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A study of Fez1 and Fez2:

Localization and knock-out

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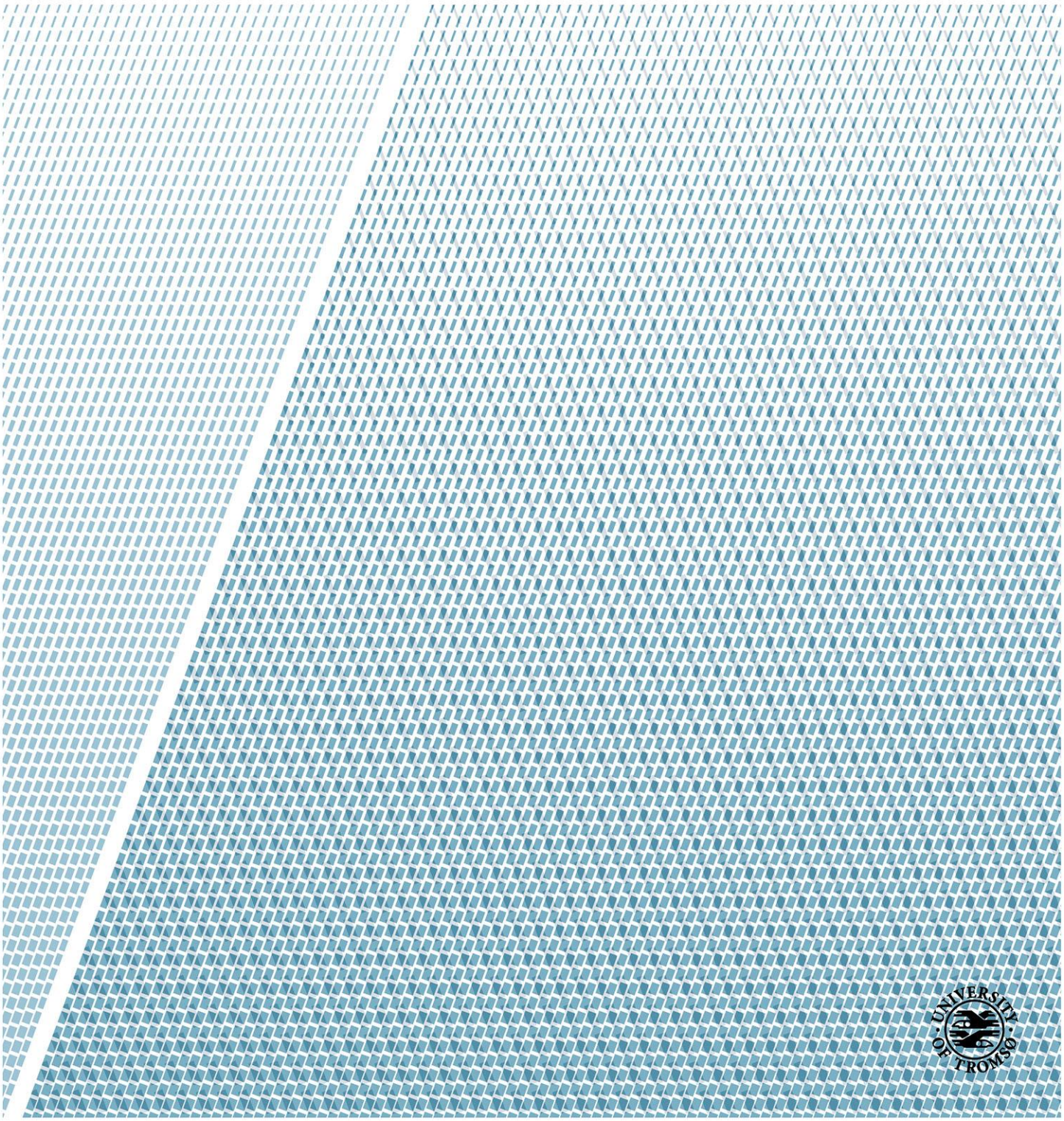

Acknowledgments

I want to thank Eva Sjøttem for helping me a lot! Thanks also to Hanne Brenne.

I also want to thank my mom for encouraging me when I did not want to write up my work.

Abstract

Autophagy is a fundamental cellular process where cell components get digested in autolysosomes and are recycled. Dysregulation of autophagy is involved in major diseases like cancer, neurodegeneration, inflammation and ischemia. In this thesis we have worked with fasciculation and elongation zeta (Fez) proteins, which are reported to inhibit autophagy. There are at least two mammalian Fez proteins, Fez1 and Fez2. Fez1 has three light chain three interaction regions (LIRs). Fez1 can use these to interact with LIR docking sites (LDS) on the autophagy Atg8 proteins. Using the Flp-In system, ten Hek293 cell lines were established. These cell lines have tetracycline inducible expression of EGFP-Fez1 mutants, and one cell line has inducible expression of EGFP-Fez2. Seven of the cell lines expressing EGFP-Fez1 are mutated in the LIR motifs. The other two express a phosphorylation mimicking mutant of Fez1 (S58E) and the un-phosphorylated form Fez1 (S58A). Fez1 binds kinesin-1. Phosphorylation of Fez1 S58 regulates the kinesin-1 binding. The second LIR is close to Fez1 S58 and phosphorylation of S58 may also regulate Atg8 interaction. The second LIR of Fez1 is recently proposed to bind to LDS in a reverse direction. As far as we know, this reverse binding is novel. The Expression and localization of Fez1 mutants and Fez2 was characterized by confocal microscopy. Immunofluorescent staining of endogenous Gabarap in the Flp-In cell lines suggest that either the reverse LIR2 is important or Fez1 Gabarap co-localization in a perinuclear dot is independent of all three Fez1 LIRs. A nuclear localization signal (NLS) is predicted in Fez1. Here the Fez1 NLS was tested experimentally. The NLS was cloned into a plasmid in front of EGFP-βgal and localization imaged by confocal microscopy. Our data indicate that the NLS is functional. Furthermore, various EGFP-Fez1 deletions constructs were made. Their localization was studied by confocal microscopy. The results indicate that Fez1 has a second NLS and also a nuclear export sequence (NES), both in the Fez1 2-130 region.

Fez1 is expressed in the brain while Fez2 is ubiquitously expressed. They are both hub proteins with many interaction partners. There is little research on Fez2. The EGFP-Fez2 cell line established here shows that Fez2 is mainly cytoplasmic, with strong enrichment in a perinuclear dot. Interestingly, immunofluorescent staining of Gabarap showed that Gabarap co-localizes with Fez2 in this dot. The Fez1 LIR2 is not conserved in Fez2.

An attempt to establish Hek293 Flp-In cell lines with the Fez1 and Fez2 genes knocked out was performed using the CRISPR/Cas9 technology. One potential Fez1 and one potential Fez2 knock out cell line was obtained. These cell lines will hopefully be useful in future research of Fez1 and Fez2.

Abbreviations and glossary

AMPK	AMP-activated protein kinase
ATG	Autophagy
ATP	Adenosine tri phosphate
BSA	Bovine Serum Albumin
CMV	Cytomegalovirus
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate also called Deoxynucleosidetriphosphates
ddNTP	Dideoxyribonucleotide triphosphate also called Dideoxynucleosidetriphosphate
DMEM	Dulbecco`s Modified Eagle`s Medium (D6046)
EGFP	Enhanced green fluorescent protein
FCS	Fetal calf serum
Fez	Fasciculation and elongation protein zeta
FRT	Flp Recombination Target
Fw	Forward
GABARAP	GABAA receptor associated protein
HEPA filter	High-efficiency particulate arrestance filter
HRP	Horseradish peroxidase
IF	Immunofluorescence
LAF bench	Laminar Air Flow
LB	Luria-Bertani
LDS	LIR docking sites
LIR	Light chain three interaction region
LMB	Leptomycin B
MAP1LC3	Microtubule associated protein one light chain three
MEME	Minimum Essential Medium Eagle (M4655)
MTOC	Microtubule organizing centre
mTORC1	Mechanistic target of rapamycin-1
MW	Molecular weight
NaOAc	Sodium Acetate
NEB	New England BioLabs
NES	Nuclear export sequence
NLS	Nuclear localization sequence
PE	Phosphatidylethanolamine
Rev	Reverse
RNA	Ribonucleic acid
RNase	Ribonuclease
Rpm	Revolutions per minute
PCR	Polymerase chain reaction
rSAP	Shrimp alkaline phosphatase
SCOC	Short coiled coil protein
SDS	Sodium Dodecyl Sulfate
ULK	Unc-51 like kinase
UV	Ultra violet
WB	Western Blot
#	Catalog number

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Introduction

Autophagy

The word autophagy comes from the Greek language and means “self-eating”. Autophagy is a way for cells to remove and recycle cell contents. Eukaryotic cells have two major ways to get rid of proteins. These are autophagy and the ubiquitin- proteasome system. Cells eat their own contents by autophagy for example: when they need energy, new building blocks, to get rid of accumulated proteins or even faulty organelles that are toxic to them. There are three types of autophagy: macroautophagy, microautophagy and chaperone mediated autophagy. The topic of this thesis is macroautophagy. In macroautophagy a phagophore forms around components also called cargo. The phagophore turns into an autophagosome when it closes in upon itself to form a closed double membrane. The components/cargo inside it are trapped and transported to either an endosome or a lysosome. The autophagosome then fuses with the endosome or with the lysosome. This turns it into an amphisome or an autolysosome. Early endosomes can be seen as sorting stations where their contents destination are decided (Jovic et al, 2010). Cell components that remain inside endosomes end up inside lysosomes when late endosomes fuse with lysosomes. Lysosomes contain enzymes that digest/break down the organic material inside them. This process delivers the cargo of the autophagosome for digestion and the contents are recycled. Figure 1 shows one phagophore, two autophagosomes and an endosome inside a cell.

Autophagy is a fundamental process. It is very important in cellular homeostasis and cellular signaling (Birgisdottir et al, 2013). Dysregulation of autophagy and mutations in the autophagy genes is involved in a lot of serious diseases like cancer, neurodegeneration, infection and cardiac failure (Jiang, P. Mizushima, N. 2014; Marx, V. 2015; Nishida, K et. al 2009). Autophagy is also involved in development, aging and immunity (Marx, V. 2015).

Autophagy is constitutively active, but can be increased. It is normal to distinguish between basal, also called housekeeping autophagy and starvation or stress induced autophagy. Autophagy can be selective, and only digest specific components. It can also be unselective and encompass a part of the cytosol for degradation (Birgisdottir et al, 2013). Examples of components that can get digested selectively in phagosomes include: bacteria and viruses (xenophagy), mitochondria (mitophagy), peroxisomes (pexophagy) and specific protein aggregates (aggrephagy) (Birgisdottir et al, 2013).

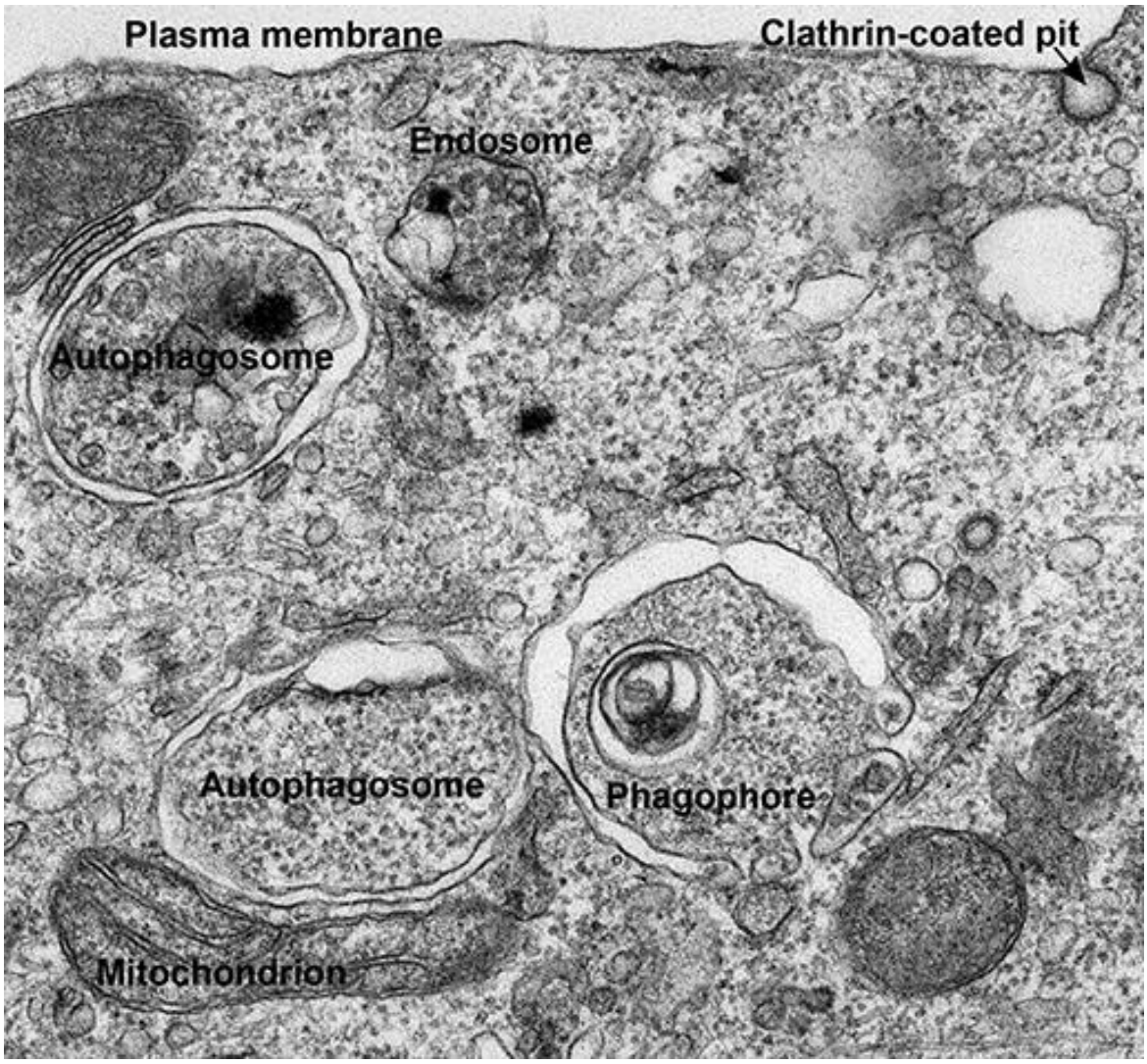

Figure 1. The image is an electron micrograph. It shows a phagophore, two autophagosomes and an endosome inside a cell. A phagophore is the beginning of a forming autophagosome. Phagophores are open instead of closed circular like the autophagosome. Autophagosomes vary in diameter from 0,5 to 1,5 um (Birgisdottir et al, 2013). The image was obtained with permission from Eeva-Liisa Eskelinen, University of Helsinki, Finland.

Regulation of autophagy

The regulation of autophagy is complex and much remains to be understood. The core autophagic machinery in humans consists of more than 40 proteins (Lin et al, 2016). These are called autophagy (Atg) proteins. Unc-51 like kinase (ULK) is one of them and it has 4 mammalian homologs (Lin et al, 2016). In humans ULK1 forms a complex with FIP200, Atg101 and Atg13. This complex is involved in the initial events of autophagosome formation (Lin et al, 2016). It initiates autophagosome formation when cells are starved, and may also

induce selective autophagy without starvation (Lin et al, 2016). To promote autophagy ULK1 phosphorylates BECN1, activating the PI3KC3 complex (Russel et al, 2013). There is a link between ULK to pro-autophagic lipid kinase VPS34 (Russel et al, 2013).

Bulk autophagy can be induced as a response to signals depending on for example amino acid depletion and energy status. Bulk autophagy is regulated by mTOR and AMP-activated protein kinase (AMPK) (Lin et al, 2016). mTOR reduces initiation of autophagy by phosphorylating ULK1 and ATG13 (Lin et al, 2016). AMPK inactivates mTOR. In addition, AMPK phosphorylates ULK1 directly at multiple sites. This phosphorylation stimulates autophagy in most cases (Lin et al, 2016). Under non starved conditions selective autophagy can still occur, even if mTOR is active. The ULK complex can be competed away from mTOR by direct binding of Huntingtin (Rui et al, 2015). Mitophagy, xenophagy and lipophagy (three selective autophagy pathways) can be promoted by the scaffold protein Huntingtin (Lin et al, 2016; Rui et al, 2015). Huntingtin also interacts directly with p62 (also called sequestosome1), an autophagy cargo receptor (Rui et al, 2015). Figure 2 shows selective autophagy on the left side and bulk autophagy on the right side.

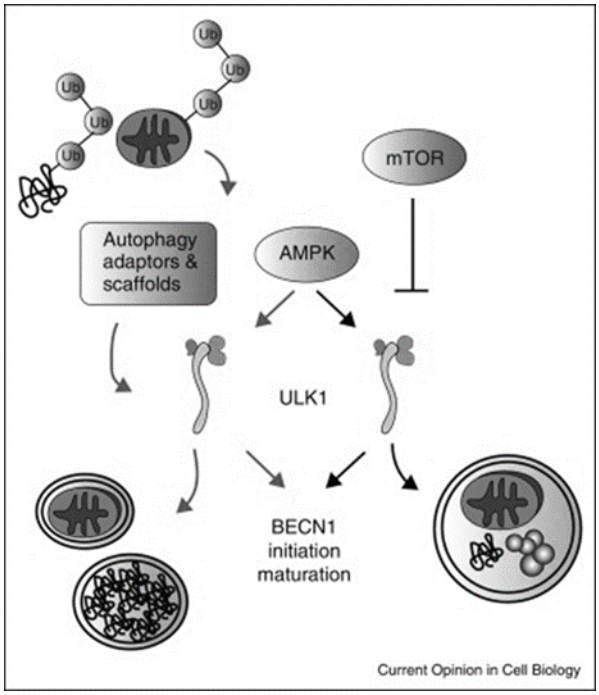

Figure 2. A schematic of the selective autophagy and the bulk autophagy processes. The green structure is a mitochondria and the black threadlike structure is representing an ubiquitinated protein aggregate. The mitochondria and protein aggregate end up being digested selectively or together in bulk autophagy. mTOR inhibits autophagy while AMPK stimulates autophagy in most cases (Lin et al, 2016). This image was adapted from Lin et al, 2016.

