP-Fez2 cell lines, in which the Fez1 and Fez2 expression could be turned on by tetracycline, were used. LC3B-I and LC3B-II levels in cell extracts from cells tetracycline treated or not treated were analyzed by Western blotting (Figure 30). Figure 30 shows that expression of all EGFP-Fez1/Fez2 proteins were successfully induced by tetracycline. However, the LC3B-II/LC3B-I ration within each lane seems relatively unchanged. This indicates that the Fez1/Fez2 overexpression in these cell lines do not affect the autophagic flux.

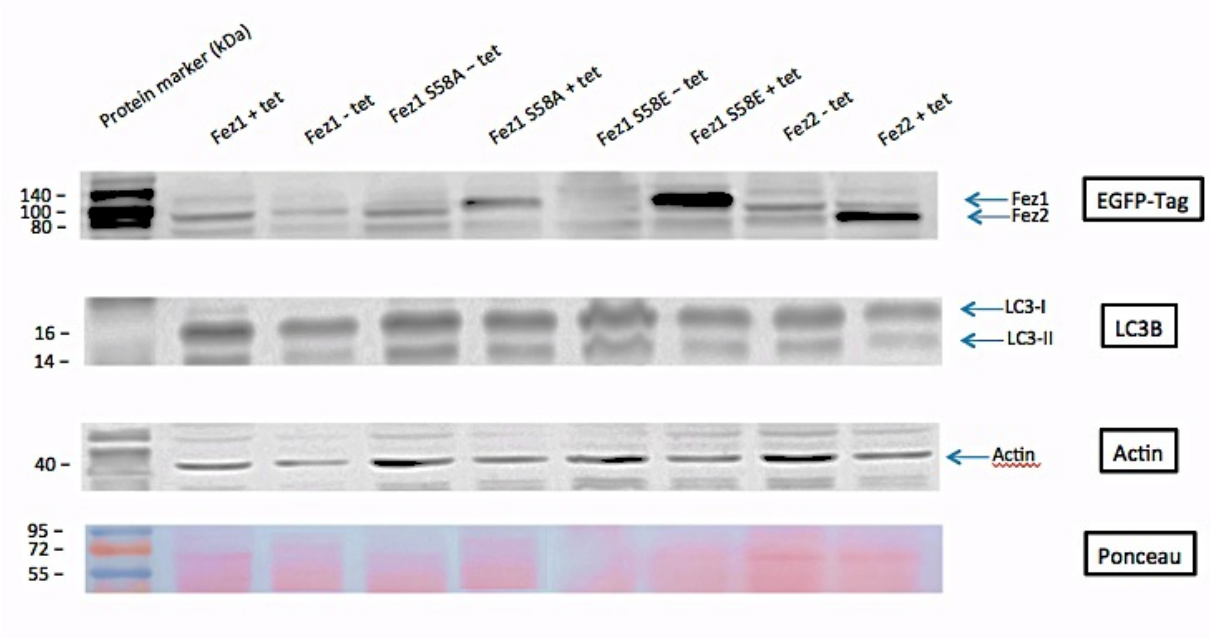


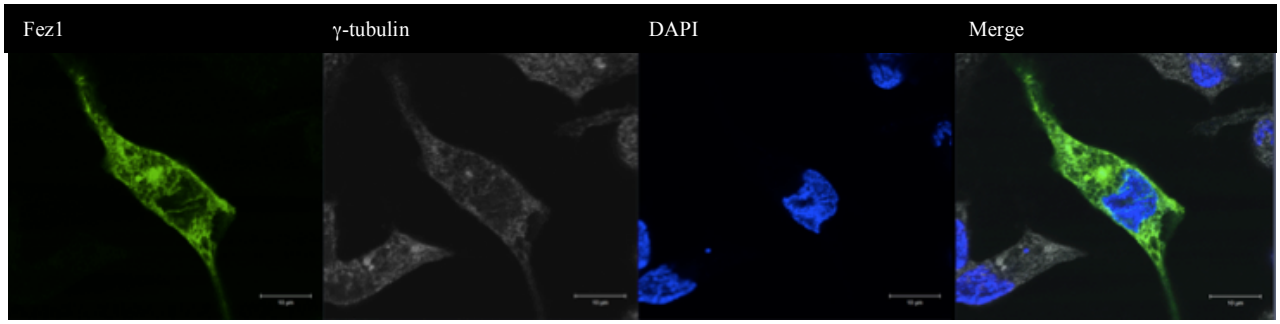
Figure 30. Western blot analysis of extracts from various Hek293 Flp-In cell types. *Extracts from Hek293 Flp-In cell lines induced with tetracycline (EGFP-Tag) detected for autophagy activity (LC3B) and loading control(Actin).*

Fez1 colocalized with endogenous γ-tubulin

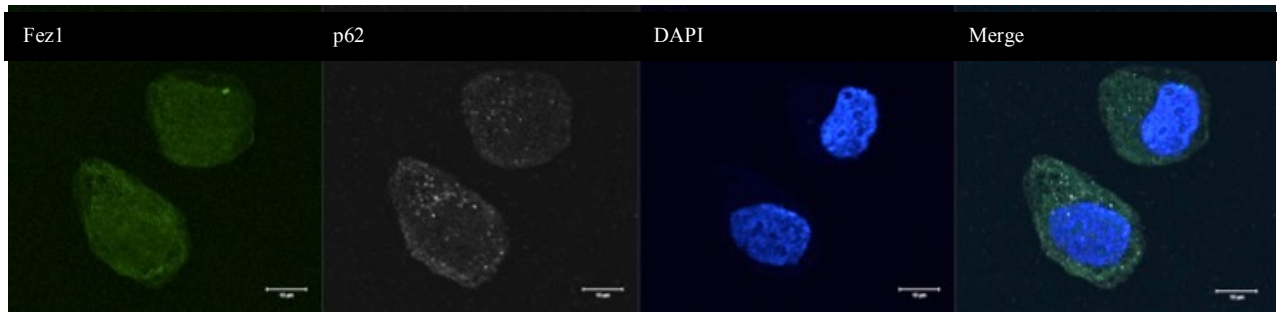
The results presented above, indicated that Fez1 colocalize both with autophagy proteins and transport proteins when it is over-expressed in cells. To verify this at a more endogenous level, localization studies were performed in the Hek293 Flp-In EGFP-Fez2 and EGFP-Fez2 expression cell lines. Instead of over-expressing the proteins we found to colocalize with Fez1/Fez2, the localizations of the endogenous proteins were analyzed by immunohistochemistry. The various Hek293 Flp-In cell lines were seeded on coverslips, expression of EGFP-Fez1 or EGFP-Fez2 proteins induced by Tetracycline for 24 hours, before the cells were fixed and stained with different antibodies. The DNA was stained with DAPI, and localization studies performed by fluorescence confocal microscopy. Only cells incubated at normal conditions were included in the results, since starvation led to a massive cell death leaving very few cells on the coverslips.

First, the localization of WT Fez1 was compared with the localization of p62, γ-tubulin and Gabarap. EGFP-Fez1 was cytoplasmic localized in most cells, but in some cells it was observed in the nucleus, as shown in Figure 31B. Similar to Fez1, both γ-tubulin and p62 were mainly cytoplasmic localized. Gabarap, on the other hand, seems to be localized both in the nucleus and in the cytoplasm throughout the cell (Figure 31C). Interestingly, Fez1 is strongly colocalized with γ-tubulin, and is enriched in the perinuclear γ-tubulin dot (Figure 31A). p62 accumulates into cytoplasmic dots, but these were not present for Fez1 (Figure 31B). Furthermore, there was no clear colocalization between Fez1 and Gabarap (Figure 31C). These results indicate that at the endogenous level and under normal conditions, Fez1 colocalize strongly with γ-tubulin, while colocalization with p62 and GABARAP was not detected.

A)



B)



C)

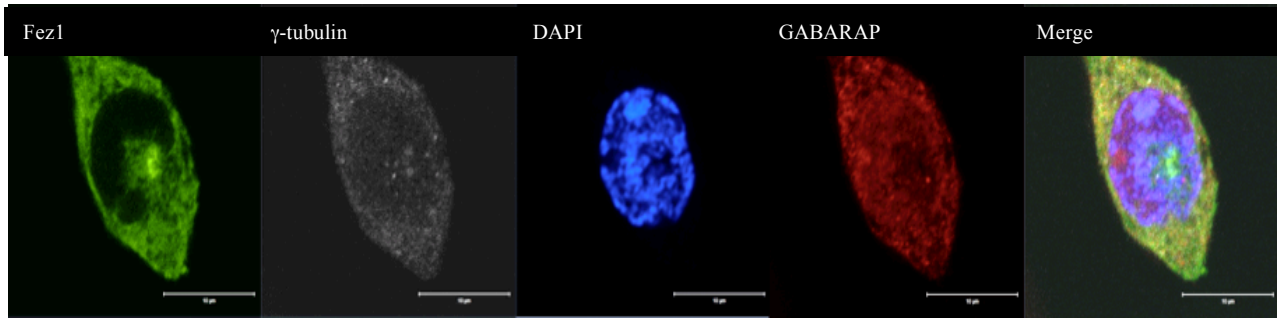
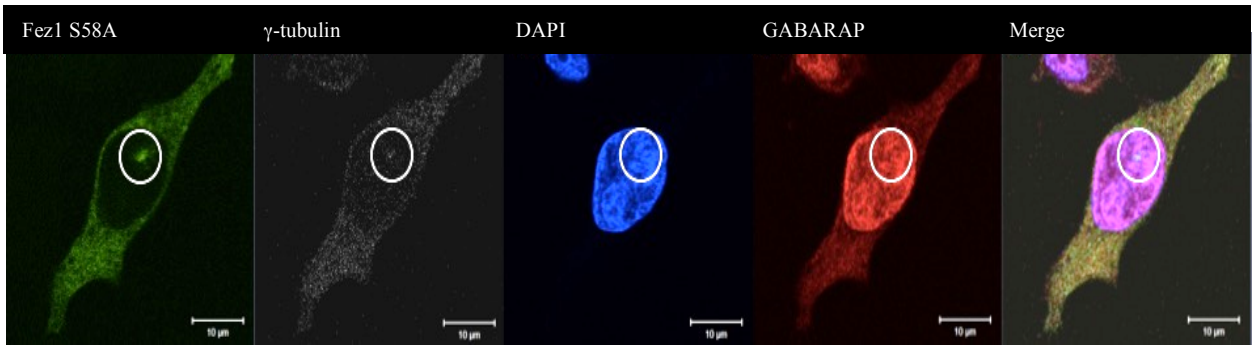


Figure 31. Fez1 colocalize with γ-tubulin, and is enriched in the perinuclear γ-tubulin dot. *Hek293 Flp-In EGFP-Fez1 cells stained with DAPI (blue), A) γ-tubulin (white). B) p62 (white). C) γ-tubulin (white) and Gabarap (red). D) γ-tubulin (white)*

Both Fez1 S58A and S58E colocalize strongly with γ-tubulin

As mentioned above, the role of Fez1 in intracellular transport is regulated by phosphorylation of the S58 site. Since Fez1 was found to colocalize strongly with γ-tubulin, it was next investigated if mutation of the S58 site would affect this co-localization. However, as displayed in Figure 32 and Figure 33, the Fez1 S58A and S58E mutants displayed a very similar localization pattern as the WT Fez1. Both mutants colocalized with γ-tubulin, and was accumulated in the perinuclear γ-tubulin dot. Interestingly, in the Fez1 S58E cell line, also Gabarap was recruited to the γ-tubulin dot, partially overlapping with Fez1 S58E.