Atg8 proteins and their role in autophagy

The ULK complex (ULK1, ULK2, FIP200 and Atg13) interacts with Atg8 proteins. Of the Atg8 proteins they prefer binding to Gabarap (Alemu et al, 2012). Gabarap can activate ULK1 (and autophagy) (Joachim et al, 2016).

Yeast only have a single Atg8 protein, while mammals have six. These six belong to three different families. The three mammalian Atg8 families are: gamma-aminobutyric acid receptor associated protein (Gabarap), microtubule associated protein one light chain three (MAP1LC3) and golgi-associated ATPase enhancer of 16 kDA (GATE16) (Shpilka,T. et. al. 2011). Atg8 proteins are expressed in various tissues (Shpilka et al, 2011). Their structure is similar, consisting of two amino terminal α helices and an ubiquitin like core. Atg8 homologues are translated as full length precursors. Then a part of their C-terminals are cleaved off by Atg4 cysteine proteases. This transforms the Atg8 proteins to the I form (Kabeya et al, 2000). This I form can be conjugated to phosphatidylethanolamine (PE) (by their C terminal), which is on the membrane of the phagophore. This turns the Atg8 proteins to the II form that is covalently bound to the membrane. Conjugated Atg8-PE is present on both the inner and outer membranes of the phagophore (Birgisdottir et al, 2013). On the inner membrane PE-Atg8 is important for recruitment of cargo. On the outer membrane PE-Atg8 recruit effector proteins and these mediate transport and maturation (fusion to lysosomes) of autophagosomes (Birgisdottir et al, 2013).
It is common to use LC3 and p62 as markers for autophagy. The amount of LC3 in the II form is correlated to the extent of autophagosome formation (Kabeya et al, 2000). The conjugation of Atg8 proteins to the autophagic membrane is essential for autophagosome formation (Shpilka et al, 2011). Atg4 de-conjugates Atg8 from the outer membrane of the phagophore during autophagosome maturation and that is necessary for autophagosome biogenesis (Birgisdottri et al, 2013). Atg8 proteins on the inside of the autophagosome are digested.

Knock down studies suggest that Gabarap, Gate16 and LC3s have unique roles but are all needed in autophagy (Weidberg et al, 2010). There is a light chain 3 interaction motif (LIR), also called Atg8- interaction motif (AIM), in many proteins (see below). Atg8 proteins have LIR docking sites (LDS) that they use to interact with proteins that have LIRs. This motif is needed for ULK1 interaction with autophagosomes after starvation (Alemu et al, 2012). Atg8

proteins may be important for assembly of the ULK complex at the phagophore (Alemu et al, 2012).

LIR also known as ATG8-interaction motif (AIM)

Atg8 proteins conjugated to PE on the phagophore use LDS to interact with proteins that have LIRs. On the inside of the phagophore the Atg8-PE use LIRs to interact with autophagy cargo receptors/adaptors such as p62. p62 can bind to for example ubiquitinated protein aggregates that are to be digested (Johansen et al, 2011; Birgisdottir et al, 2013). p62 brings the cargo to the autophagosome by interacting with Atg8-PE. The cargo then ends up on the inside of the autophagosome. Selective autophagy relies on this interaction between Atg8 proteins and the LIR motifs in the cargo receptors/adaptors. On the outside of the phagophore Atg8-PE use their LDS to scaffold proteins to the membrane of the autophagosome.

The consensus of the core LIR motif consists of amino acids: (W/F/Y)XX(L/I/V), where X can be any amino acid (Birgisdottir et al, 2013). LIRs are believed to form an extended β-conformation and to interact with the β2 strand of Atg8 homologues (Noda et al, 2010). Figure 3 shows the formation of an autolysosome.

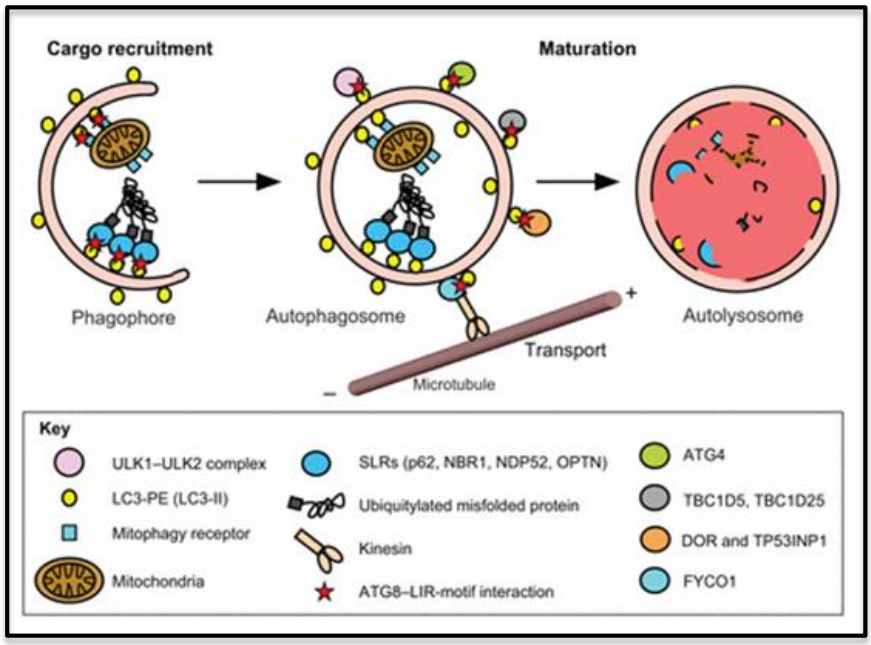

Figure 3. Selective autophagy- the formation of an autolysosome. Conjugated Atg8 proteins are both on the inside and outside of the phagophore. Atg8 proteins on the inside of the phagophore are involved in degradation of specific cargo. Atg8 proteins on the outside are involved in transport. FYCO1 uses its LIR motif to interact with with LC3-PE on the outer membrane of the autophagosome. This interaction is involved in the transport of autophagosomes toward the plus end of microtubules (Birgisdottir et al, 2013). This image was adapted from Birgisdottir et al (2013).

LIR motifs are found in many proteins and have many functions. In addition to target cargo receptors to autophagosomes they are involved in regulating autophagosome formation and maturation (Birgisdottir et al, 2013). Examples of proteins that contain LIRs are: cargo receptors, proteins associated with vesicles and of their transport, specific signaling proteins that are degraded by selective autophagy, Rab GTPase- activating proteins (GAPs) and members of the basal autophagy apparatus (Birgisdottir et al, 2013). There are LIRs that only interact with a specific Atg8 homologues. The F-type LIRs of ULK1 and ATG13 for example, prefer to bind to the Gabarap subfamily (Birgisdottir et al, 2013). In this thesis I have worked with fasciculation and elongation protein zeta (Fez), and it has three LIRs.

Fez1 and Fez2

There are at least two Fez proteins, Fez1 and 2. They both have multiple isoforms. Fez is short for fasciculation elongation protein zeta. Bloom and Horvitz named them this because they are similar to unc-76 gene in *C.elegans* (Bloom et al, 1997).

Fascicles are bundles of axons (Bloom et al, 1997). Unc-76 is important for normal axon fasciculation in *C.elegans* (Bloom et al, 1997). Unc-76 is required for axon-axon interactions and loss of Unc-76 function results in defects in axonal transport (Alborghetti et al, 2011). Fez1 is involved in axonal outgrowth (Kuroda et al, 1999). In a study it has been shown that the Fez1 gene is able to rescue nematodes with mutations in Unc-76 gene (Alborghetti et al, 2011). This suggests that Fez1 is evolutionary quite conserved. Fez2 is considered to be more different from Fez1 and Unc-76. Fez2 has additional protein interaction partners compared to Fez1 (Alborghetti et al, 2011).

In rats studies have shown that Fez1 mRNA is found only in the brain (Ikuta et al, 2007). Fez2 is expressed in all tissues. Fez proteins all have a conserved coiled coil domain in their C-terminal half. In this thesis the Fez1 isoform of 392 amino acids and the Fez2 isoform of 380 amino acids were studied. Figure 4 and Figure 5 show three LIRs in Fez1 that previously have been mapped in our group (Alemu, unpublished data). They are located at LIR1: 4-23, LIR2: 48-67 and LIR3: 98-117. Fez1 interacts with Atg8 proteins (Alemu, unpublished data). Fez1 is partly localized in the nucleus (Lanzaa et al, 2008) and interacts with transcription regulatory proteins (Assmann et al, 2006). Fez1 has a putative bipartite nuclear localization signal (NLS) at positions 289-292 and 290-293 (Lanzaa et al, 2008).

Figure 4. A schematic representation of the Fez1 isoform. The localization of a predicted NLS and the LIRs are indicated.

Fez1 was classified as a hub protein in 2006 (Assmann et al, 2006). The classification was extended to the entire family of Fez proteins in 2011 (Alborghetti et al, 2011). Hub proteins are proteins that have more than typically 30 interaction partners. 59 interaction partners have been found for Fez2, and of these 40 interacted with Fez1 (Alborghetti et al, 2011). The conserved coiled coil region close to the C-terminal is involved in many of the protein interactions (Alborghetti et al, 2011). Fez1 forms homodimers with itself, and this is mediated by the N-terminus (Alborghetti et al, 2010; Lanza et al, 2009). Fez2 has not been studied as much as Fez1.

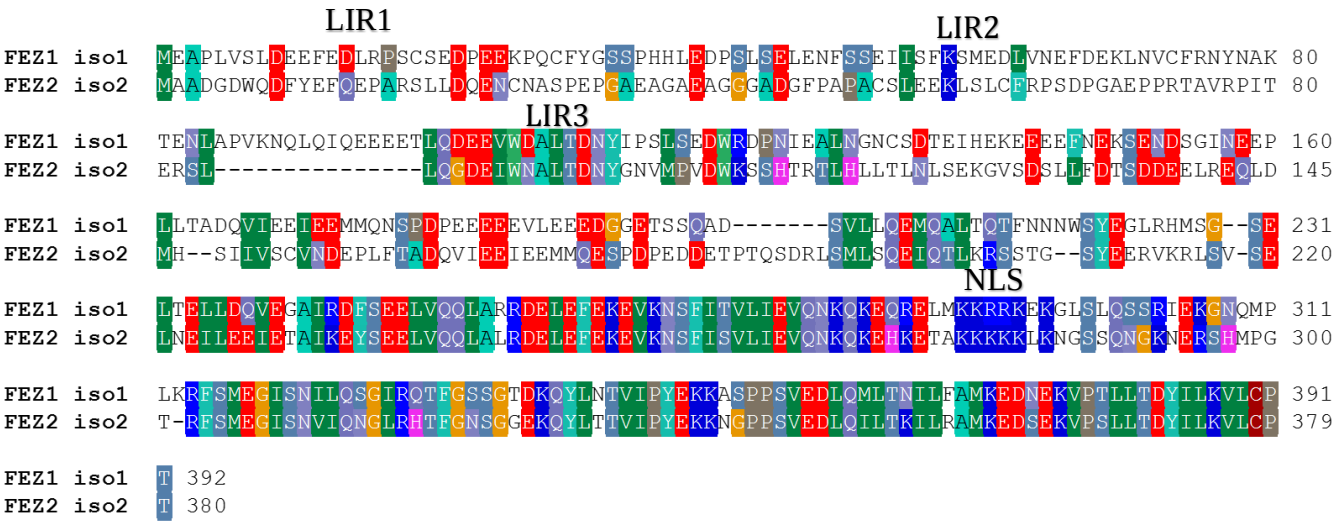

Figure 5. Fez1 and Fez2 isoforms, aligned using ClustalX. Putative LIRs and a nuclear localization signal (NLS) are indicated.

Fez1

Fez1 and c-Jun N-terminal kinase–interacting protein 1 (JIPI) are involved in the regulation of kinesin-1

Kinesin-1 is a microtubule based motor protein that transports cargo and hydrolyses adenosine tri phosphate (ATP) to move (Blasius et al, 2007). Microtubules consist of α and β tubulin whose minus ends are usually anchored at the centrosome (Kobayashi et al, 1998; Malikov et al, 2015). Their plus ends are usually toward the periphery and the centrosome is a microtubule organizing center (MTOC). Anterograde, plus-end directed motion is generally driven by kinesin-1 (Welte et al, 2004). Retrograde transport, toward the minus end is driven by dynein (Welte et al, 2004).

In order not to waste ATP, by moving without cargo, kinesin-1 is thought to have a folded conformation that keeps it inactive. Binding of JIPI alone to kinesin-1 is not sufficient to activate kinesin-1 (Blasius et al, 2007). Binding of both Fez1 and JIPI to kinesin-1 is sufficient to activate kinesin-1 for microtubule binding and motility (Blasius et al, 2007). Phosphorylation of Fez1 serine 58 (S58) has been shown to impact Fez1 binding to Kinesin-1 (Chua et al, 2012). Fez1 has a direct interaction with tubulin and the conserved C terminal half of Fez1 is necessary for this interaction (Fujita et al, 2007). Fez1 is involved in the anterograde transport of mitochondria from soma to axon or dendrites, which is essential for neuronal differentiation (Ikuta et al, 2007; Fujita et al, 2007).

Fez1 and human immunodeficiency virus type 1 (HIV-1) transport

Kinesin and dynein transport of viruses on microtubules is important if not critical in the replication and spread of many different viruses (Dodding et al, 2011). Neurons are resistant to HIV type 1, and the resistance is caused by Fez1 (Haedicke et al, 2009). Neurons naturally express high levels of Fez1 compared to other brain cells (Haedicke et al, 2009). Exogenous Fez1 expression promotes HIV-1 infection in non-neuronal cells (Malikov et al, 2015), while Fez1 S58 mutant that cannot bind kinesin-1 does not (Malikov et al, 2015). Fez1 binds HIV-1 capsids (a shell of protein that protects the nucleic acids of a virus) and this accomplishes net retrograde movement to the nucleus (Malikov et al, 2015).

Currently it is believed that Fez1 negatively regulates autophagy

Fez1 forms a complex with short coiled coil protein (SCOC) that is involved in the regulation of autophagy (Behrens et al, 2013). A hypothesis of how this works is that two Fez1 proteins are bound to the ULK1 complex. When two SCOC molecules arrive they bind to the two Fez1 proteins, competing binding of the ULK1 complex. The ULK1 complex is released from Fez1 and initiate autophagy. Hence, Fez1 inhibits autophagy by binding ULK1 complex, while SCOC promote autophagy by binding to Fez1 and releasing the ULK1 complex (McKnight et al, 2012).

Essential methods

T-Rex system

The T-rex system is a mammalian tetracycline controlled expression system. HEK Flp-In T-rex cells are made for this system. The CMV promoter contains 2 copies of a Tet operator in tandem. The Tet operators are binding sites for Tet repressor. After transfection with pDEST Flp-In EGFP plasmid with the gene of interest, the CMV promoter is incorporated into the genome with the gene of interest and EGFP. This is done by the Flp-In system described in the methods section. Expression of the gene of interest is depressed by a Tet repressor that binds to the Tet operator. Tetracycline binds to the Tet repressor and causes a conformational change in the Tet repressor which makes it unable to bind to the Tet operator. This induces transcription.

Crispr/ Cas9 knock out

Crisp/Cas9 endonuclease is originally believed to be a bacterial adaptive immune defense against viruses. Crisp is short for clustered regularly interspaced short palindromic repeats. These repeats exist naturally in many different kinds of bacteria. Close to the Crisp region is Cas (crisp associated genes) coding for endonucleases and maybe also other proteins. There are spacers between the Crisp repeats. These spacers resemble the DNA of viruses. RNA is made from these spacers and can bind to viruses with complementary DNA. This RNA is a RNA "guide" because it can be used to recognize viruses. The RNA guide forms a complex with the nuclease and guides it to the virus. The nuclease then cuts the virus genome.

By changing the guide RNA to target a wished gene in the human genome instead of a virus, we take advantage of this system. The guide will direct the nuclease to a specific place in the human genome. There the nuclease will cut the DNA. A double strand break will appear and the DNA repair machinery will start either non homologous end joining or homology directed repair. If it starts non homologous end joining, indels (insertions and or deletions) can occur. This will lead to mutations in the DNA and in some cases no expression of the protein.

The human genome contains around 3 billion base pairs within 23 pairs of chromosomes. With this much DNA it is difficult to obtain specificity. There are different types of Crisp. In this thesis a Crisp mechanism that works like described below was used:
Instead of making a double strand cut in one place like Cas9 (the nuclease) usually does, the cas9 used had been modified. This cas9 only nicks the DNA (makes a single strand cut). We used two different guides, one for each strand in DNA, so that we got two single strand cuts close to each other. This way one does end up with a double strand cut. Higher specificity is reached this way because the RNA guides may lead the Cas9 nuclease to cut more than one place in the genome, but they are unlikely to be close enough to each other for it to matter. The DNA repair machinery will repair the single strand cuts. In our target we get a double strand cut and non-homologous end joining will happen some times.