A)



B)

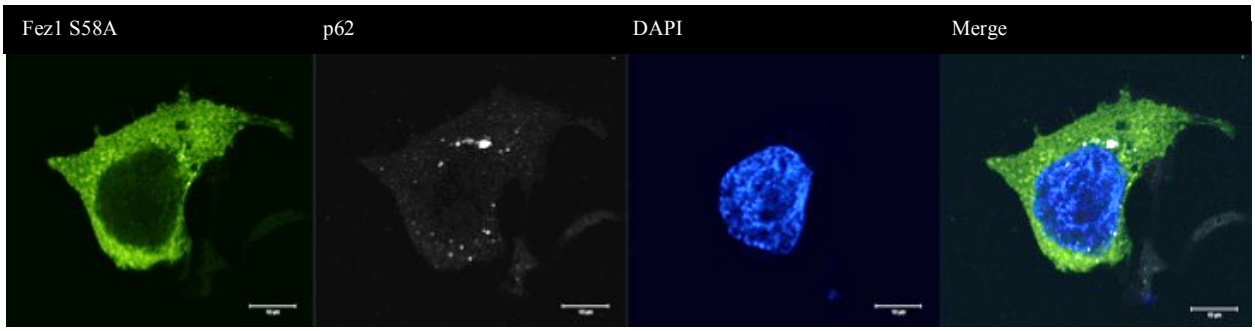
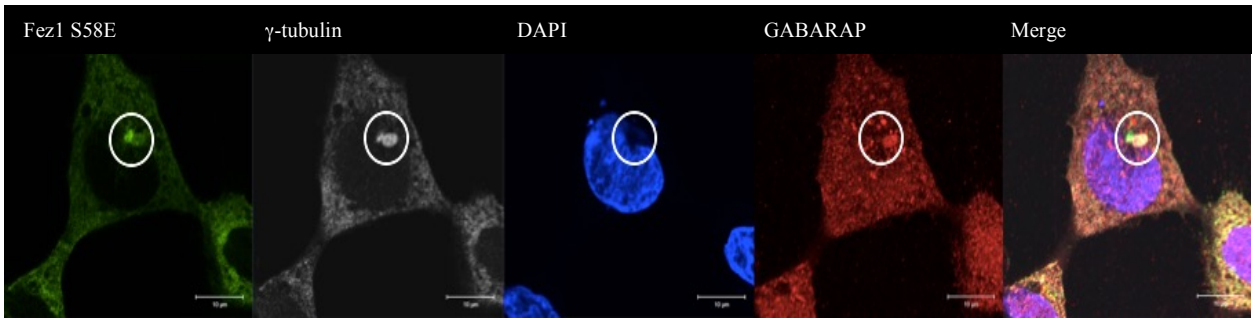


Figure 32. Fez1 S58A colocalize with γ-tubulin, and is enriched in the perinuclear γ-tubulin dot. *Hek293 Flp-In cells EGFP-Fez1 S58A (green) and A) γ-tubulin (white) and Gabarap (red). B) p62 (white).*

A)



B)

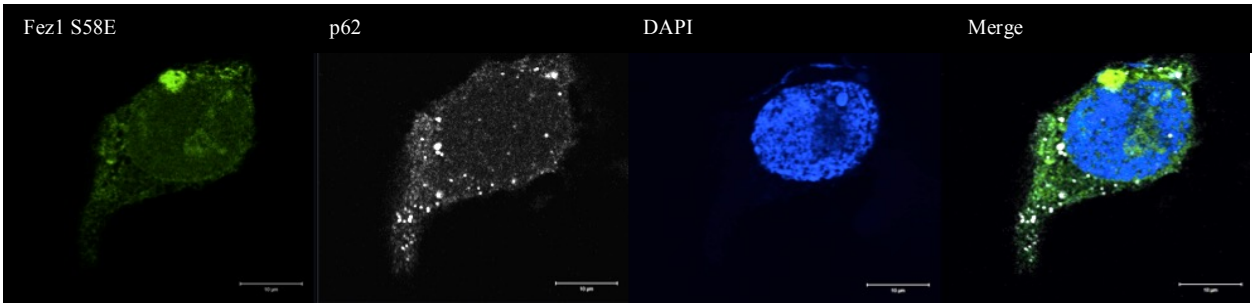


Figure 33. Fez1 S58E colocalize with γ-tubulin, and is enriched in the perinuclear γ-tubulin dot. *Hek293 Flp-In Fez1 S58E (green) and A) normal condition; γ-tubulin (white). B) normal condition; p62 (white) and C) starved condition; p62 (white).*

Fez2 is strongly enriched in the perinuclear γ-tubulin dot

The results presented above indicated that Fez1 and its homologue fez2 is similarly localized in the cell. In order to investigate if Fez2 co-localize with γ-tubulin as Fez1, and not with p62 and Gabarap, the Hek293 Flp-In EGFP-Fez2 cell line was immunostained with antibodies against γ-tubulin, p62 or Gabarap. According to confocal analysis (Figure 34), Fez2, γ-tubulin and p62 are cytoplasmic localized, while GABARAP displays both nuclear and cytoplasmic localization. Interestingly, Fez2 is strongly enriched in the perinuclear γ-tubulin structures, and seem to be perinuclear colocalized (Figure 34A and 34B). p62 forms any cytoplasmic dots as seen before, and many of these are localized in or close to the perinuclear γ-tubulin structure (Figure34C).

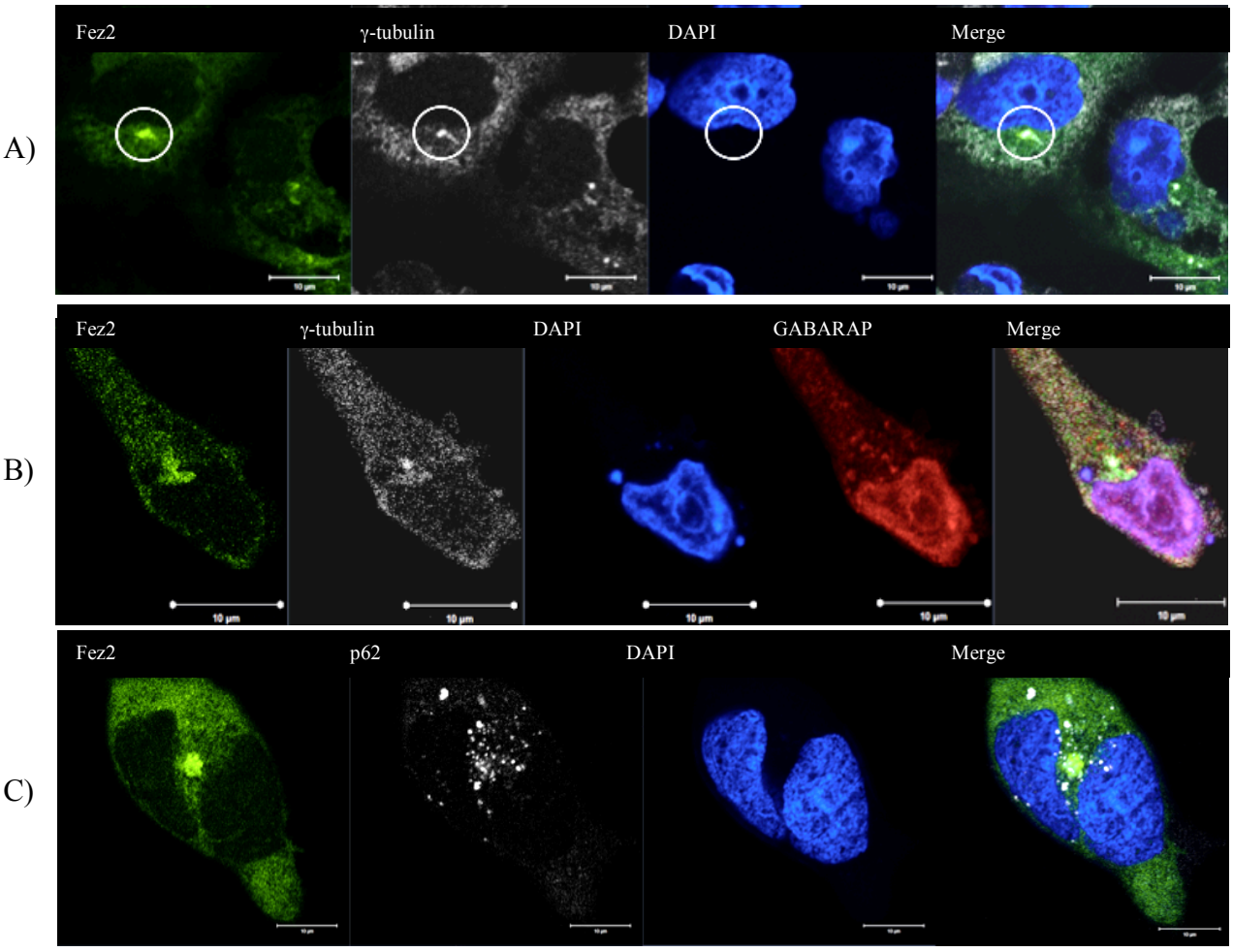


Figure 34. **Fez2 is enriched in the perinuclear γ-tubulin structure.** *Hek293 Flp-In EGFP-Fez2 (green) and A) γ-tubulin (white) B) γ-tubulin (white) and Gabarap (red) C) p62 (white)*

To summarize, Fez1 or Fez2 colocalized with p62, γ-tubulin, and Gabarap when these proteins were over-expressed together with Fez1 or Fez2. However, in Hek293 Flp-In cells with stable, low-level expression of Fez1 or Fez2, both proteins were found to colocalize strongly with γ-tubulin – but the colocalization with p62 and Gabarap was not clearly observed.

Discussion

Knock out of Fez1 or Fez2 did not succeed after testing around 200 cell clones

In this study, the CRISPR/Cas9 technology was used in an attempt to knock out Fez1 and/or Fez2 in Hek293 Flp-In cells and SH-SY5Y cells. CRISPR/Cas9 plasmids specifically targeting the Fez1 or Fez2 gene were established by cloning, and transfected into Hek293 Flp-In cells and SH-SY5Y cells. A cell-sorting machine was then used to sort successfully transfected cells into 96-well dishes, one individual cell per well. Clones that grew were cultured further in 6-well dishes, before cell extracts were harvested and genomic DNA isolated. Western blotting and genomic PCR were thereafter used to screen for knock out clones. However, none of the around 200 clones tested were successfully knock out.

The result suggests that the Fez1 and Fez2 genes may be essential for cell survival and/or cell growth. This is in line with a previous attempt to establish Fez1 and/or Fez2 knock out (Schäfer (2016). In the previous experiment setup, a more inefficient Cas9 enzyme was used, the Cas9n enzyme that is less expected to generate off-target effects. The Cas9n enzyme makes a nick instead of a double-strand cut as the Cas9 enzyme makes. In this study the efficient Cas9 enzyme was used. However, no knock out cells were established even if a large amount of clones was screened. This strengthens the view that Fez1 and Fez2 are necessary for cell survival or cell growth. Another explanation for the unsuccessful knock out may be that the Fez1/Fez2 genomic regions targeted by the guide sequences are inaccessible for the Cas9 enzyme due to conformational structures or bound proteins. However, this is less reasonable since four different guide sequences were used for each of the two proteins. Anyway, if a new attempt to establish knock out cell lines will be performed in future studies, other guide sequences should be selected. Furthermore, future works need to be done to verify if the Fez genes are really essential for cell survival and growth.

Confocal imaging showed colocalization of Fez1 with autophagy and transport proteins

The presence of two or more fluorescence signal on the same physical structure in a cell indicates colocalization at subcellular level. The colocalization in multiple structures throughout a cell might demonstrate correlation between the proteins and increase the probability that they are involved in the same cellular processes.