Aims of this thesis

Autophagy is a fundamental process for cellular homeostasis. However, the regulation of autophagy is far from understood. The Fez1 protein is reported as a negative regulator of autophagy. Previous studies have mapped three Atg8 binding motifs in Fez1. Fez1 is involved in intracellular transport via kinesin-1 binding. Phosphorylation of Fez1 S58 residue is important for this binding. Fez1 is also reported to be a nuclear protein. A putative Fez1 NLS has been described but not tested experimentally. Fez2 has not been studied much. The aim of this thesis was:

- to verify the predicted Fez1 NLS by experimental approaches

- to establish EGFP-Fez1 mutant cell lines to study the impact of the Fez1 LIRs for subcellular localization and co-localization with Atg8 proteins

- to establish EGFP-Fez1 mutant cell lines to study the impact of the Fez1 S58 phosphorylation for subcellular localization and co-localization with Atg8 proteins

- to establish a cell line with inducible EGFP-Fez2 expression

- to establish knock out cell lines of Fez1, Fez2 and double Fez1-Fez2 knock out, using the CRISPR/Cas9n technology

Materials

The bacteria used was *E.coli* DH5α (Bethesda Research Laboratories Inc.), made chemically competent by the technicians in the lab.

Table 1. Growth media for bacteria

Luria bertani (LB) medium	10 g Bacto Trypton 5g Bacto yeast extract 10 g NaCl dH2O to 1L pH adjusted to 7.5 with NaOH Antibiotic: Ampicillin100 ug/ml or Kanamycin 50ug/ml
LB agar plate	10 g Bacto Trypton 5g Bacto yeast extract 10 g NaCl 15g agar dH2O to 1L pH adjusted to 7.5 with NaOH Antibiotic: Ampicillin 100 ug/ml or kanamycin 50ug/ml
Super optimal broth with catabolite repression medium (SOC)	20 g Bacto Trypton 5g Bacto yeast extract 10 ml 250mM KCL 5mg MgCl2 20mM glucose dH20 to 1L pH adjusted to 7.5 with NaOH

The media is bought from "mediekjøkken" at UNN SUMP.

Table 2. Human cells and growth media

Cells	Growth media	Antibiotics
HeLa (Henrietta Lacks), Source: Leibniz-Institut DSMZ, # ACC 57.	Minimum Essential Medium Eagle, 10% (vol/vol) Standardized fetal calf serum	100 U/ml Penicillin 100 ug/ml Steptomycin
HEK293 (human embryonic kidney cells) Source: ATCC CRL-1573	Dulbecco`s Modified Eagle`s Medium, 10% (vol/vol) Standardized fetal calf serum	100 U/ml Penicillin 100 ug/ml Steptomycin
HEK293 Flp-In T-rex Source: Thermo Scientific	Dulbecco`s Modified Eagle`s Medium, 10% (vol/vol) Standardized fetal calf serum	100 U/ml Penicillin 100 ug/ml Steptomycin

Table 3. Selection media for transfection

Cells	Selection media, For 100 ml:
HEK293 Flp-In T-rex	90 ml Dulbecco`s Modified Eagle`s Medium 10 ml Standardized fetal calf serum 400 uL Hygromycin B (50mg/ml) (Gibco) 7,5 uL Blasticidin S HCL (10mg/ml) (Gibco)

Solutions and antibiotics for human cell lab:

- Dulbecco`s Modified Eagle`s Medium, Sigma Aldrich, #
- Minimum Essential Medium Eagle`s medium, Sigma Aldrich, #
- Hank`s Balanced salt solution, Sigma Aldrich, #H8264
- Penicillin-Streptomycin (10,000 Unic penicillin, 10 mg steptomycin per ml in 0,9% NaCl), Sigma Aldrich, #P0781
- Standardized fetal calf serum, Biochrom AG, #S0615
- 0,25 Trypsin-EDTA solution, Sigma Aldrich, #T4049
- Phosphate 10xbuffered saline (PBS), Gibco, lot 1721740 (diluted 1:10 before use and also autoclaved before use in cell lab)

Table 4. ENTRY plasmids of Fez1 mutant constructs

Plasmids
pENTR Fez1 (F12A/L15A) (LIR1 mutation)
pENTR Fez1 (S58E)
pENTR Fez1 (S58A)
pENTR Fez1 (W106A/L109A) (LIR3 mutation)
pENTR Fez1 (F12A/L15A/W106A/L109A) (LIR1 and 3 mutation)
pENTR Fez2
pENTR Fez1 (F56A)
pENTR Fez1 (I52A/F56A)
pENTR Fez1 LIR1 and LIR3 mut (I52A/F56A)
pENTR Fez1 LIR1 and LIR3 mut (F56A)

The source of the 6 first plasmids are: Alemu, E. A. 2011. The 4 last plasmids were made in this thesis.

Table 5. ENTRY plasmids of Fez1 deletion constructs

Plasmids	
pDest EGFP Fez1	pENTR Fez1 (276-392)
pDEST EGFP Fez1 (1-308)	pENTR Fez1 (99-111)
pDEST EGFP Fez1 (309-392)	pENTR 1A Fez1 (98-117)
pENTR1A end Fez1 (1-39)	pENTR Fez1 (131-392)
pENTR1A Fez1 (4-23)	pENTR Fez1 (2-270)
pENTR1A Fez1 (4-67)	pENTR1A Fez1 (48-117)
pENTR1A Fez1 (4-117)	pENTR1A Fez1 (48-67)
pENTR Fez1 (1-98)	

The source of these plasmids is: Alemu, E. A. 2011

Table 6. Plasmids for expressing of Fez1 deletion constructs fused to EGFP, made in this study

Plasmids	
pDEST EGFP Fez1 (1-39)	pDEST EGFP Fez1 (1-98)
pDEST EGFP Fez1 (4-23)	pDEST EGFP Fez1 (276-392)
pDEST EGFP Fez (4-67)	pDEST EGFP Fez1 (99-111)
pDEST EGFP Fez1 (4-117)	pDEST EGFP Fez1 (98-117)
pDEST EGFP Fez1(48-67)	pDEST EGFP Fez1 (2-270)
pDEST EGFP Fez1 (48-117)	pDEST EGFP Fez1 (131-392)

Table 7. Plasmids for expressing the putative NLS of Fez1 fused to EGFP-βgal

Plasmid	**Source**
pEGFP-betagal-N1	Pankiv et. al. 2010
pEGFP-betagal-N1 Fez1 NLS	This thesis

Table 8. Other plasmids

Plasmid	**Source**
pDEST-EGFP-C1	Bjorkoy et al (2005)
pDEST Flp-In EGFP	ThermoFisher
pOG44	ThermoFisher

Table 9. Plasmid for CRISPR

Plasmid	**Source**
pX461 (PSpCas9n (BB) 2A GFP)	Ran et. al. 2013

Table 10. Plasmids for CRISPR, made in this study

Plasmid	
pX461 Fez1 T3B	pX461 Fez2 T1A
pX461 Fez1 T2B	pX461 Fez2 T2A
pX461 Fez1 T1B	pX461 Fez2 T3A
pX461 Fez1 T3A	pX461 Fez2 T1B
pX461 Fez1 T2A	pX461 Fez2 T2B
pX461 Fez1 T1A	pX461 Fez2 T3B

Table 11. Plasmids for establishment of stable cell lines, made in this thesis

Plasmid	
pDEST Flp-In EGFP Fez1 (F56A)	pDEST Flp-In EGFP Fez1 (I52A/F56A)
pDEST Flp-In EGFP Fez1 LIR1 and LIR3 mut (F56A)	pDEST Flp-In EGFP Fez1 LIR1 and LIR3 mut (I52A/F56A)
pDEST Flp-In EGFP Fez1 S58A	pDEST Flp-In EGFP Fez1 S58E
pDEST Flp-In EGFP Fez1 LIR1 mut	pDEST Flp-In EGFP Fez1 LIR3 mut
pDEST Flp-In EGFP Fez1 LIR1 and 3mut	pDEST Flp-In EGFP Fez2

Table 12. Oligos from Invitrogen

Method	**Oligo**
Site directed mutag-enese	Fez1 F56A fw GAGAATTTTTCTTCCGAAATAATCAGCGCCAAGTCCATGGAGGAC
	Fez1 F56A rev GTCCTCCATGGACTTGGCGCTGATTATTTCGGAAGAAAAATTCTC
	Fez1 I52A F56A fw GAGAATTTTTCTTCCGAAGCAATCAGCGCCAAGTCCATGGAGGAC
	Fez1 I52A F56A rev GTCCTCCATGGACTTGGCGCTGATTGCTTCGGAAGAAAAATTCTC
Cloning of Fez1 NLS into EGFP beta gal vector	Fez1 NLS fw TCGAGGAACTGATGAAAAAGAGGCGGAAAGAGAAAGGGGGTAC
	Fez 1 NLS rev CCCCTTTCTCTTTCCGCCTCTTTTTCATCAGTTCC
CRISPR guide sequences	Fez1 T1A (fw) CACCGTTCATCCAGACTCACCAGTG
	Fez1 T1A (rev) AAACCACTGGTGAGTCTGGATGAAC
	Fez2 T2B fw CACCGTGGCTTTTAAAATACGGTCT
	Fez2 T2B rev AAACAGACCGTATTTTAAAAGCCAC
	Fez2 T1A fw CACCGTACAATACCAGGATGTACAC
	Fez2 T1A rev AAACGTGTACATCCTGGTATTGTAC
	Fez1 T2B fw CACCGTTCATCTCCCCACCATCTCG
	Fez1 T2B rev AAACCGAGATGGTGGGGAGATGAAC
	Fez2 T1B fw CACCGAAGTGTGAGCACGAGTGGT

	Fez2 T1B rev AAACACCACTCGTGCTCACACTTC
	Fez2 T2A fw CACCGAAAGGTTCTCCAGTATTCAC
	Fez2 T2A rev AAACGTGAATACTGGAGAACCTTTC
	Fez1 T1B fw CACCGCCTTCGACCCTCCTGCTCGG
	Fez1 T1B rev AAACCCGAGCAGGAGGGTCGAAGGC
	Fez1 T2A fw CACCGCTCCTCCGGGTCCTCCGAGC
	Fez1 T2A rev AAACGCTCGGAGGACCCGGAGGAGC

Table 13. Sequencing primers

Primer	**Sequence**	**Source**
ENTER fw	-	Invitrogen
pX330-seq-fw (10uM)	AGGGATGGTTGGTTGGTGGG	Invitrogen.
GFP-N1 primer 10 uM	-	Invitrogen

Table 14. Antibodies

Antibody	Method	Animal	Diluted	Source
Fez1	WB*	Rabbit	1:500	Chua, J et. al (2012)
Fez1	WB*	Rabbit	1: 500	Cell signaling
Fez2	WB*	Rabbit	1:1000	Sigma Aldrich
αGFP	WB*	Rabbit	1:5000	Abcam
Gabarap	IF**	Rabbit	1:300	Abgent
LC3B	IF**	Rabbit	1:500	Sigma Aldrich
HRP Anti mouse	WB*	-	1:1500	Abinova
HRP Anti rabbit	WB*	-	1:1500	Sigma Aldrich
HRP antibiotin	WB*	-	1:1500	Cell Signaling
Alexa 555 rabbit	IF**	-	1:5000	Life technologies

*western blot, **immunofluorescence

Table 15. Buffers, solutions, enzymes and chemicals used in different methods

Method	Buffer, solution, enzyme etc	Source or content
Oligo cloning (CRISPR)	10xT4 Ligation buffer	New England Biolabs (NEB)
	T4 Polynucleotide kinase	NEB
	10 x ligation buffer	NEB
	10x NEB 2.1 buffer	NEB
	T4 DNA ligase	NEB
	BbsI	NEB
	Shrimp alkaline phosphatase	Biotec pharmacon
	10xCutSmart buffer	NEB
	Agel	NEB
Sodium dodecyl sulfate-polyacrylamide gel elect rophoresis (SDS PAGE) And Western blot	ProSieve Quad Color Protein marker 4,6-300kDa	Lonza
	Biotinylated protein ladder	Cell signaling
	Tris buffered saline with tween-20 (TBS-T)	For 1 Liter: 10 ml Tris pH 8.0 75 ml 2M NaCl 1 ml Tween 20 914 ml dH2O
	5 % milk in TBS-T	For 50 ml: 2.5g dried milk (Magermilch/Non-fat milk powder) 50ml TBS-T
	Gel blotting paper	GEHelthcare life sciences
	nitrocellulose blotting membrane 0,45 um	GEhealthcare life science.
	Transfer buffer	For 1 Liter: 1,08 g Tris 28,55g glycine 150 ml methanol H2O to 1 Liter SDS running buffer

		75g Glycine (Sigma Aldrich) 15g Tris 25 ml SDS (20%) (Sigma Aldrich) H2O to 5 L
	Ponceau S dye	Sigma Aldrich
	10% separating gel	For 10 ml: 4,9 ml H20 2,5 ml 40% AcrylAmide (Applichen) 2,5 ml 4x Separating buffer 100 uL 10% APS 10 uL TEMED (N,N,N,N-Tetramethylethylenediamine, Sigma Aldrich)
	Concentrating 4% Gel	For 10 ml: 6,4 ml H2O 1 ml 40% AcrylAmide (Applichen) 2,5 ml 4x Concentrating buffer 100 uL 10% APS 10 uL TEMED
	4xSeparating gel buffer	For 1 liter: 181,65g Tris-base 4g SDS dH2O to 1 liter pH adjusted to 8,8 with HCl
	4xConcentrating gel buffer	For 1 liter: 60,55g Tris base 4g SDS dH2O to 1 liter pH adjusted to 6,8 with HCl

	2xSDS gel loading buffer	100mM Tris-HCl pH 6,8 200mM DTT 4% SDS (w/v) 0,2% Bromophenol Blue (w/v) 20% glycerol (w/v)
	Chemiluminescent Peroxidase Substrate-3	Sigma Aldrich
Cloning of Fez1 NLS oligo into EGFP-beta gal vector	10x CutSmart buffer	NEB
	10x T4 Ligation buffer	NEB
	T4 polynucleotide kinase	NEB
	Shrimp alkaline phosphatase	Biotec Pharmacon
	Xho1	NEB
	KpnI	NEB
	10x Ligation buffer	NEB
	T4 DNA Ligase	NEB
Site directed Mutagenesis	dNTP	Sigma Aldrich
	DMSO	NEB
	10x Cloned Pfu reaction buffer	NEB
	10x Pfu Turbo polymerase	NEB
	Dpn I	NEB
Agarose gel electrophoresis	Agarose powder	SeaKem LE
	DNA ladder 1 kb	NEB
	DNA ladder 100 bp	NEB
	6 X T Gel Loading buffer	NEB
	Gel red	Biotium
	20x Minigel buffer	Tris/base 193,76 ml NaOAc 27,22 ml EDTA 14,9 ml dH2O up to 2 Liter pH adjusted to pH 8 with HAc
	BsrGI	NEB

Restriction enzyme digestion	NEB 2.1 buffer	NEB
Sanger sequencing	BigDye 3.1	Applied Biosystems
	Sequencing Buffer 5x	Our in house sequencing lab
Fixating and staining of cells on coverslips	Formaldehyde 4%	VWR
	Methanol >99,8%	Sigma Aldrich
	5 % Bovine Serum Albumin (BSA) in PBS	2,5g BSA (Sigma Aldrich) in 50 ml PBS
	DAPI	Sigma Aldrich. Diluted 1:1000 in PBS
LR Gateway reaction	LR Clonase EnzymeMix	Invitrogen
	Proteinase K	NEB
Transfection	TransIT-LT1	Mirus Bio LLC
	Metafectene Pro	Biontex laboratories
Freezing E.coli	50% Glycerol	Sigma Aldrich
Human cell lab	Fibronectin	20% fibronectin (Sigma Aldrich) in PBS
	Dimethyl sulfoxide (DMSO)	Sigma Aldrich
General	TE buffer	10 mM Tris-HCL pH 8.0 1mM EDTA
	Phosphate buffered saline	Gibco (diluted before use)
	GenElute Plasmid miniprep Kit	Sigma Aldrich
	Chemiluminescent Peroxidase Substrate-3 Kit	Sigma Aldrich
	Qiagen Plasmid midi prep Kit	Qiagen

Table 16. Instruments

Instrument	**Source**
NanoDrop 2000	Thermo Scientific
2720Thermal Cycler	Applied Biosystems
Electrophoresis power supply model 200/2.0	Bio-Rad
Confocal Microscope LSM780	Zeiss
BioRad power (pac 300) (Run 30 mA per gel)	BioRad
Trans-Blot Turbo transfer system Run standard SD midi (25V 0,3A 30 min)	BioRad.
Termaks incubator	FormaScientific
LAF cabinet	ScanLaf
Cell Counting Chamber	Bürker.

Methods

Growing E.coli on agar petri dish

The frozen E.coli were taken out of the archive and placed in a box that had -20°C. A sterile wire inoculation loop was used to inoculate the E.coli onto an agar plat with correct antibiotics. This was done by gently moving the inoculation loop around on the petri dish. The petri dish was placed into the cell culture room that had 37°C, overnight for growth.

Picking colony for further growth in broth

It was important to not leave the broth inside the incubator for too long. Ampicillin has a half-life of around 16-17 hours. The shaker inside the incubator moves the air so that the E.coli get more oxygen, more even distribution of nutrients and also prevents sedimentation.

3-4 ml LB broth with the correct antibiotic was pipetted into a 15 ml sterile glass tube with a lid. A single colony was touched with the tip of a micropipette. The micropipette was put into the glass tube and the lid closed. This was incubated overnight inside the 37°C incubator with shaking.

Miniprep

Miniprep is a method to isolate the amplified plasmid DNA from chromosomal DNA and other E.coli contents. To make this possible the plasmid DNA needs to be separated from chromosomal DNA and RNA.