Fez1 is involved in regulation of autophagy and it interacts with KIF5B, which is involved in transport on microtubule filaments in cells. Furthermore, it is reported that tubulin is essential for fusion of autophagosomes and lysosomes. This study found that Fez1 colocalize with both KIF5B and tubulin, which indicates that they may function in concert, and indicating that all three are involved in regulation of autophagy.

S58 in Fez1 is reported to regulate the Fez1-KIF5B interaction via phosphorylation. Here the localization patterns of two Fez1 mutants were studied. Fez1 S58A, a phosphorylated defective protein that is not able to activate the Fez1-KIF5B interaction. Fez1 S58E mimics the phosphorylated form of Fez1 reported to be able to activate Fez1-KIF5B interaction. Surprisingly, the subcellular distribution of both Fez1 mutants overlapped completely with the pattern of WT Fez1. This may be explained by dimerization. Fez1 bind to itself as dimers. The mutants were expressed together with WT Fez1. Hence, a mutant and a WT protein can bind to each other and thereby colocalize.

The involvement of Fez1 in regulation of autophagy was further supported by the finding that Fez1 colocalized with the autophagy proteins p62, GABARAP, LC3B and WIPI2. WIPI2 initiates autophagosome formation and enables LC3B lipidation, which is important for autophagosome formation. LC3B is used as a marker for autophagy since the number of autophagosomes usually correlates with LC3B lipidation. Gabarap, another Atg8 protein studied here, is important for autophagosome maturation. The interactions of Fez1 with WIPI2, LC3B and GABARAP suggest that Fez1 has a role in regulation of autophagosome formation and/or maturation. The autophagy receptor protein p62 is strongly associated with LC3B at the autophagosome. Hence, the colocalization of Fez1 and p62 strengthen the hypothesis that Fez1 has a regulatory role associated with the autophagosome. This will be an interesting question to address in further studies.

Syntaxin17 is a SNARE protein that is involved in the autophagosome-lysosome fusion, but the exact mechanism is still unknown. In this study no colocalization was observed between Fez1 and Syntaxin17. This may indicate that Fez1 is not involved in the autophagosome-lysosome fusion step. However, further investigation is needed before in can be concluded.

Fez1 and Fez2 colocalize in cells

Interestingly, we found Fez2 to be colocalized with Fez1. This indicates that Fez2 might have overlapping functions with Fez1. Not only is Fez1 inhibiting autophagy, it is also involved in bundling and elongation of axons, and participates in the transport of mitochondria and other cargos along microtubules. Very little is known about the function of Fez2, which is well expressed in all cell types. Whether Fez2 has overlapping functions with Fez1 will be an important question to address in further studies.

Over-expression of Fez1 or Fez2 do not impair LC3B lipidation

Since both Fez1 and Fez2 were found to colocalize with LC3B, the effect of overexpressed Fez1 or Fez2 on LC3B lipidation was investigated by Western blotting. The findings indicate that under normal conditions over-expressing of Fez1 and Fez2 does not impair LC3B lipidation, since the LC3-I/LC3-II ratio was unchanged upon tetracycline induced expression of Fez1 or Fez2 in the Hek293 Flp-In cell lines. However, whether Fez1 and Fez2 affect LC3B lipidation under starved conditions was not measured her, but will be interesting for future studies. LC3-I/LC3-II ratio is used to monitor autophagy flux. This means that under normal conditions (fed conditions) this ratio should remain unchanged, since autophagy is suppressed. Whereas under starved conditions, the flux ratio should increase as a result of increased autophagy.

Over-expression of proteins and limitations

Most of the colocalization studies performed in this work were based on protein overexpression. High protein concentrations cells can lead to non-physiological interactions that will not happen under physiological levels. It is easier to detect interactions under over-expressed conditions, than at endogenous levels. Detecting endogenous proteins require specific antibodies. Here, some of the proteins found to colocalize with Fez1 and Fez2, were next studied at endogenous level using specific antibodies. Only the Fez1/Fez2 colocalization with tubulin was verified at the endogenous level. Hence, further work need to be done to validate the colocalizations presented in this study. The most important limitation with confocal imaging technique is the fact that it is visually based. Thus, making it highly prone to random error or bias. In addition, the numbers of cells examined were too low, so the results from this study alone are not enough to conclude anything, but serve as a base for future studies to identify the roles of Fez1/Fez2 in regulation of autophagy, and if they really are essential for cell survival.

Clinical or pharmacological significance of the findings

Autophagy is essential for maintaining cellular homeostasis and Fez1 has been reported to negatively regulate this process (Joachim et al., 2012). In addition, it is reported that Fez1 is involved in neuronal resistance against viral infections (Chua et al., 2013; Haedicke et al., 2009; Malikov et al., 2015). Hence, improving the understanding of Fez1 and its homolog Fez2 will be of great value to improve the knowledge on underlying causes of neuronal diseases and development. This may lead to the identification of new drugs targets and biomarkers, and may also predict whether drug candidates are likely to work.

Conclusion

A gene knockout is a genetic technique in which one of the genes in a the genome is made non-functional. Knock out cell lines are primarily used to understand the role of a specific protein by comparing the knockout cell to a wild type. Here the CRISPR/Cas9 technology was used in an attempt to knock out the expression of Fez1 and Fez2. However, no complete knock out was obtained. This can have various explanations. First, Fez1 and Fez2 might be essential for cell survival and/or cell growth. This theory is supported by an earlier experiment that also attempted to establish Fez1 and/or Fez2 knock out cells, but was unsuccessful. A less reasonable explanation is that due to conformational structures or bound proteins the Fez1/Fez2 genomic regions targeted by the guide sequences are inaccessible for the Cas9 enzyme. The reason why this explanation is less reasonable is because four different guide sequences were used for each of the two proteins.