RNA is removed by RNase A, a ribonuclease that catalyzes degradation of RNA. The resuspension solution most likely contains a chelating agent like EDTA. The chelating agent chelates calcium (Ca2+) and magnesium (Mg2+). Ca2+ and Mg2+ are essential for deoxyribonuclease (DNase) activity. The miniprep method relies on the chromosomal DNA denaturing at alkaline pH and being unsolvable when the solution is neutralized (Birnboim et al, 1979). The solution becomes less alkaline when the neutralization solution is added. The plasmid DNA can then anneal, while the chromosomal DNA is too big to properly anneal. The chromosomal DNA forms a pellet on the bottom of the tube when centrifuged. Annealed

plasmid DNA is solvable and remains in the supernatant. The lysis solution probably contains Sodium Dodecyl Sulfate (SDS). SDS is a detergent that dissolves the cell membrane, and also denatures many proteins. Denatured proteins form hydrophobic interactions and end at the bottom of the tube by centrifugation. It is important to be gentle in the lysis step, to avoid shearing of genomic DNA making it solvable in the supernatant. The column washes away contaminants while the plasmids remain bound until eluted.

Before the kit is ready for use RNase A solution was added to the resuspension solution, and 300 ml 95-100 % ethanol to the wash solution. To isolate the plasmids the instructions in the miniprep user guide were followed.

Procedure

1. 1,5 ml overnight culture was harvested by centrifugation at 13,000g. The broth was poured into a suitable container. The E.coli were resuspended with 200 uL resuspension solution by pipetting up and down. 200uL lysis solution was added, and the tube inverted gently to mix once. 350 uL neutralization solution was added and the tube inverted 4-6 times to mix. This was then centrifuged at 13,000g for 10 minutes.
2. The binding column was prepared by spinning it at 13,000g with 500 uL column preparation solution. The flow through was discarded. The supernatant from centrifugation was transferred to the column and spun 1 minute at 13,000g. The flow through was discarded. 500 uL optional wash solution was added and the column centrifuged 1 minute at 13,000g. The flow through was discarded. 750 uL wash solution was added and the column centrifuged 1 minute at 13,000g. The flow through was discarded.
3. The column was spun 1 minute at 13,000g to remove more liquid. The column was transferred to a new collecting tube and the DNA was eluted with 50 uL elution solution, by centrifuging 1 minute at 13,000g. The plasmid concentration was measured with the nano-drop machine.

Midiprep

Midiprep was performed to isolate the low copy pOG44 plasmid from E.coli.

Some plasmids are low copy plasmids and bigger amounts of E.coli culture are needed to get a good concentration. The Qiagen quick-start protocol was followed. At the end of the protocol the DNA is precipitated, so that it is ready for transfection. Before use the RNase A solution was added to buffer P1.

Procedure

1. 100 ml E.coli culture was centrifuged at 6000 g for 15 minutes. The supernatant was discarded and the E.coli resuspended in 4 ml buffer P1. 4 ml Buffer P2 was added and the tube inverted 4-6 times. This was incubated at room temperature for 5 minutes. 4 ml cold Buffer P3 was added, the tube inverted 4-6 times and incubated 15 minutes on ice.
2. This was then centrifuged at 20,000g for 30 minutes at 4°C. The supernatant was filtered through filter paper.
3. The Qiagen-tip was equilibrated with 100 ml buffer QBT. Supernatant was applied to the Qiagen-tip. Then the Qiagen-tip was washed with 10 ml Buffer QC twice. The DNA was eluted with 5 ml buffer QF.
4. 3,5 ml room temperature isopropanol was added to the eluted DNA. This was centrifuged at 15,000 g for 30 minutes at 4°C to precipitate the DNA. The supernatant was discarded.
5. 2 ml room temperature 70% ethanol was added. This was centrifuged for 10 minutes at 15,000 g to wash the pellet. The supernatant was discarded. The DNA was dried overnight in room temperature.
6. The DNA was resuspended in TE buffer.

Agarose gel electrophoresis

Agarose gel electrophoresis was used to see if LR gateway reactions had worked, oligoes had become inserted or if plasmids had become linearized.

It is a procedure where DNA is run on an agarose gel with a ladder. The gel is covered in buffer which conducts current when the power is turned on. This current makes the negatively charged DNA move toward the positive pole. The overall negative charge of DNA comes from the

charged oxygens on their phosphate backbone. This charge is distributed uniformly to all DNA because all DNA has a phosphate backbone. This means that the DNA is not separated by charge, but by mass (Lee et. Al. 2012). What separates the DNA by mass is the gel. A theory of how this works is that the pores in the gel work like a sieve. The DNA is said to moves like a snake through the pores. Smaller fragments of DNA go faster and therefore further over a given period of time. Bigger fragments are held back more, and therefore move slower and less over a given period of time. The higher the voltage applied, the quicker the DNA fragments move. However, the resolution might go down if too high voltage is applied. The more concentrated the agarose gel is, the smaller the pores in the gel are likely to be. Larger DNA fragments are separated better using a low concentration of agarose. Smaller DNA fragments are separated best in a higher concentration of agarose.

To make the plasmids visible on the gel, gel red and ultra violet (UV) light were used. The gel red intercalates between the DNA helix and is visible in UV light when bound to DNA. The ladder also becomes visible and is used as a reference to see how many base pairs the DNA has.

Procedure

1. 1 g agarose powder was put into 100 mL minigel buffer. This was then heated in a microwave until the solution became clear. 50 ml of this solution was poured into a gel form sealed with autoclave tape. The form stood on a straight surface. The gel comb was placed.
2. The samples were prepared by mixing the following:
   1 uL 6xT loading buffer**,** 100-300 ng plasmid DNA and dH2O to 6 uL.
3. The polymerized gel was covered completely with minigel buffer. The comb was removed and 5 uL ladder and 6 uL sample loaded into the wells. The gel was run for one hour at 90V.
4. The gel was placed in a gel red bath for 10 minutes. Then a picture was taken using BioDoc-It imaging system (a UV transilluminator machine).

LR Gateway reactions

LR reactions were used to move inserts from pENTR vectors into pDEST vectors.

The insert inside the pENTR plasmid was flanked by two attL sites. With help of a site specific recombination reaction done by lambda integrase family of recombinases (Hartley et. al. 2000), the insert was moved into the pDEST vector which had attR recombination sites. This is why it is called an LR reaction. The two plasmids carry different antibiotic resistance (kanamycin and ampicillin). In addition the pDEST plasmids contains a ccdB gene (Hartley et. al. 2000). This gene inhibits growth of E.coli (Bernard et al, 1992; Miki et. al.1992). The ccdB gene is removed when the insert is inserted into the pDEST vector. This selects inserted plasmids after the E.coli are transformed and plated on ampicillin plates.

After an LR reaction was done and amplified in E.coli purified plasmid was run on agarose gel to get an indication of whether the LR reaction worked.

Procedure

1. The following was mixed by vortexing it a few seconds:
   100 ng pENTER plasmid, 150 ng destination vector (pDEST plasmid),
   TE buffer up to 9 uL and then 1 uL LR Clonase Enzyme Mix to yield 10 uL altogether. This was then incubated in a 25°C water bath for one hour.
2. 1 uL Proteinse K was added, mixed by pipetting and incubated at 37°C water bath for 10 minutes. This was transformed into E.coli and plated on ampicillin plates.

Heat shock transformation of E.coli

Transformation is a process where bacteria take up new DNA. Bacteria that can do this are competent. Some bacteria are naturally competent (Timothy et. al.), but most are not (Yoshida, N. Sato, M. 2009). The E.coli used are made chemically competent by the technicians in the lab. Changing the temperature, giving the competent E.coli a heat shock brings the plasmid into the E.coli. After this the E.coli need time to express the antibiotic resistance carried on the plasmid. They are incubated in nutrient rich SOC for one hour. If the resistance is ampicillin the incubation time can be shorter. When the E.coli were plated with the correct antibiotic the transformed E.coli were able to grow.

Procedure

1. The frozen E.coli was thawed on ice for 20 minutes. 50 uL E.coli was transferred into falcon tubes on ice. 5 uL LR reaction or 50 ng plasmid DNA was added into the E.coli. This was mixed by pipetting up and down carefully to avoid harming the E.coli.
2. After incubating this for another 20 minutes on ice, the E.coli were heat shocked. This was done by putting them in a 37°C water bath for 2 minutes. The tubes were put back on ice for two minutes. 1 ml SOC was added and the E.coli were incubated in the 37°C culture room for one hour.
3. The E.coli was transferred to eppendorf tubes and spun at 2500 g for 5 minutes. 750 uL supernatant was discarded and the E.coli were resuspended in the remaining 250 uL by pipetting up and down. These 250 uL were plated on a plate with the correct antibiotic, and incubated overnight.

Restriction enzyme digestion

Restriction enzymes are endonucleases that cut DNA at specific sequences. In this thesis this was done to linearize DNA or before running DNA on gel electrophoresis to see if LR reactions had worked. BsrGI is a restriction endonuclease that will cut out inserts from pDEST EGFP plasmids. After an LR reaction and restriction digestion by BsrGI two bands were expected on the gel, one for the plasmid and one for the insert.

Procedure for cutting with BsrGI

1. 1 uL NEB 2.1 buffer, 1 uL/~300ng plasmid, 0,5 uL BsrGI and 7,5 uL dH2O were incubated in a 37°C water bath for 1 hour.

Sanger sequencing

Sanger sequencing was done to verify cloned and mutated plasmids.

The DNA is denatured to single stranded DNA, the primer binds, and the DNA polymerase adds deoxyribonucleotide triphosphate (dNTP) onto the growing strand. DNA polymerase depends on the 3` OH group being present on the sugar ring, because it connects the alfa phosphate with the new nucleotide to it. In Sanger sequencing some dNTP is replaced by

dideoxyribonucleotide triphosphate (ddNTP). The ddNTP doesn't have the 3` OH and therefore terminates chain elongation. This is done many times at random places on the strand. The ddNTP is fluorescently labeled with different colors for each nucleotide, and our in house sequencing lab uses this to sequence the DNA.

Procedure

1. The following was mixed: 2 uL 5x Sequencing buffer, 1 uL BigDye 3.1, 1 uL primer (10uM), 200-500 ng plasmid DNA and dH20 to 10 uL. The mix was put into a PCR machine running the following program:
   96 °C 20 seconds, 51°C 15 seconds, 60°C 4 minutes, repeat 34 times and then hold at 4°C.
2. When the program was done the tube was handed to the sequencing lab. The DNA sequences were analyzed bioinformatically using ExPASy (ExPASy) and BLAST (NCBI).

Site directed mutagenesis

Site directed mutagenesis is a way of making specific mutations in DNA. Oligonucleotide primers containing the desired base change were used in a PCR reaction. In the PCR machine DNA denatures, the primers anneal to one strand each and DNA polymerase elongates the strands. After many repeats the resulting plasmids will contain the desired base change. The wild type plasmid is digested by the restriction enzyme DpnI.

Procedure

1. The primers (Fez1 F56A fw, Fez1 I52A F56A fw, Fez1 F56A rev and Fez1 I52A F56A rev) were diluted with dH2O to a final concentration of 100uM.

2. A PCR reaction was done, the following was mixed in a tube: 1 uL forward primer (Fez1 F56A fw, Fez1 I52A F56A fw) diluted to 10 uM with water, 1 uL reverse primer (Fez1 F56A rev, Fez1 I52A F56A rev) diluted to 10 uM with water, 1 uL DNA template (pENTR Fez1 and pENTR Fez1 LIR1 and LIR3 mut) with a concentration of 25 ng/uL, 4 uL dNTP (10uM), 0,5 uL DMSO , 2,5 uL 10x Cloned Pfu reaction buffer, 0,5 uL Pfu Turbo polumerase and dH2O to a final volume of 25 uL. The PCR program was: (96°C 2 minutes (96°C 30 seconds, 54°C 1 minute. 68°C 7 minutes)x 20 repetitions, 68°C 10 minutes and then hold on 4°C .

3. 1uL DpnI was added, this was mixed by centrifuging in a mini-centrifuge for 5 seconds and put onto a 37 °C water bath for one hour. 5 uL of the reaction transformed into 50 uL E.coli, plated on kanamycin LB plates and incubated overnight.
4. Three colonies were picked from each plate and grown overnight in LB kanamycin broth in the culturing room. Miniprep was done. The plasmids were sent to the sequencing lab for DNA sequencing.
5. Correctly mutated Fez1 constructs were transferred into pDEST EGFP Flp-In by LR reaction. Correct recombination was verified by DNA sequencing.

Cloning of the putative Fez1 NLS into the pEGFP-βgal N1 vector

Two oligoes, Fez1 NLS fw and rev, encoding the putative Fez1 NLS, were annealed. These had to be phosphorylated so that they could become ligated in frame with EGFP βgal in a mammalian expression vector. DNA without 5`phosphates cannot be ligated.

The restriction enzymes XhoI and KpnI were used to make two double strand cuts in the vector. Shrimp alkaline phosphatase dephosphorylated the plasmid and the small piece that was cut out, preventing relegation. There was now space and a place for the NLS to be ligated to, and so it was ligated (the oligoes were phosphorylated).

Procedure

1. The EGFP- βgal plasmid was plated on ampicillin plates, grown overnight, a colony picked, and grown overnight in ampicillin LB broth. Miniprep was done.
2. The oligoes (Fez1 NLS fw and Fez1 NLS rev) were resuspended in dH2O to a final concentration of 100uM. The oligoes were phosphorylated and annealed by incubating the following for half an hour in a 37°C water bath, and then run in a PCR machine with the following program: 95 °C 5 minutes and ramp down to 25 °C at 5 °C per minute :
1 uL Oligo Fez1 NLS fw (100uM), 1 uL Oligo Fez1 NLS rev (100uM), 1 uL 10xT4 Ligation buffer, 6 uL dH2O and 1 uL T4 Polynucleotide kinase.
3. The oligo was then diluted 1:100 in dH2O. Linearization of the EGFP-βgal plasmid was done by incubating the following in a 37°C water bath for 45 minutes: 2uL 10xCutsmart buffer, 1 ug pEGFP-betagal-N1 vector, 0,5 uL Xhol , 0,5 uL KpnI and dH2O to 20 uL.

4. 0,5 uL SAP was added and incubation continued for 15 minutes. The digested plasmids were purified with PCR clean up kit. 100 ng plasmid was run on agarose gel to verify the linearization.
5. The oligoes were ligated into the linearized EGFP-βgal plasmid, by incubating the following in a 25°C water bath for two hours:
1 uL 10xligation buffer, 50 ng linearized EGFP-βgal plasmid, 1 uL diluted, phosphorylated and annealed oligo, 1 uL T4 DNA ligase and dH2O to 10 uL.
6. The plasmids were transformed into E.coli, plated on ampicillin plates and incubated overnight. A colony was picked for further growth in LB ampicillin broth overnight. Miniprep was done.
7. The plasmids were verified by DNA sequencing. Fresh E.coli culture with the plasmid was cryopreserved.

Precipitating plasmid DNA

This was done to purify plasmids before transfected into mammalian cells.

Precipitation is about solubility. For the DNA to precipitate it needs to be poorly solvable in the solvent. DNA is polar and solvable in water, but not in ethanol.

Ethanol is added two times, once to precipitate DNA and once to wash away salts (salts are solvable in 70% ethanol). When the cold 96% ethanol is added in the procedure, the amount added is the volume of the solution times two. This gives 2/3 ethanol = 0,667 which rounds up to 70% ethanol.

In the procedure sodium acetate (NaOAc) is added to the DNA. NaOAc breaks down to: Na+ and CH3COO-. Na+ is drawn to the negative charge on DNA. In water there are hydration shells which stops this interaction. When ethanol which has a much lower dielectric constant is added, the hydration shells are interrupted. Na+ is able to condense DNA in the presence of ethanol (Flock et. al. 1996). The ions are important to neutralize the phosphate backbone charge of DNA (Flock et. al 1996).

Procedure

1. The 96% ethanol was put into the -20°C freezer for 5 minutes. The volume of the solution to be precipitated was estimated with pipet, if not already known.
2. 10% of the sample volume, of Sodium Acetate (NaOAc) with pH 5,2 was added to the plasmid solution.
3. The new volume was calculated, and the equivalent of the new volume times two of 96% percent ethanol from the -20°C freezer was added. This was vortexed 10 seconds and incubated in room temperature for 20 minutes.
4. It was then centrifuged at 13,000 g for 30 minutes. The supernatant was discarded carefully, by pouring it out on the opposite side from the DNA. The centrifugal force causes most of the DNA to be on the side of the eppendorf tube that pointing away from the middle of the centrifuge. The eppendorf tube was always placed with the lid pointing out of the middle inside the centrifuge.
5. 200 uL 70% ethanol was added and spun at 13,000g for 10 minutes. The supernatant was discarded.
6. The DNA was dried with vacuum or overnight in room temperature. It was then resuspended in TE buffer

Cryopreserving E.coli

E.coli were cryopreserved for long term storage of plasmids.

Cryopreserved means that very low temperature (-70 °C in the freezer) was used to preserve cells. When the water inside cells starts to freeze the solutes dissolved in the remaining liquid phase concentrate. The cells could become damaged by ice crystals or the changed composition of the remaining liquid phase (Pegg, D. E. 2007). To protect the cells during freezing and whilst frozen, glycerol, a cryoprotectant was used. Glycerol penetrates the cell membrane and decreases the freezing-point of biological fluids and water to a minimum of -46 °C (Hubálek, Z. 2003).

Procedure

1. 300 uL 50% glycerol was added to a cryotube. 1,2 mL fresh overnight E.coli culture was added. This was mixed by pipetting and put inside the -70°C freezer.

Cell culturing

Cells were splitted routinely to avoid apoptosis and senescence. The cells were incubated in a 37 °C, 5% CO2 incubator with the correct amounts of O2 and humidity. The cells were split when they were around 90% confluent. To starve the cells they were put in hanks balanced salt solution for two hours. When the cells were treated with leptomycin B for two hours, 2,3 uL was used per 500 uL media (the leptomycin B from Sigma Aldrich was diluted 1:4 in methanol before use). Leptomycin B inhibits proteins with a nuclear export signal from exiting the nucleus. To induce protein expression with tetracycline media with 1ug/ml tetracycline was used for 12 hours.

Procedure

1. Media was removed with the aspirator. The cells were washed with PBS before trypsin was added. The cells were incubated until loosened. This took around 1 minute for Hek293 cells and 3 minutes for HeLa cells. The flask was hit gently to help loosen the cells. The cells were watched in the microscope to make certain that they really had become non- adherent.
2. Media was added (Table 17) to a new flask, and cells diluted between 1:10 to 1:5 times.

Table 17. Volumes added to adherent cells in flasks

Flask size	PBS	Trypsin	Media
25 ml	5 ml	0,5 ml	At least 5 ml
75 ml	8 ml	1 ml	At least 8 ml

Coating microscope cover slips

Cover slips were coated in order to obtain the cells to adhere better – especially the HEK293 cells.

Procedure

1. Sterile cover slips (1,5mm) were put in a 24 well dish. If they were not sterile, they were sterilized by incubating them in enough ethanol to cover them completely. The incubation lasted 20 minutes and afterwards the cells were washed with PBS twice.

2. 100 uL PBS with 20 % fibronectin was pipetted onto each cover slip. This was incubated for 20 minutes. The cover slips were washed with PBS.

Transfection of mammalian cells

Transfection is a method to transfer an expression plasmid into a eukaryotic cell.

Establishing inducible stable cell lines

HEK293 Flp-In T-rex cells were used to establish stable cell lines. The transfected DNA is incorporated into the cell genome, with help of a Flp-recombinase that has its DNA carried by pOG44. The plasmids cross the cell membrane with help of the liposomes from metafectene. The pOG44 DNA is expressed so that the Flp-recombinase is made. The HEK293 Flp-In T-rex cells have a Flp Recombination Target in their genome, and this is where the Flp-recombinase integrates the DNA. When the plasmid is inside the genome the HEK Flp-In cells gain hygromycin resistance.

Cell division is critical for the transport of the DNA into the nucleus. We therefore wanted the cells to grow exponentially when transfecting. In this thesis transfection was done when the cells were around 50-70 % confluent.

Transient transfection

In transient transfection the DNA of interest is transcripted and translated, but not incorporated into the genome. When the cells divide the transfected DNA disappears over time. HeLa cells were used and transfected at 70% confluency. TransIT was used as a transfection reagent to get less background fluorescence.

In both procedures it was important that the media used in the tubes where DNA and transfection reagent were, had no antibiotics or FCS. The media used on the cells after the liposomes were formed had antibiotics and FCS.

Procedure

1. The cells were seeded out as described in table 18.
2. Metafectene was thawed completely. Eppendorf tube 1 and 2 was prepared as described in table 18. It was only pipetted once to mix. When using transIT everything was put directly into the same tube.
3. The contents of eppendorf tube 1 was added to eppendorf tube 2. This was pipetted once to mix. When using transIT there was only one tube. This was incubated 20 minutes in

room temperature. It was then transferred drop wise, with a pipet to the cells. The cells were incubated overnight in the CO2 cell incubator.

4. 24 hours later, the cells were fixated on their cover slips when doing transient transfection. When making inducible stable cell lines selection media was added 24 or 48 hours after transfection, depending on confluence. The media was however always changed 24 hours after transfection to remove metafectene/TransIT.

Table 18. The amounts used in transfection

Making new inducible cell lines	
50 000 or 100 000 cells were seeded in a 6cm culture dish with 4 ml media, the day before transfection.	
Eppendorf Tube 1	**Eppendorf Tube 2**
500 ng pDEST Flp-In plasmid 1,5 ug pOG44 100 uL DMEM without antibiotic and Fetal calf serum (FCS)	4 uL metafectene 100 uL media without antibiotic and FCS
Transfection with metafectene	
20 000 cells were seeded in 24 well culture dish with 0,5 ml media the day before transfection	
Eppendorf Tube 1	**Eppendorf Tube 2**
50 ng DNA 25 uL MEME without antibiotics or FCS	1 uL metafectene 25 uL MEME without antibiotics or FCS
Transfection with TransIT	
The cells were in 0,5ml media in a 24 cm well culture tray (20 000)	
Eppendorf Tube	
100 ng pDEST EGFP plasmid 1 uL TransIT 50 uL MEME without antibiotics and FCS	

Cryopreserving mammalian cells

For mammalian cell lines glycerol and DMSO were used as cryoprotectants. DMSO penetrates the cell membrane (Hubálek, Z. 2003).

Procedure

1. The cells were washed with PBS and loosened by trypsination. They were pelleted by centrifugation for 3-5 minutes 1000 revolutions per minute (rpm). The media was removed.
2. The cells were resuspended in 0,9 ml FCS and 0,1 ml DMSO. This was transferred to a cryotube and put on ice or directly into the -70 degrees freezer. Later the cells were transferred to a liquid nitrogen freezer.

Fixation and staining of cells on coverslips

Cells on coverslips were fixated with paraformaldehyde (PFA). PFA reacts with primary amines on proteins and nucleic acids to form partially-reversible methylene bridges. The cells die but their structure should be preserved. Timing is important because too long and too short exposure to PFA will impair the result.

Procedure

1. The media was removed and 0,5 ml 4% paraformaldehyde (PFA) was added to each well inside a ventilated bench. This was incubated for 20 minutes. The PFA was removed and the coverslips washed twice with 0,5 ml PBS. The cover slips were sometimes stored in PBS, in the fridge a few days before continuing.
2. When incubating the cells with antibodies the following was done: 0,5 ml room temperature methanol was added to each well and incubated for 5 minutes. The cells were washed twice with PBS and then blocked with 0,5 ml 5% BSA for 20 minutes. 50 uL primary antibody (LC3 or GABARAP), diluted in PBS, was pipetted onto parafilm. The cover slips were mounted onto the drop, with help of a cannula and tweezers. They were put inside a box with a wet paper to keep the cover slips from drying out. This was incubated for one hour. Then the cover slips were washed in 1% BSA in PBS. 50 uL diluted secondary antibody (Alexa555) was pipetted onto parafilm and the cover slips mounted. This was incubated for another hour inside the box with the wet paper. The cover slips were washed in 1% BSA in PBS before continuing.
3. 10 uL diluted DAPI in PBS was pipetted onto parafilm. The coverslips were mounted onto the drop. This was incubated for 5 minutes. The cover slips were washed by dipping them in first PBS, then dH2O 2-3 times with help of tweezers. The cover slips were dried by letting them touch a paper, before they were mounted on 10 uL mowiol on the microscope slide. They were dried overnight in a drawer in room temperature. After this they were stored in the fridge.

Confocal Microscope

Confocal microscopy was used to study the localization of Fez2, Fez1 and various mutated forms of Fez1.

Fluorescence is when atoms or molecules absorb light of a particular wavelength and then emit light with a lower wavelength. In fluorescent microscopy only molecules that fluorescence become visible. In order to see various Fez1 mutants and Fez2, they were tagged with enhanced green fluorescent protein (EGFP), chromatin was stained by DAPI and endogenous proteins (Microtubule associated protein 1 light chain 3B (LC3B) or GABAA receptor associated protein (GABARAP)) were marked with fluorescent antibodies.

Crispr/ Cas9 knockout

The CRISPR/cas9 technology was used to make Fez1 and Fez2 knock out cell lines.

The pX461 plasmid (Ran et. al. 2013) contains the Cas9 enzyme. Oligoes containing the DNA guide sequences were phosphorylated and annealed and then ligated into the pX461 plasmid. The pX461 plasmid was linearized beforehand to make ligation possible. Shrimp alkaline phosphatase was used to prevent relegation.
To see if the pX461 plasmid had become linearized it was run on a gel. Round and linearized plasmids migrate differently through the gel and one band at around 9 kb was expected for a linearized plasmid. The pX461 plasmid is around 9300 bp. By experience non-linearized plasmids turned up at around 6000 bp.

Procedure

1. The guide oligoes (see table 12) were diluted in dH2O to 100uM. To phosphorylate and anneal the oligoes the following was mixed in a PCR tube and incubated in the PCR machine: 1 uL oligo forward, 1 uL oligo reverse, 1 uL 10x T4 Ligation buffer, 6 uL dH2O and 1 ul T4 Polynucleotide kinase. The PCR machine had the following program: 37°C 30 minutes, 95°C 5 minutes, ramped down to 25°C at 5°C per minute.
2. The pX461 plasmid was linearized by incubation the following in a 37 °C water bath for 30 minutes: 2 uL 10xNEB 2 buffer, 1 ug pX461 vector, 0,5 uL BbsI and dH2O to 20 uL

3. 0,5 uL SAP was added and it was incubated for another 15 minutes in the water bath.
4. The linearized pX461 was cleaned up with the PCR clean up kit. 100 ng was then run on a 1% agarose gel to see if the plasmid had become linearized.
5. The phosphorylated and annealed oligoes were diluted 1:200. They were then ligated to the linearized, cleaned pX461 plasmid by incubating the following on a 25 ℃ water bath for 2 hours: 1 ul 10x ligation buffer, 50 ng linearized PX461 vector, 1 uL phosphorylated and annealed oligo (diluted 1:200 in dH2O first), 1 uL T4 DNA ligase and dH2O to 10 uL.
6. The ligation mix was transformed into E.coli, plated onto ampicillin plates and incubate overnight in the 37 °C room. 2 colonies were picked for further growth overnight in LB broth with ampicillin. Miniprep was done. The plasmids were incubated in a 37 °C water bath for one hour with the following: 1 uL CutSmart buffer, 3 uL purified plasmid, 0,5 uL BbsI, 0,5 uL Agel and 5 uL dH2O. This was then run on agarose gel (2 uL 6XT loading buffer were added).
7. Plasmids with correct restriction pattern were sequenced, using 150 ng plasmids and 1 uL pX330-seq-U6 primer. Verified plasmids was precipitated to prepare it for transfection and fresh E.coli culture with the plasmid was used to cryopreserve the plasmids.
8. 300 000 Hek Flp-In cells were seeded out in 6 well plates and 2 ml media was used in each well. After 24 hours the cells were transfected with the Crispr plasmids (Table 19), using metafectene.

Table19. Showing the amount of plasmid to put into each tube.

Fez1:	
Tube 1: T1A+T1B (1 ug+1ug)	Tube 2: T2A+T2B (1ug+1ug)
Fez2:	
Tube 3: T1A+T1B (1ug+1ug)	Tube 4: T2A+T2B (1ug+1ug)
Fez1 and Fez2:	
Tube 5: (T1A+T1B) Fez1 + (T1A+T1B) Fez2 (500 ng+500ng) + (500ng+500ng)	
Tube 6: (T2A+T2B) Fez1 + (T2A+T2B) Fez2 (500ng+500ng) + (500ng+500ng)	

9. After 24 hours the cells were looked at in a fluorescent microscope to ascertain that there were green transfected cells before proceeding. 96 well plates were prepared by

adding 100 uL media to each well. The media used in these contained 20% FCS instead of 10%. The transfected cells were loosened properly with trypsin. A glass tube with a filter cork was used to filter the cells into the tube. They were put on ice and carried to the BD FACSARIA iii cell sorter. This machine put a single green cell into each well in the 96 well plates. The cells were incubated in the cell incubator and 100 uL normal media was added once a week until the wells were confluent. To find the wells where the cells were growing the plates were held towards light to look for a slightly different color (pinker color) in the media or white spots at the bottom of the well. These wells were then checked in a microscope and transferred into 1 well in a 24 well plates. When these cells were confluent they were split from the well in the 24 well plate into 3 wells in a 6 well plate. When these were ready one well was cryopreserved, one harvested for western blot and one split into 75 cm cell culture flasks. When the 75 cm culture flasks were 70% confluent two more samples were cryopreserved.

Western Blot

Western Blot is a method were antibodies are used to detect a specific protein in a cell extract or protein mix. Here Western Blot was used to investigate if Fez1 or Fez2 was successfully knocked out by CRISPR/Cas9 and whether the various EGFP Fez1 constructs and EGFP Fez2 was expressed in the Flp-In cells.

Acrylamide is a potent neurotoxin so it was important to wear gloves and to work inside a ventilated cabinet. When the gel has polymerized it is no longer as dangerous as it is in its liquid state. The acrylamide gel the samples are run on is a form of gel electrophoresis like described in the agarose gel electrophoresis section. The difference is that we are separating proteins according to both size and charge. The proteins are denatured (the 2x SDS gel loading buffer lysed the cells and then they were boiled for 10 minutes). SDS binds noncovalent to proteins and gives them a negative charge because SDS is negatively charged. Around one SDS binds per two amino acids in the protein which yields about the same amount of SDS per amount protein. The proteins are transferred from the gel onto a membrane in the Trans-Blot Turbo transfer system. The SDS gives the proteins a negative charge which causes them to move toward the positive charge when the current is turned on. This makes the proteins migrate

downward in the machine, which is why it was important to put the membrane underneath the gel. The Ponceau S dye stains proteins red and makes them visible on the membrane. Blocking with 5% non-fat milk in TBS-T was done to reduce non-specific binding of the primary antibody. Antibodies are proteins and the membranes has high affinity for proteins. 5% non-fat milk in TBS-T contains proteins that saturate the membrane. The first time the membrane is washed with TBS-T it is to remove excess unbound primary antibody. The second time it is to remove excess unbound secondary antibody. This reduces "noise" on the final picture.

The secondary antibody is conjugated to horseradish peroxidase (HRP), which is a chemiluminescent substrate. Luminescence means emission of light, and chemiluminescence is emission of light because of a chemical reaction. This light can be detected in the ImageQuant LAS 4000. The HRP linked biotin antibody binds to the biotin ladder and makes it visible also. When applying the Chemiluminescent Peroxidase Substrate-3 timing is important. The bands on the picture can become too dark or the substrate for the chemiluminescent reaction (HRP) can become used up.

Preparing samples for Western blot procedure

1. The cells were washed with PBS, trypsinated, media was added and the cells transferred to eppendorf tubes where they were centrifuged 3 minutes, to pellet the cells. The media was removed and 60 uL 2xSDS gel loading buffer with 20%DTT was added. Sometimes the media was removed and 60 uL 2xSDS gel loading buffer with 20% DTT was put directly into the well in the 6 well plate. After this the cells were mixed until they became more viscous, and put into eppendorf tubes before they were boiled 10 minutes at 100°C.

Procedure

1. The dual gel caster was assembled and filled up to three fourths with 10% separating gel solution. Water was put on top of this and incubated for 20-30 minutes until the gel polymerized. The water was removed and a gel comb was added. The rest of the gel caster was filled with concentrating gel, and it was tried to avoid air bubbles. This was incubated for 20-30 minutes until the gel had polymerized.
2. The gel was put into the BioRad power chamber with SDS-gel running buffer. 5 uL ProSieve Quad Color Protein marker and 5 uL Biotinylated protein ladder were put into the same well. 5-20 uL samples were loaded. The BioRad power supply was

turned on and programmed to run with 30 mA per gel. The gels were run for 1,5 hour until the "migration front" had reached the bottom.

3. Membrane and gel blotting paper was cut into 6,5 x 8,5 cm pieces and put into transfer buffer. A "sandwich" gel blotting paper, gel, membrane and gel blotting paper was made. The membrane was always underneath the gel. Trans-Blot Turbo transfer system was turned on and the standard SD midi program was run (25V and 0,3A for 30 minutes).
4. The membrane was put into a box with Ponceau S dye for 3 minutes. The membrane was washed with dH2O by letting dH2O run over it in the sink. A picture was taken. The membrane was put into a box with 5% milk in TBS-T for 30 minutes on gentle shaking. 1,5 ml with 5% non-fat milk in TBS-T or TBS-T was put into tubes. Primary antibody was added to the tube and then the membrane. This was incubated overnight at 4°C on a rotater. The membrane was washed in TBS-T for 30 minutes, while changing the TBS-T 4-6 times.
5. A new tube with 1,5 ml 5% non-fat milk in TBS-T or TBS-T, and secondary antibody (HRP anti rabbit) in addition to HRP antibiotin was prepared and the membrane added. This was rotated for one hour at room temperature. The membrane was then washed in TBS-T for 30 minutes while changing the TBS-T 4-6 times.
6. 1 ml of each of the reagents in the Chemiluminescent Peroxidase Substrate-3 Kit (chemiluminescent reagent and chemiluminescent reaction buffer) was mixed and 1 ml put onto each membrane. This was incubated in a dark place for 5 minutes. Chemiluminescent detection and images obtained using ImageQuant LAS 4000.