One of the most common applications of fluorescence microscopy is to compare the subcellular distributions of two fluorescently labeled proteins. Such comparisons can be used to understand the function of a protein, since colocalization of proteins might demonstrate that they are involved in the same cellular processes. This study demonstrated that Fez1 and Fez2 colocalize with proteins involved in autophagosome formation and maturation, and with proteins involved in intracellular transport. This may suggest that Fez1 and Fez2 have a role in the formation and transport of autophagosomes. This will be an important topic to address in future studies of Fez1 and Fez2.

References

Addgene. (n.d). Genome Engineering Guide. Retrieved October 3, 2016 from https://www.addgene.org/genome-engineering/

Alborghetti, M. R., Furlan Ada, S., da Silva, J. C., Sforca, M. L., Honorato, R. V., Granato, D. C., . . . Kobarg, J. (2013). Structural analysis of intermolecular interactions in the kinesin adaptor complex fasciculation and elongation protein zeta 1/ short coiled-coil protein (FEZ1/SCOCO). *PLoS One, 8*(10), e76602. doi:10.1371/journal.pone.0076602

Alborghetti, M. R., Furlan, A. S., & Kobarg, J. (2011). FEZ2 has acquired additional protein interaction partners relative to FEZ1: functional and evolutionary implications. *PLoS One, 6*(3), e17426. doi:10.1371/journal.pone.0017426

Ariosa, A. R., & Klionsky, D. J. (2016). Autophagy core machinery: overcoming spatial barriers in neurons. *J Mol Med (Berl)*. doi:10.1007/s00109-016-1461-9

Banreti, A., Sass, M., & Graba, Y. (2013). The emerging role of acetylation in the regulation of autophagy. *Autophagy, 9*(6), 819-829. doi:10.4161/auto.23908

Berg JM, Tymoczko JL, & L., S. (2002). BiochemistrySection 9.3, Restriction Enzymes: Performing Highly Specific DNA-Cleavage Reactions. (5th ed.): New York: W H Freemanhttps://www.ncbi.nlm.nih.gov/books/NBK22528/.

Biotium. (2013). GelRedTM Nucleic Acid Gel Stain, 10,000X in Water product information, Retrieved from https://biotium.com/wp-content/uploads/2013/07/PI-41003.pdf

Birgisdottir, A. B., Lamark, T., & Johansen, T. (2013). The LIR motif - crucial for selective autophagy. *J Cell Sci, 126*(Pt 15), 3237-3247. doi:10.1242/jcs.126128

Bjørkøy, G., Lamark, T., Pankiv, S., Øvervatn, A., Brech, A., & Johansen, T. (2009). Chapter 12 Monitoring Autophagic Degradation of p62/SQSTM1. *452*, 181-197. doi:10.1016/s0076-6879(08)03612-4

Blasius, T. L., Cai, D., Jih, G. T., Toret, C. P., & Verhey, K. J. (2007). Two binding partners cooperate to activate the molecular motor Kinesin-1. *J Cell Biol, 176*(1), 11-17. doi:10.1083/jcb.200605099

Bloom, L., & Horvitz, H. R. (1997). The Caenorhabditis elegans gene unc-76 and its human homologs define a new gene family involved in axonal outgrowth and fasciculation. *Proceedings of the National Academy of Sciences of the United States of America*.

Boya, P., Reggiori, F., & Codogno, P. (2013). Emerging regulation and functions of autophagy *Nat Cell Biol* (Vol. 15, pp. 713-720): Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.

Burkhard, P., Stetefeld, J., & Strelkov, S. V. (2001). Coiled coils: a highly versatile protein folding motif. *Trends in Cell Biology, 11*(2), 82-88. doi:10.1016/s0962-8924(00)01898-5

Chen, D., & Dou, Q. P. (2010). The ubiquitin-proteasome system as a prospective molecular target for cancer treatment and prevention. *Curr Protein Pept Sci, 11*(6), 459-470.

Chua, J. J., Butkevich, E., Worseck, J. M., Kittelmann, M., Gronborg, M., Behrmann, E., . . . Jahn, R. (2012). Phosphorylation-regulated axonal dependent transport of syntaxin 1 is mediated by a Kinesin-1 adapter. *Proc Natl Acad Sci U S A, 109*(15), 5862-5867. doi:10.1073/pnas.1113819109

Chua, J. J., Jahn, R., & Klopfenstein, D. R. (2013). Managing intracellular transport. *Worm, 2*(1), e21564. doi:10.4161/worm.21564

Chylinski, K., Makarova, K. S., Charpentier, E., & Koonin, E. V. (2014). Classification and evolution of type II CRISPR-Cas systems. *Nucleic Acids Res, 42*(10), 6091-6105. doi:10.1093/nar/gku241

Cooper, G. M., & Hausman, R. E. (2004). *The cell : a molecular approach* (3rd ed.). Washington, D.C: ASM Press.

Cuervo, A. M., & Wong, E. (2014). Chaperone-mediated autophagy: roles in disease and aging. *Cell Res, 24*(1), 92-104. doi:10.1038/cr.2013.153

Dooley, H. C., Wilson, M. I., & Tooze, S. A. (2015). WIPI2B links PtdIns3P to LC3 lipidation through binding ATG16L1. *Autophagy, 11*(1), 190-191. doi:10.1080/15548627.2014.996029

European Collection of Cell Cultures, & Sigma. (2010). Fundamental Techniques in Cell Cultures Laboratory Handbook Retrieved from https://www.phe-culturecollections.org.uk/media/101902/ecacc_lab_handbook.pdf

FAO. (2012). Cryoconservation of animal genetic resources. FAO Animal Production and Health Guidelines No. 12 (pp. 85, 87). doi:10.1017/S2078633612000628

Finley, E. B. a. L. P. a. D. (2013). Mycoplasma Detection and Elimination. Retrieved March 29, 2017, from http://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma-Aldrich/General_Information/1/mycoplasma-detection-and-elimination.pdf

Fujioka, Y., Suzuki, S. W., Yamamoto, H., Kondo-Kakuta, C., Kimura, Y., Hirano, H., . . . Noda, N. N. (2014). Structural basis of starvation-induced assembly of the autophagy initiation complex. *Nat Struct Mol Biol, 21*(6), 513-521. doi:10.1038/nsmb.2822

Fujita, T., Ikuta, J., Hamada, J., Okajima, T., Tatematsu, K., Tanizawa, K., & Kuroda, S. i. (2004). Identification of a tissue-non-specific homologue of axonal fasciculation and elongation protein zeta-1. *Biochemical and Biophysical Research Communications, 313*(3), 738-744. doi:doi.org/10.1016/j.bbrc.2003.12.006

Fujita, T., Maturana, A. D., Ikuta, J., Hamada, J., Walchli, S., Suzuki, T., . . . Kuroda, S. (2007). Axonal guidance protein FEZ1 associates with tubulin and kinesin motor protein to transport mitochondria in neurites of NGF-stimulated PC12 cells. *Biochem Biophys Res Commun, 361*(3), 605-610. doi:10.1016/j.bbrc.2007.07.050

Geeraert, C., Ratier, A., Pfisterer, S. G., Perdiz, D., Cantaloube, I., Rouault, A., . . . Pous, C. (2010). Starvation-induced hyperacetylation of tubulin is required for the stimulation of autophagy by nutrient deprivation. *J Biol Chem, 285*(31), 24184-24194. doi:10.1074/jbc.M109.091553

Gindhart, J. G., Chen, J., Faulkner, M., Gandhi, R., Doerner, K., Wisniewski, T., & Nandlestadt, A. (2003). The kinesin-associated protein UNC-76 is required for axonal transport in the Drosophila nervous system. *Mol Biol Cell, 14*(8), 3356-3365. doi:10.1091/mbc.E02-12-0800

Glick, D., Barth, S., & Macleod, K. F. (2010). Autophagy: cellular and molecular mechanisms. *J Pathol, 221*(1), 3-12. doi:10.1002/path.2697

Haedicke, J., Brown, C., & Naghavi, M. H. (2009). The brain-specific factor FEZ1 is a determinant of neuronal susceptibility to HIV-1 infection. *Proc Natl Acad Sci U S A, 106*(33), 14040-14045. doi:10.1073/pnas.0900502106

Heather, J. M., & Chain, B. (2016). The sequence of sequencers: The history of sequencing DNA. *Genomics, 107*(1), 1-8. doi:doi.org/10.1016/j.ygeno.2015.11.003

Hirokawa, N., Noda, Y., Tanaka, Y., & Niwa, S. (2009). Kinesin superfamily motor proteins and intracellular transport. *Nat Rev Mol Cell Biol, 10*(10), 682-696. doi:10.1038/nrm2774

Ikuta, J., Maturana, A., Fujita, T., Okajima, T., Tatematsu, K., Tanizawa, K., & Kuroda, S. i. (2007). Fasciculation and elongation protein zeta-1 (FEZ1) participates in the polarization of hippocampal neuron by controlling the mitochondrial motility. *Biochemical and Biophysical Research Communications, 353*(1), 127-132. doi:doi.org/10.1016/j.bbrc.2006.11.142

Isbir, T., Kirac, D., Demircan, B., & Dalan, B. (2013). Gel Electrophoresis A2 - Maloy, Stanley. In K. Hughes (Ed.), *Brenner's Encyclopedia of Genetics (Second Edition)* (pp. 165-167). San Diego: Academic Press.

JM, B., JL, T., & L., S. (2002). Biochemistry. 5th edition *Section 9.3, Restriction Enzymes: Performing Highly Specific DNA-Cleavage Reactions.*: New York: W H Freeman.

Joachim, J., Wirth, M., McKnight, N. C., & Tooze, S. A. (2012). Coiling up with SCOC and WAC: two new regulators of starvation-induced autophagy. *Autophagy, 8*(9), 1397-1400. doi:10.4161/auto.21043

Johansen, T., & Lamark, T. (2014). Selective autophagy mediated by autophagic adapter proteins. *Autophagy, 7*(3), 279-296. doi:10.4161/auto.7.3.14487

Kaushik, S., & Cuervo, A. M. (2012). Chaperones in autophagy. *Pharmacol Res, 66*(6), 484-493. doi:10.1016/j.phrs.2012.10.002

Kim, T. K., & Eberwine, J. H. (2010). Mammalian cell transfection: the present and the future. *Analytical and Bioanalytical Chemistry*. doi:10.1007/s00216-010-3821-6

Le Douce, V., Herbein, G., Rohr, O., & Schwartz, C. (2010). Molecular mechanisms of HIV-1 persistence in the monocyte-macrophage lineage. *Retrovirology, 7*, 32. doi:10.1186/1742-4690-7-32

Lee, P. Y., Costumbrado, J., Hsu, C. Y., & Kim, Y. H. (2012). Agarose gel electrophoresis for the separation of DNA fragments. *J Vis Exp*(62). doi:10.3791/3923

Lee, Y. K., & Lee, J. A. (2016). Role of the mammalian ATG8/LC3 family in autophagy: differential and compensatory roles in the spatiotemporal regulation of autophagy. *BMB Reports, 49*(8), 424-430. doi:10.5483/BMBRep.2016.49.8.081

Liu, X., & Klionsky, D. J. (2016). The Atg17-Atg31-Atg29 complex and Atg11 regulate autophagosome-vacuole fusion. *Autophagy, 12*(5), 894-895. doi:10.1080/15548627.2016.1162364

Lonza. (2007). MetaPhor® Agarose, The high resolution agarose that challenges polyacrylamide. Retrieved March 27, 2017, from http://www.takara.co.kr/file/manual/pdf/MetaPhor_Agarose_-_Protocol.pdf

Lonza. (2017a). Mycoplasma – Products for Detection and Removal. Retrieved March 29, 2017, from http://www.lonza.com/products-services/bio-research/cell-culture-products/mycoplasma-detection-and-removal.aspx

Lonza. (2017b). Mycoplasma Testing Webinar. Retrieved March 29, 2017, from http://www.lonza.com/campaigns/bioresearch/mycoplasma-testing-webinar-2016.aspx