Firms used as references in methods applied:

- Addgene, available from: https://www.addgene.org/
- Biontex, available from: http://www.biontex.com/con_4_6_4/cms/front_content.php?changelang=1
- Bio-Rad available from: http://www.bio-rad.com/?WT.srch=1&WT.mc_id=aw-corp-EU-brand&WT.knsh_id=e5bed7e3-a70d-4e93-ba41-4c2e499d0266
- Biotium, available from: https://biotium.com/
- NCBI, available from: http://www.ncbi.nlm.nih.gov/
- ExPASy translate tool available from: http://web.expasy.org/translate/
- ThermoFisher, available from: https://www.thermofisher.com/us/en/home.html

RESULTS

The predicted NLS in Fez1 is functional

It is known that Fez1 goes into the nucleus (Lanzaa et al, 2008). A bipartite nuclear localization signal (NLS) has been reported in Fez1 at positions 289-292 and 290-293 (Lanzaa et al, 2008). The nucleus is separated from the cytosol by the nuclear envelope. The nuclear envelope has nuclear pores that allows small proteins and salts to pass by passive diffusion. Proteins bigger than ~40 kDa cannot pass the nuclear pores through passive diffusion (Pankiv et. al. 2010). Instead the proteins pass the nuclear pores to get into and out of the nucleus directed by NLS and nuclear export sequence (NES) in the respective protein. Usually importin-α or exportin1/CRM1 binds to NLS or NES, respectively. This interaction transports the proteins into or out of the nucleus (Terry et al, 2007).

Fez1 is larger than 40 kDa (Table 20). However, the function of the Fez1 NLS has not been verified experimentally. In order to test the Fez1 NLS experimentally the cNLS predictor available from: http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_y.cgi was used. We used it to to verify the prediction of a Fez1 NLS in the coiled coil region of the Fez1 C-terminus. The result supported the predicted NLS at position 286 with the sequence ELMKKRRKEKG and with cut-off score 6 (Figure 6A). The higher the cut of score the stronger the predicted NLS activity. Cut off-score 7-8 predict that a GUS-GFP reporter protein fused to a NLS sequence would be partially localized to the nucleus. Cut off score 3, 4 and 5 predict that a GUS-GFP reporter protein fused to a NLS would be localized to both the nucleus and the cytoplasm.

Pankiev et al, (2010) used EGFP-βgal-N1 plasmid to study the NLS of p62/SQSTM1. In order to test the predicted Fez1 NLS experimentally, the 289-EELMKKRRKEKGG-293 sequence of Fez1 was cloned in frame in front of EGFP-βgal fusion protein (Figure 6B). This was done to see if it would be sufficient to make βgal go into the nucleus. The construct was transiently transfected into HeLa cells. 24 hours after transfection the cells were treated with LMB for two hours, starved in Hanks balanced salt solution for two hours, or left untreated for two hours. Leptomycin B/LMB is a nuclear export inhibitor that inactivates CRM1/exportin1, a nuclear export sequence (NES) receptor in eukaryotes (Kudo et al, 1999). When proteins (that were too big for passive diffusion), went into the nucleus during the two hour incubation time, they were kept there by LMB. An EGFP-βgal construct without Fez1 NLS was transfected in parallel as

control. After fixation, DNA was stained by DAPI, and the localization studied by confocal fluorescent microscopy. The images presented in Figure 6C shows that the NLS-EGFP-βgal protein is cytoplasmic localized under normal and starved conditions, while treatment with LMB results in nuclear accumulation. The EGFP-βgal control shows a weak nuclear staining with LMB, but much less prominent than the NLS-EGFP-βgal. This indicates that the Fez1 NLS is functional, and has the potential to direct Fez1 to the nucleus. The very weak nuclear staining during normal and starved conditions, may indicate that this is a weak NLS, or that the nuclear export is very rapid.

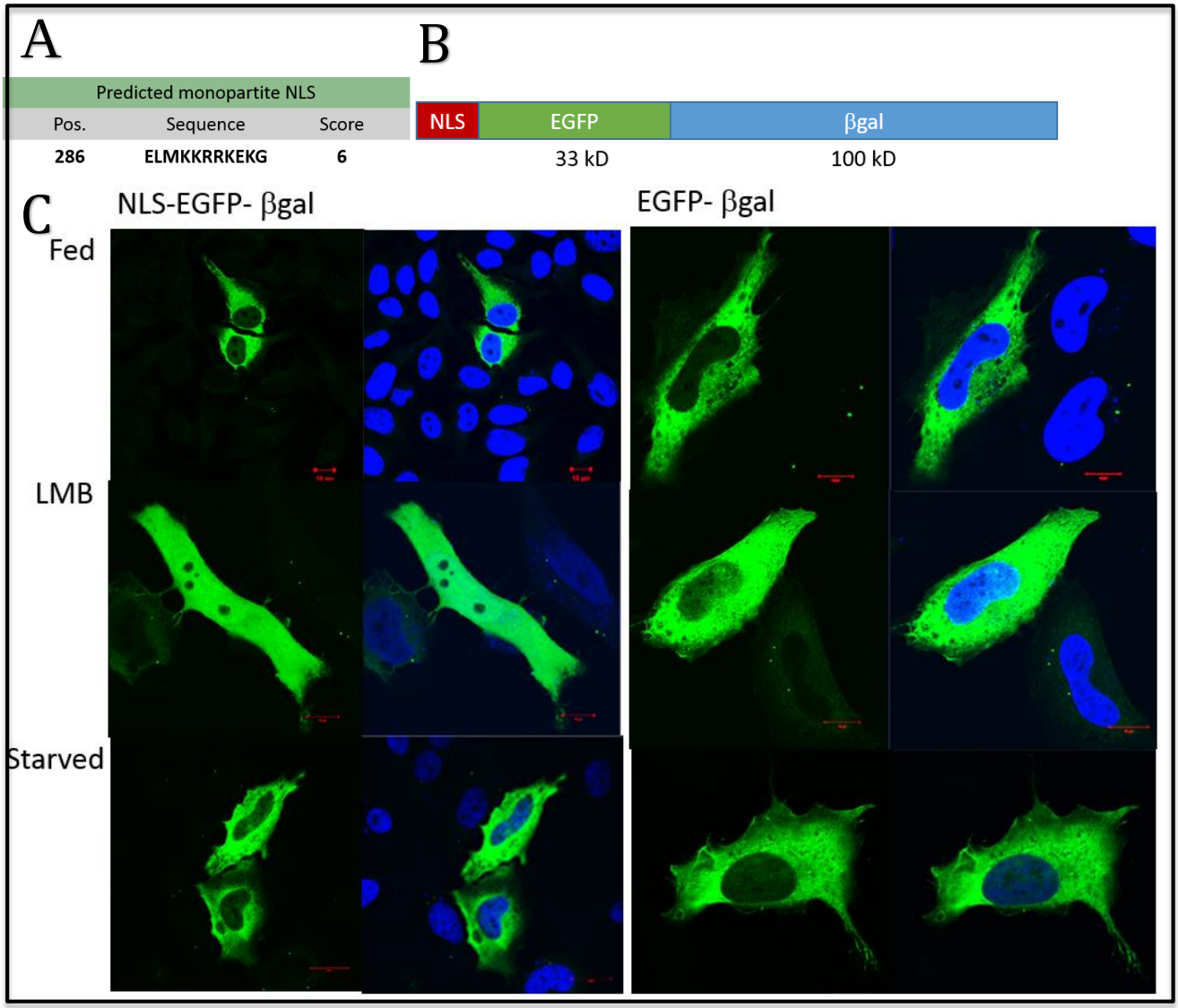

Figure 6. The Fez1 NLS is functional. A) The cNLS predictor predicted that Fez1 has a NLS at position 286 with a cut off score 6. **B)** Presentation of the NLS-EGFP-βgal protein, with the putative NLS of Fez1. **C)** Confocal fluorescent microscope images of NLS-EGFP-βgal and EGFP-βgal transiently transfected into HeLa cells and treated with LMB, starved in hanks balanced salt solution or under normal growth conditions for 2 hours, 24 hours after transfection. NLS-EGFP-βgal is accumulated more in the nucleus than EGFP-βgal after LMB treatment.

Fez1 has two regions that direct nuclear localization

The finding that the predicted Fez1 NLS seems to be relatively weak, prompted us to investigate whether Fez1 contains more than one NLS. To this end, four deletion constructs of Fez1 were cloned behind EGFP into a pDEST EGFP plasmid (Figure 7).

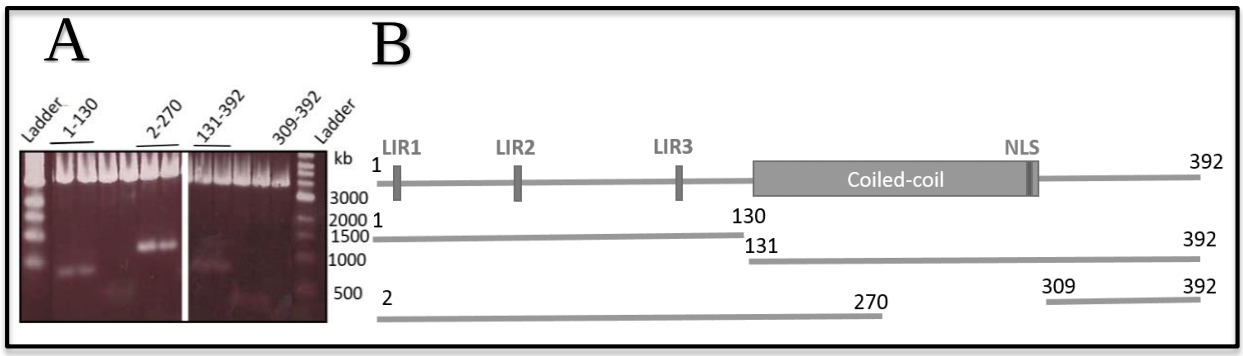

Figure 7. The Fez1 deletion constructs were succesfully cloned into a pDEST EGFP plasmid. A) The pDEST EGFP plasmids contained the Fez1 deletions after LR reaction. The pDEST EGFP plasmids with Fez1 deletions were treated with BsrGI and analyzed on a 1% agarose gel followed by gel red incubation. The pDEST EGFP plasmids were also verifyed by sequencing. **B)** Schematic representation of the Fez1 deletions. LIR1, LIR2, LIR3: Atg8 interaction motifs mapped by Alemu (unpublished). NLS: Nuclear localization signal.

The deletion constructs of Fez1 were transiently transfected into HeLa cells. 24 hours after transfection they were treated with LMB for two hours, starved in hanks balanced salt solution for two hours or left untreated for two hours. Proteins bigger than ~40 kDa have to be actively transported to cross the nuclear envelope (Pankiv et. al. 2010). The Fez1 deletions molecular weights (MW) are listed in Table 20. After fixation the cells DNA was stained by DAPI and studied by confocal fluorescent microscopy (Figure 8).

Table 20. The MW in kDa of the different EGFP Fez1 deletions.

Fez1 and deletions	Amino acids	*0,11	+33 kDa for EGFP	Weight kDa
Fez1	392	43,12	33	76,12
1-130	130	14,3	33	47,3
131-392	261	28,71	33	61,71
309-392	83	9,13	33	42,13
2-270	268	29,48	33	62,48

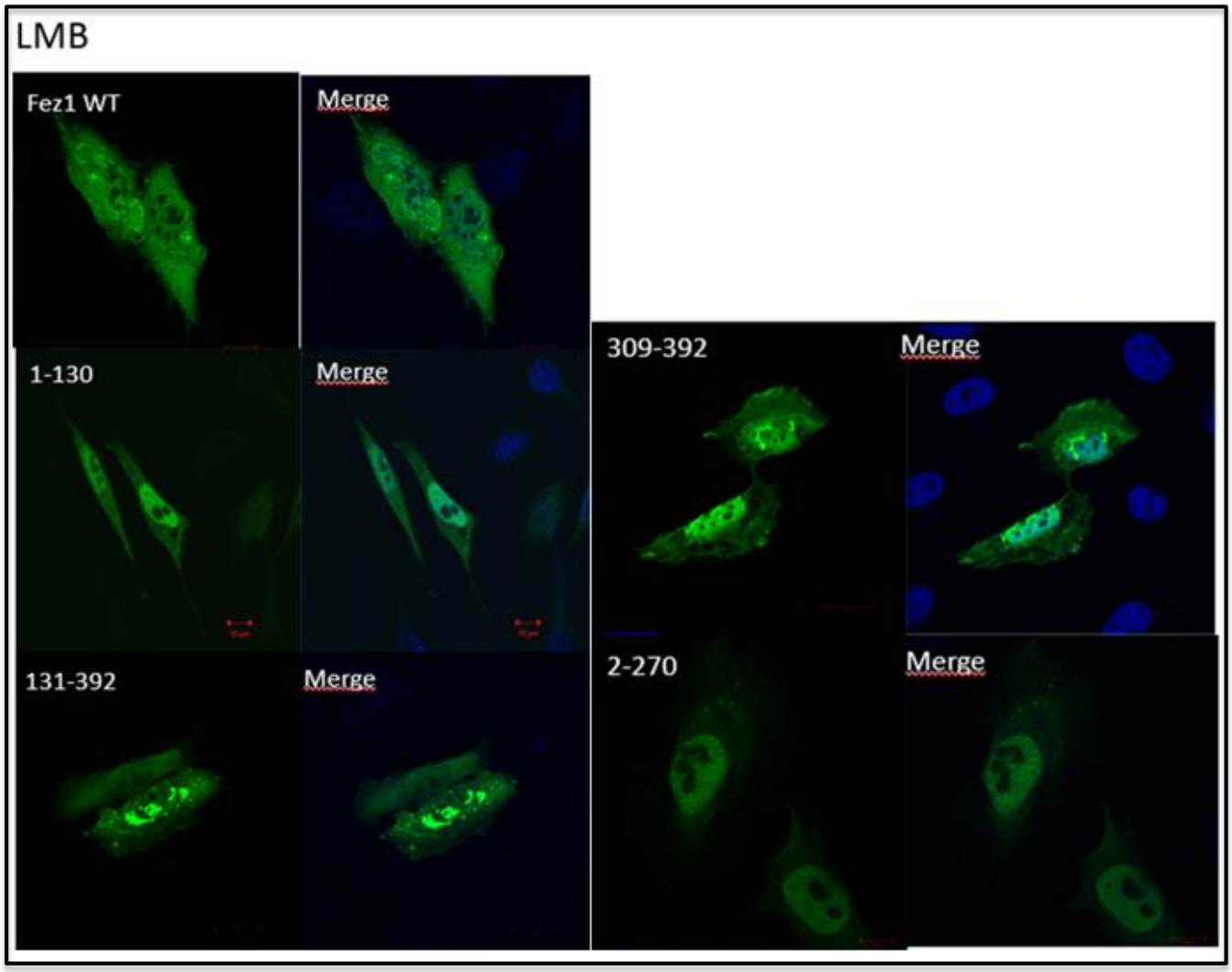

Figure 8. All Fez1 deletion constructs were accumulated in the nucleus after treatment with LMB. HeLa cells were transfected with EGFP Fez1 deletion constructs and treated with LMB for 2 hours, 24 hours after transfection. They were fixed and stained by DAPI before studied in the confocal fluorescent microscope.

EGFP Fez1 2-270 was clearly accumulated in the nucleus in all cells after LMB treatment. This indicates that Fez1 may have a second NLS, since the predicted NLS is not present in 2-270. EGFP Fez1 1-130 is also accumulated in the nucleus, suggesting that the second putative NLS of Fez1 may be in the Fez1 2-130 region.

Fez1 is mainly cytoplasmic localized in both normal and starved conditions

Autophagy is induced when cells are exposed to various stresses like starvation or low oxygen levels. To test wether Fez1 relocalises to the nucleus during stress, HeLa cells transfected with the Fez1 deletion constructs were starved in hanks balanced salt solution for 2 hours, 24 hours after transfection. After fixation the cells were stained by DAPI and studied by confocal fluorescent microscopy. Figure 9 shows that EGFP Fez1 131-392 and 309-392 were nuclear localized in both normal and starved conditions. Wild type (WT), EGFP Fez1 1-130 and 2-270 were mainly cytoplasmic in both conditions. This supports the function of the predicted NLS at position 286, while the 2-130 region may contain a strong NES. Since Fez1 seems to have two NLS it needs a way to exit the nucleus so it is logical to assume that it also has a NES. The fact that Fez1 WT was not in the nucleus while EGFP Fez1 131-392 was, suggests that the NES is not in the 131-392 region. We tried to predict a NES with two online NES predictors. One predictor predicted no NES and the other predicted several but they had very low scores.

EGFP Fez1 309-392 was enriched in the nucleus on all the confocal fluorescence microscopy images taken in this thesis. We assume that this is due to its small size, meaning that it crosses the nuclear pores by passive diffusion. Assuming that it is passive diffusion we expect there to be an equilibrium between the nuclear and cytoplasmid EGFP Fez1 309-392, and the amount of EGFP Fez1 309-392 inside the nucleus should not be affected by LMB. The other EGFP Fez1 deltions should all be to big for passive diffusion (Table 20).