Mahmood, T., & Yang, P. C. (2012). Western blot: technique, theory, and trouble shooting. *N Am J Med Sci, 4*(9), 429-434. doi:10.4103/1947-2714.100998

Malikov, V., da Silva, E. S., Jovasevic, V., Bennett, G., de Souza Aranha Vieira, D. A., Schulte, B., . . . Naghavi, M. H. (2015). HIV-1 capsids bind and exploit the kinesin-1 adaptor FEZ1 for inward movement to the nucleus. *Nat Commun, 6*, 6660. doi:10.1038/ncomms7660

Mason, J. M., & Arndt, K. M. (2004). Coiled coil domains: stability, specificity, and biological implications. *Chembiochem, 5*(2), 170-176. doi:10.1002/cbic.200300781

Maturana, A. D., Fujita, T., & Kuroda, S. (2010). Functions of fasciculation and elongation protein zeta-1 (FEZ1) in the brain. *ScientificWorldJournal, 10*, 1646-1654. doi:10.1100/tsw.2010.151

Mijaljica, D., Prescott, M., & Devenish, R. J. (2014). Microautophagy in mammalian cells: Revisiting a 40-year-old conundrum. *Autophagy, 7*(7), 673-682. doi:10.4161/auto.7.7.14733

Nguyen, T. N., Padman, B. S., Usher, J., Oorschot, V., Ramm, G., & Lazarou, M. (2016). Atg8 family LC3/GABARAP proteins are crucial for autophagosome-lysosome fusion but not autophagosome formation during PINK1/Parkin mitophagy and starvation. *J Cell Biol, 215*(6), 857-874. doi:10.1083/jcb.201607039

Pankiv, S., Clausen, T. H., Lamark, T., Brech, A., Bruun, J. A., Outzen, H., . . . Johansen, T. (2007). p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. *J Biol Chem, 282*(33), 24131-24145. doi:10.1074/jbc.M702824200

Papinski, D., & Kraft, C. (2016). Regulation of Autophagy By Signaling Through the Atg1/ULK1 Complex. *Journal of Molecular Biology, 428*(9, Part A), 1725-1741. doi:doi.org/10.1016/j.jmb.2016.03.030

Ran, F. A., Hsu, P. D., Wright, J., Agarwala, V., Scott, D. A., & Zhang, F. (2013). Genome engineering using the CRISPR-Cas9 system. *Nat Protoc, 8*(11), 2281-2308. doi:10.1038/nprot.2013.143

Rath, D., Amlinger, L., Rath, A., & Lundgren, M. (2015). The CRISPR-Cas immune system: biology, mechanisms and applications. *Biochimie, 117*, 119-128. doi:10.1016/j.biochi.2015.03.025

Rubinsztein, D. C., Codogno, P., & Levine, B. (2012). Autophagy modulation as a potential therapeutic target for diverse diseases. *Nat Rev Drug Discov, 11*(9), 709-730. doi:10.1038/nrd3802

Schäfer, H. B. (2016). *A study of Fez1 and Fez2: Localization and knock out.* (Master degree), UiT.

Shpilka, T., Weidberg, H., Pietrokovski, S., & Elazar, Z. (2011). Atg8: an autophagy-related ubiquitin-like protein family. *Genome Biol, 12*(7), 226. doi:10.1186/gb-2011-12-7-226

Sigma. (2016). Competent Cell Compendium:
Tools and Tips for Successful Transformations. Retrieved April 20, 2017 from http://www.sigmaaldrich.com/content/dam/sigma-aldrich/life-science/cloning-and-expression/compcellscompend.pdf

Strauss, H. M., & Keller, S. (2008). Pharmacological interference with protein-protein interactions mediated by coiled-coil motifs. *Handb Exp Pharmacol*(186), 461-482. doi:10.1007/978-3-540-72843-6_19

Svenning, S., & Johansen, T. (2013). Selective autophagy. *Essays Biochem, 55*, 79-92. doi:10.1042/bse0550079

Thermo Fisher Scientific. (n.d). How Cationic Lipid Mediated Transfection Works. Retrieved May 5, 2017 from https://www.thermofisher.com/no/en/home/references/gibco-cell-culture-basics/transfection-basics/gene-delivery-technologies/cationic-lipid-mediated-delivery/how-cationic-lipid-mediated-transfection-works.html

Truebestein, L., & Leonard, T. A. (2016). Coiled-coils: The long and short of it. *Bioessays, 38*(9), 903-916. doi:10.1002/bies.201600062

Uttenweiler, A., & Mayer, A. (2008). Microautophagy in the yeast Saccharomyces cerevisiae. *Methods Mol Biol, 445*, 245-259. doi:10.1007/978-1-59745-157-4_16

Wang, G., & Mao, Z. (2014). Chaperone-mediated autophagy: roles in neurodegeneration. *Transl Neurodegener, 3*, 20. doi:10.1186/2047-9158-3-20

Weidberg, H., Shvets, E., Shpilka, T., Shimron, F., Shinder, V., & Elazar, Z. (2010). LC3 and GATE-16/GABARAP subfamilies are both essential yet act differently in autophagosome biogenesis. *The EMBO Journal, 29*(11), 1792-1802. doi:10.1038/emboj.2010.74

Yin, Z., Pascual, C., & Klionsky, D. J. (2016). Autophagy: machinery and regulation. *Microb Cell, 3*(12), 588-596. doi:10.15698/mic2016.12.546

Young, L., Sung, J., Stacey, G., & Masters, J. R. (2010). Detection of Mycoplasma in cell cultures. *Nat Protoc, 5*(5), 929-934. doi:10.1038/nprot.2010.43

Zhang, F., Wen, Y., & Guo, X. (2014). CRISPR/Cas9 for genome editing: progress, implications and challenges. *Hum Mol Genet, 23*(R1), R40-46. doi:10.1093/hmg/ddu125