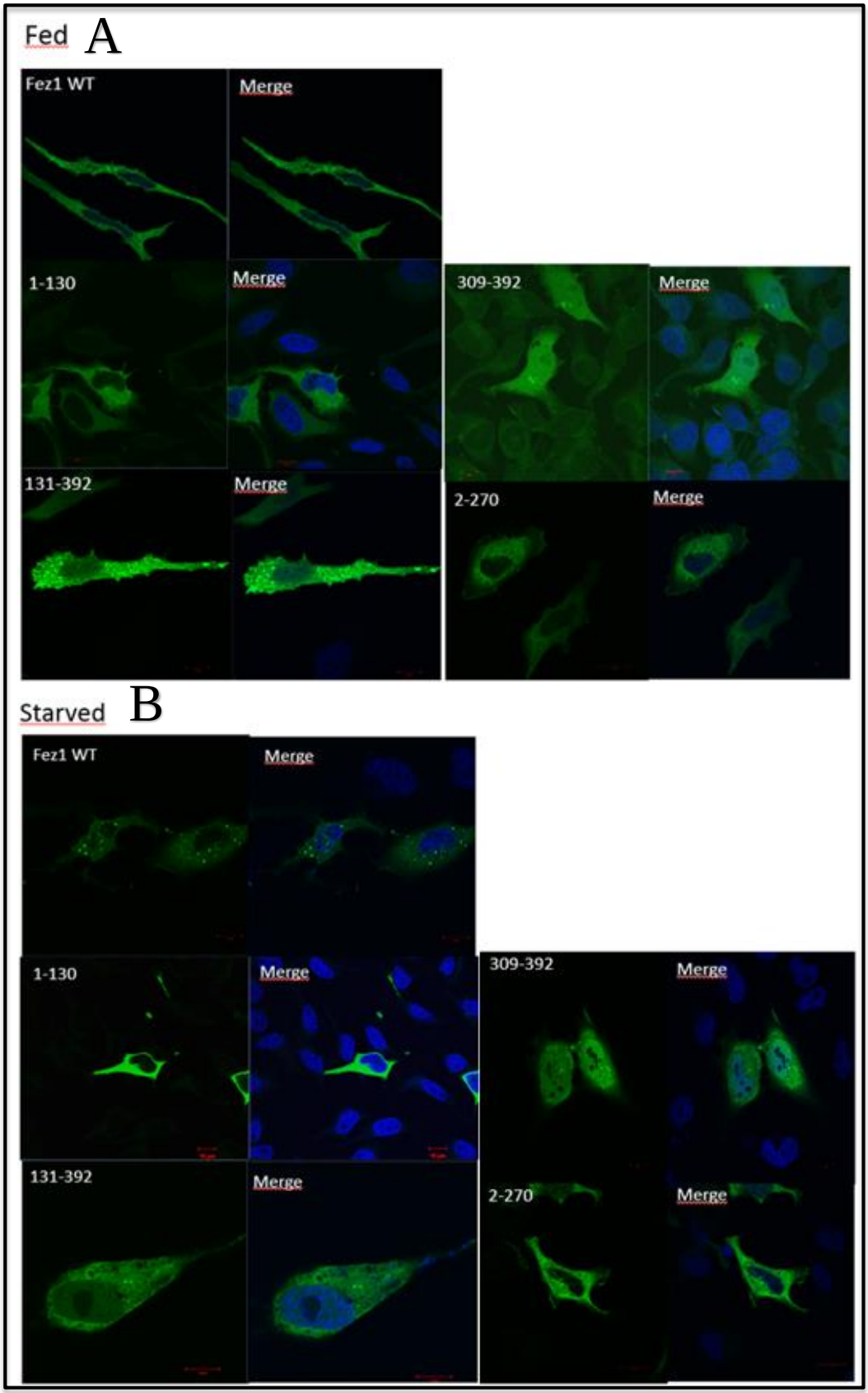

Figure 9. EGFP Fez1 131-392 was accumulated in the nucleus in both fed and starved conditions while Fez1 WT was not. HeLa cells were starved for 2 hours or left in normal growth conditions for 2 hours, 24 hours after transient transfection. After fixation DNA was stained by DAPI and the cells imaged by confocal fluorescent microscopy.

Establishment of stable cell lines with inducible expression of EGFP Fez1 mutant constructs and EGFP Fez2

Previously, three LIR motifs have been mapped in the N-terminal of Fez1 (Alemu, unpublished data). Furthermore, phosphorylation of serine 58 (S58), which is close to LIR2, has been shown to have impact on Fez1 binding to Kinesin-1. The phosphorylation induces kinesin binding and is important for sprouting of nerve cells (Chua et al, 2012). In order to study the importance of the predicted LIRs and the S58 phosphorylation on Fez1 localisation; HEK293 Flp-In T-rex stable cell lines with inducible expression of EGFP Fez1 LIR mutants, S58E and S58A mutants, were established.

First LR reaction was done to get the mutated Fez1 constructs in a pDEST Flp-In EGFP vector. The plasmids were veryfied by sequencing and transfected into HEK293 Flp-In T-rex cells. Succesfully transfected cells were selected with blasticidin and hygromycin. Expression of EGFP-Fez1 proteins was induced with tetracycline for 12 hours. Induced and not induced samples were run on western blots to confirm that the mutant constructs were expressed and that they had the right size (~80 kDa for Fez1 mutants and ~75 kDa for Fez2). Figure 10 shows that the mutant constructs were all expressed and that they had the correct size. The cell lines transfected with Fez2, LIRm (1), LIRm (1,3) and S58A have as expected, stronger bands in the induced samples than not induced samples verifying induced expression of the EGFP-fusion proteins. S58E has a stronger band for not induced than for induced, suggesting that the S58E samples were swopped by mistake.

Next, cells were seeded on cover slips, fixated and the cells DNA stained by DAPI. Localizations of the various mutants were studied by confocal fluorescent microscopy. Confocal fluorescent microscopy images displayed more green cells in the induced than not induced samples (Figure 10), verifying inducible expression of all constructs. The Fez1 mutants are localized mainly in the cytoplasma. Some of the constructs seem to be accumulated perinuclear especially EGFP Fez1 S58A and EGFP Fez2. Furthermore, EGFP Fez2 is enriched in dots, as well as EGFP Fez1 LIR3 mutant and S58E.

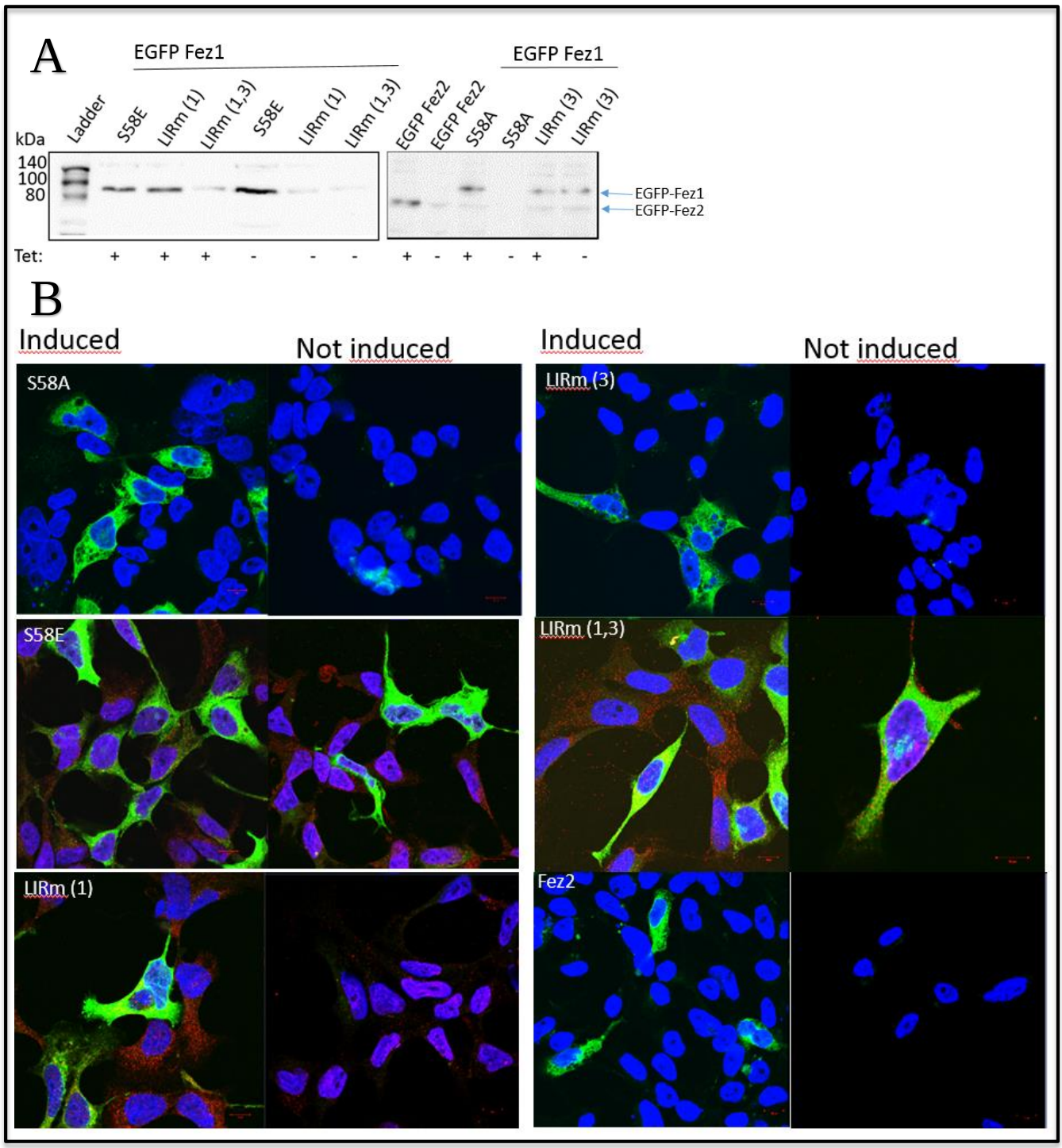

Figure 10. The established cell lines are all expressing EGFP-Fez1 or EGFP-Fez2 proteins, and the EGFP-fusion proteins have the correct size. A) The established cell lines were induced with tetracycline for 12 hours, harvested and cell extracts run on western blot with αGFP antibody. The blue arrows indicate the EGFP-Fez1 and EGFP-Fez2 bands. **B)** The established cell lines were induced or not, fixated, their DNA stained by DAPI and the cells were studied by confocal fluorescent microscopy.

Establishment of Fez1 constructs with reverse LIR2 mutations

Recently, our research group identified crystal structures of Gabarap binding to the LIR2 motif of Fez1 in a reverse orientation (Sjøttem, unpublished). In order to test this LIR, cell lines expressing EGFP Fez1 rLIR2 were established. First, the reverse LIR was mutated by site-directed mutagenesis and then an LR reaction was done to get the construct into a pDEST EGFP Flp-In plasmid. The plasmids were validated by sequencing and transfected into HEK Flp-In T-rex cells by the Flp-In method. Succesfully transfected cells were selected with blasticidin and hygromycin. The established cell lines were induced with tetracycline for 12 hours before extracts from induced and not induced samples were run on western blots to verify that the mutants were expressed and had the correct size. Figure 11 shows that the expressed mutants, all had the expected size, except LIRm(1,F56A,3), which was not visible on the western blot. LIRm(1,F56A,3) needs further testing to verify expression.

The established cell lines were fixated, DNA stained by DAPI and the cells studied by confocal fluorescent microscopy (Figure 11). All the constructs were induced, but in the case of LIRm(r2) there is no induced image. However, LIRm(r2) clearly has bands in both induced and not induced samples on the Western blot. LIRm (1,r2,3) is enriched in a perinuclear dot (Figure 11).

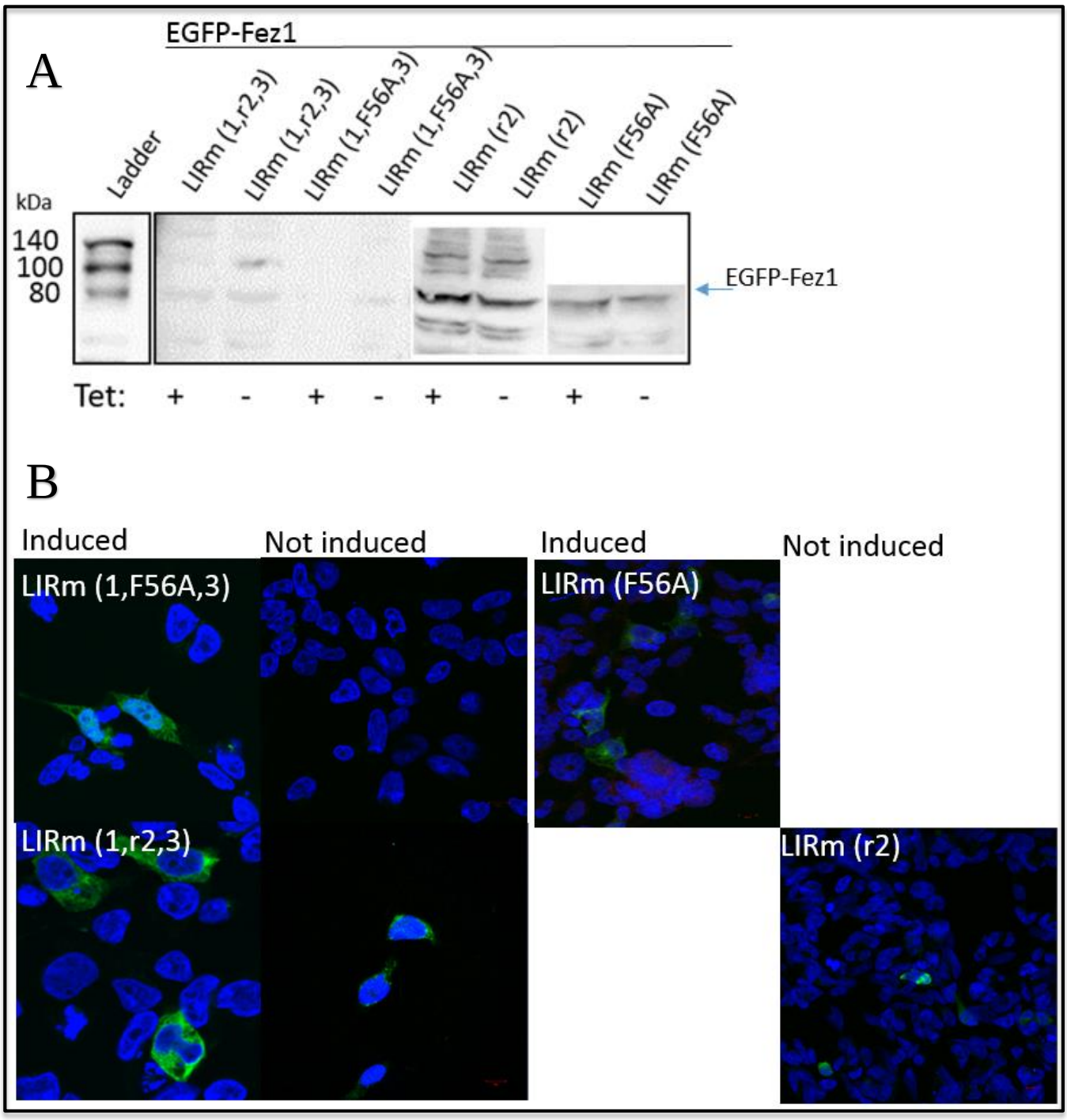

Figure 11. The established cell lines were all expressing Fez1 mutants with the correct size. A) The established cell lines were induced with tetracycline for 12 hours and extracts run on WB with αGFP antibody. The blue arrow indicates where EGFP Fez1 is. **B)** The established cell lines were fixated, the cells nucleus stained by DAPI and the cells studied by confocal fluorescent microscopy.

Fez1 co-localizes with Gabarap in certain dots

The LIR motifs in Fez1 bind strongly to Gabarap (Alemu, unpublished). Here, the EGFP-Fez1 and LIR mutants were studied for co-localization with Gabarap. The induced cell lines were fixed and stained with Gabarap antibody before imaging. Fez1 WT, LIR1 mut, and LIR1+3 mut were co-localized with Gabarap in a perinuclear dot in many cells (Figure 12). There was a very limited amount of images of Fez1 LIR1+r2+3 mut. From Figure 11 we see that Fez1 LIR1+r2+3 mut is able to form a perinuclear dot. However none of the images with Fez1 LIR1+r2+3 mut with Gabarap staining has this dot. Therefore we do not know if the reverse LIR affects co-localization of Gabarap in this dot. The reverse LIR may be important for co-localization with Gabarap in this dot. Alternatively none of the Fez1 LIRs seem to be important for co-localization with Gabarap in this perinuclear dot. More images are needed and images of Fez1 LIRm1+r2+3 with a perinuclear dot and Gabarap staining would be ideal.

Established HEK293 Flp-In T-rex cell lines with EGFP Fez1 S58 mutants were also induced with tetracycline for 12 hours, fixated, stained with Gabarap antibody and studied by confocal fluorescent microscopy (Figure 13). The EGFP Fez1 S58E mutant was co-localized with Gabarap in perinuclear dots, while no such dot was observed for the S58A mutant.

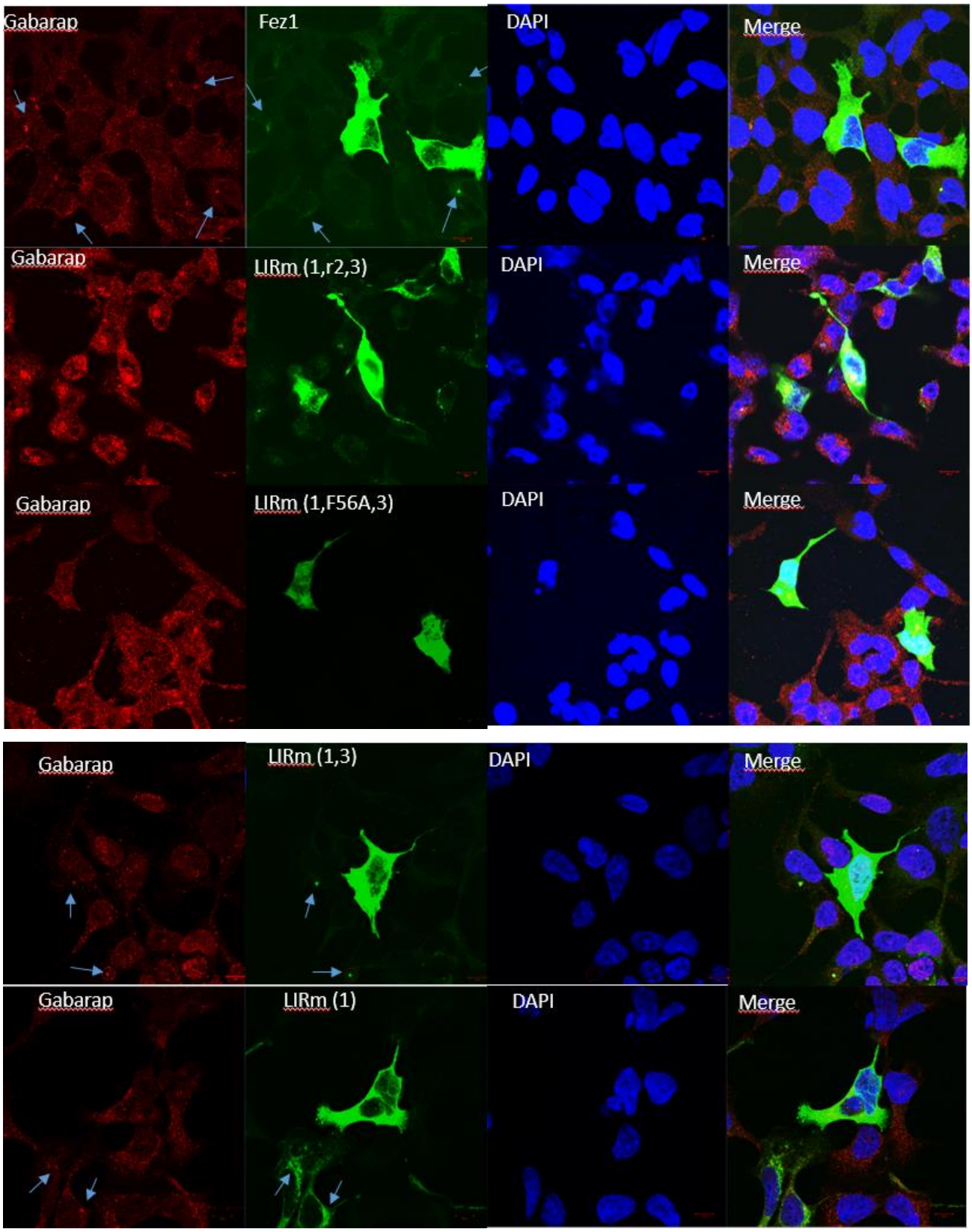

Figure 12. The co-localization of Fez1 and Gabarap in a perinuclear dot may be independent of the Fez1 LIRs. Established HEK293 Flp-In T-rex with EGFP Fez1 LIR mutants were induced with tetracycline for 12 hours, fixated and stained with a Gabarap antibody before they were studied by confocal fluorescent microscopy. A strong perinuclear dot displaying co-localization of Fez1 mutants and Gabarap is indicated by blue arrows.

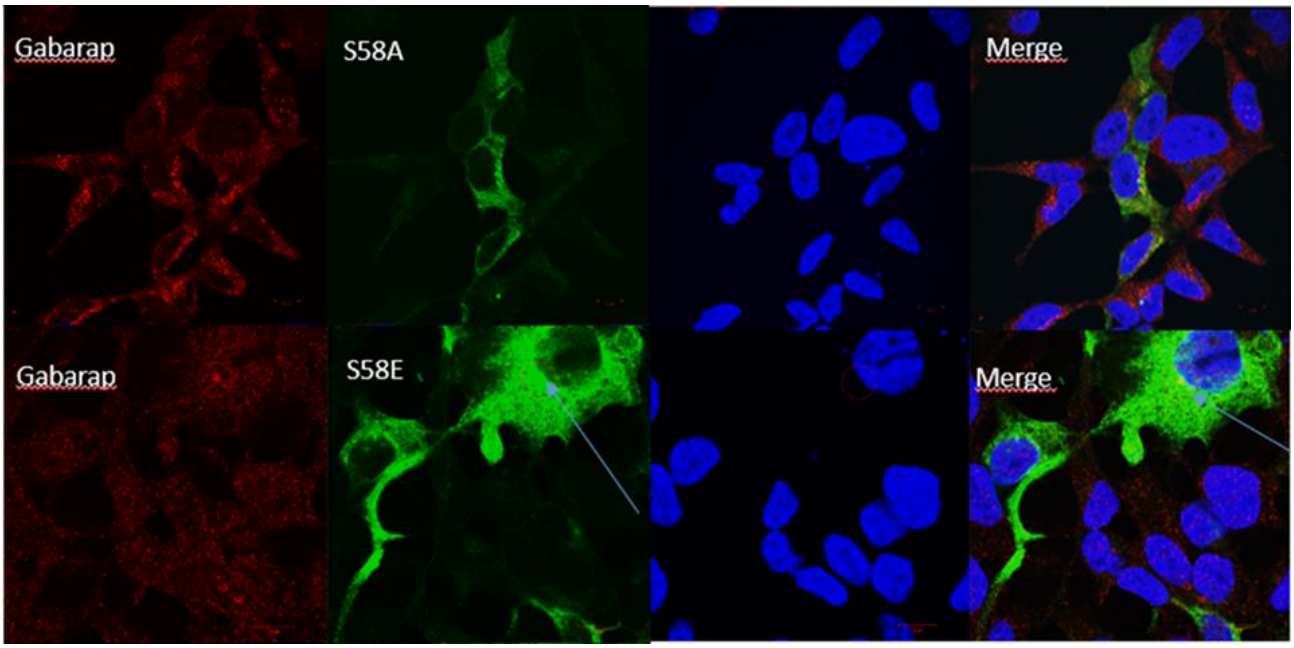

Figure 13. EGFP Fez1 S58E mutant was co localized with Gabarap in a dot. The established HEK293 Flp-In T-rex cell lines with EGFP Fez1 S58 mutants were induced with tetracycline for 12 hours, fixated and stained with Gabarap antibody and imaged by confocal fluorescent microscopy. The blue arrow indicates co-localization of Fez1 S58E and Gabarap in a dot.

Fez2 co-localizes strongly with Gabarap in a perinuclear dot

Next, the localization of EGFP Fez2 was studied using the established inducible HEK293 Flp-In T-rex EGFP Fez2 cell line and confocal fluorescent microscopy. Interestingly, Fez2 and Gabarap are enriched and co-localize in a large, perinuclear dot (Figure 14). The Fez1 LIR2 is not conserved in Fez2.

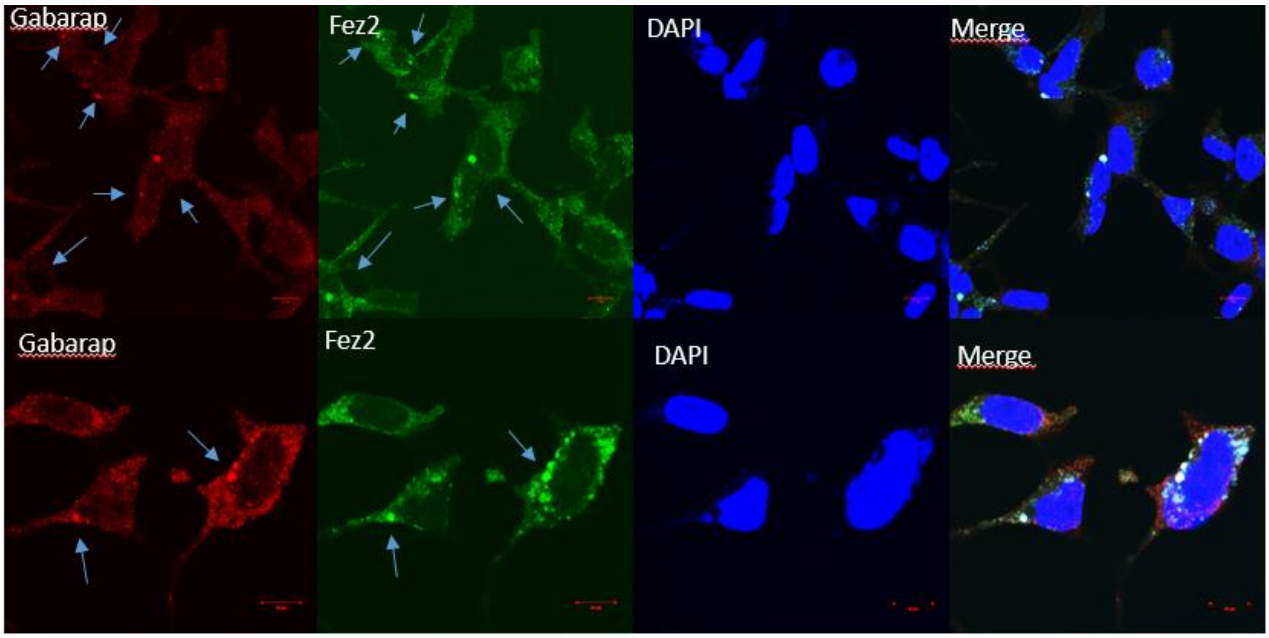

Figure 14. EGFP Fez2 co-localizes strongly with Gabarap in a perinuclear dot. Established HEK293 Flp-In T-rex EGFP Fez2 cells were induced with tetracycline for 12 hours, fixed and Gabarap was stained with an antibody before imaging by confocal fluorescent microscopy. Blue arrows indicate co-localization of Fez2 and Gabarap in a perinuclear dot.

Fez1 and Fez2 partially co-localize with LC3B

LC3 was originally identified as a microtubule-associated protein (Mann et al, 1994). JIPI binds to dynein (Fu et al, 2014). JIPI has a LIR and can bind to LC3 and this regulates dynein-driven transport of autophagosomes (Fu et al, 2014). Since Fez1, together with JIPI, has been found to be sufficient to activate kinesin-1 for microtubule binding and motility (Blasius et al, 2007), it would be very interesting if Fez1 was also involved in the regulation of dynein. Therefore we wanted to see if Fez1 would be co-localized with LC3B.

HEK293 Flp-In T-rex cells with EGFP Fez1 and Fez2 were induced for 12 hours with tetracycline and fixated. LC3B was stained with an antibody before imaging by confocal fluorescent microscopy (Figure 15). In certain cells, both Fez1 and Fez2 co-localized with LC3B in dots or aggregates. However, these dots do not seem to be the same structure as the perinuclear dot seen in the Gabarap staining.

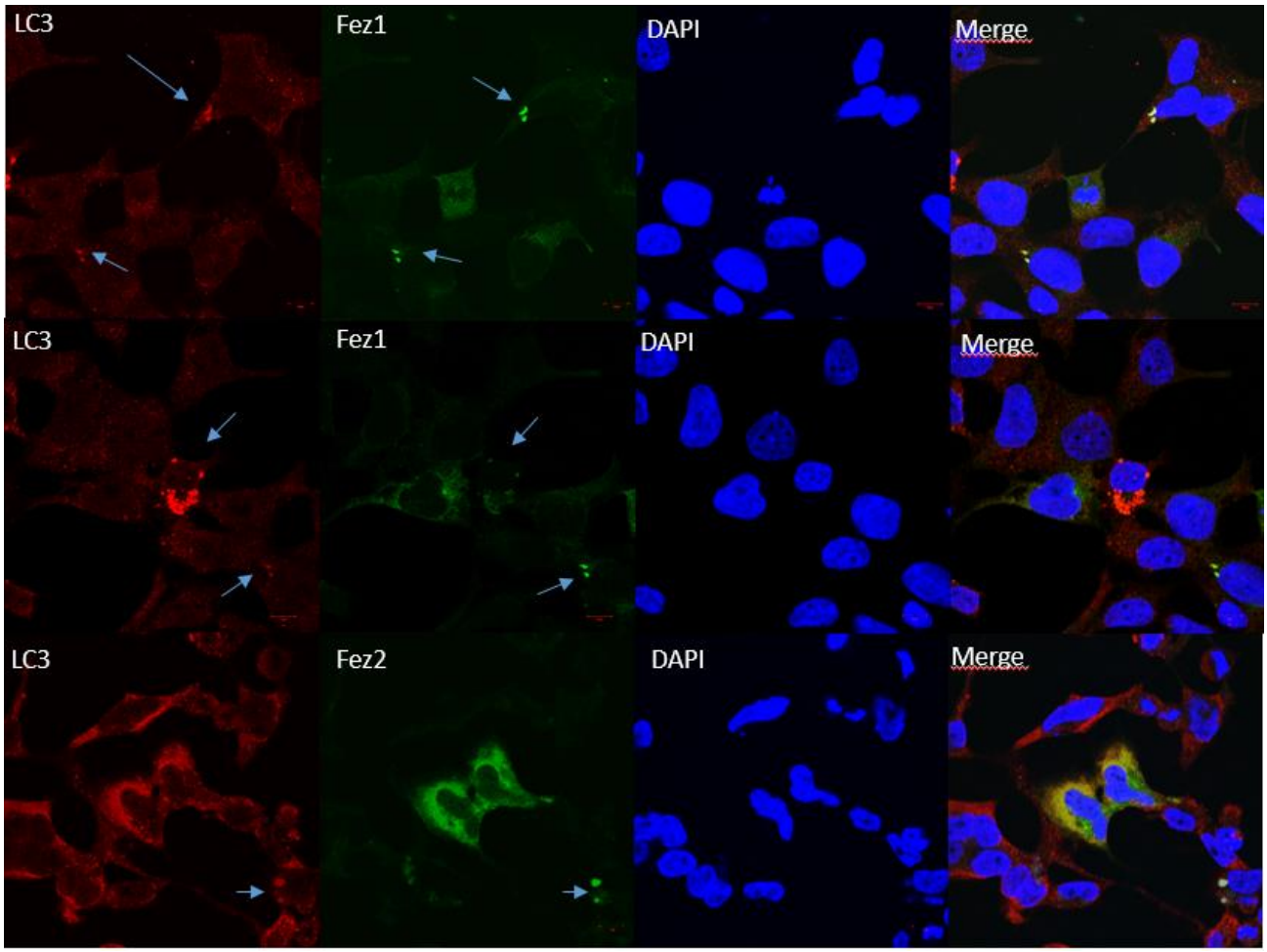

Figure 15. EGFP Fez1 and EGFP Fez2 co-localized with LC3B in certain cells. HEK293 Flp-In T-Rex cells with EGFP Fez1 or Fez2 were induced with tetracycline for 12 hours, fixated, LC3B stained with an antibody and imaged by confocal fluorescent microscopy. The blue arrows indicate co-localization of EGFP- Fez1/Fez2 and LC3B.

Two potential knock out cell lines were established using CRISPR/Cas9

Knock out cell lines are important tools for functional studies of proteins. One can use normal cell lines as a control and look for differences in the knock out cell lines and this can give information about the protein. Fez1 and Fez2 are homologues that probably have different functions. It would be interesting to see if Fez2 can compensate for the lack of Fez1 when it is knocked out. Fez2 has not been studied much. Therefore we wanted to make knock out cell lines of Fez1, Fez2 and double Fez1- Fez2 knocked out.

The guide sequence in CRISPR needs to target an exon, and not an intron. It is advantageous to target an exon as early in the protein as possible because otherwise the beginning of the

protein could be translated and one can end up with a truncated Fez instead of a knocked out Fez. Fez1 has multiple isoforms. The two sets of Fez1 guide sequences used both had targets in the first Fez1 exon. This exon is the same in all Fez1 isoforms (Figure 17). Fez2 also has multiple isoforms and the two sets of Fez2 guide sequences used both targeted an exon that was the same in all Fez2 isoforms. The ordered oligoes with the guide sequences were phosphorylated and annealed into the pX461 vector (Ran et. al. 2013). The plasmids were validated by sequencing before transfection into HEK293 Flp-In T-rex cells. By using Flp-In cells one can utilize the Flp-In system to Flp-In other genes of interest into the knock out cells later. 24 hours after transfection the EGFP-expressing cells were sorted with BD FACSARIA iii cell sorter, and one cell was seeded in each well in a 96-well plate. When the cells were confluent they were splitted into 6-well dishes. On confluency, samples were frozen and tested by running western blots. 54 cell lines were tested for Fez1 and/or Fez2 knock out with western blot. The result was two potential knock out cell lines: F1T1C2 and F2T1C20 (Figure 17 and Figure 18). F stands for Fez, T for which set of targets was used, and C for which well in the 96 well plate the cells came from.

The antibodies used were tested to verify that they could recognize Fez1 or Fez2. The Fez1 antibody from Chua et al (2012) had been tested before. Cells transfected with pDEST EGFP, pDEST EGFP Fez1 or pDEST EGFP Fez2 and cells treated with Fez1 or Fez2 siRNA, were run on western blot for antibody testing. Figure 16 shows that both antibodies stain bands of expected size for the endogenous and overexpressed proteins (~50 kDa and ~80 kDa). The bands are weakened upon siRNA transfections, clearly indicating that they are specific.

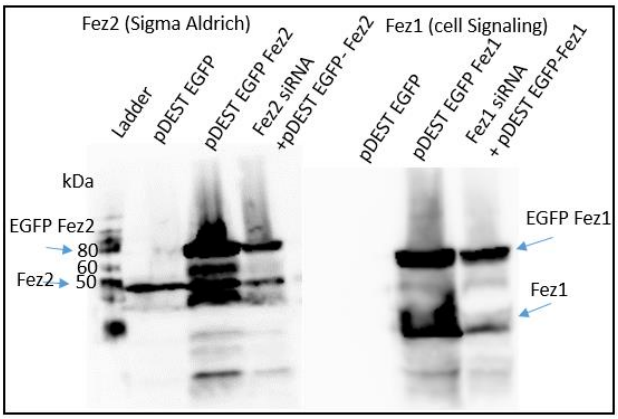

Figure 16. Verification of the Fez1 and Fez2 antibodies. Samples of pDEST EGFP, pDEST EGFP Fez1 or pDEST EGFP Fez2 and cells treated with siRNA against Fez1 or Fez2 were run on 10% SDS-gel, and blotted against the indicated antibodies. The arrows indicate where the Fez1, Fez2, EGFP- Fez1 and EGFP-Fez2 bands are.

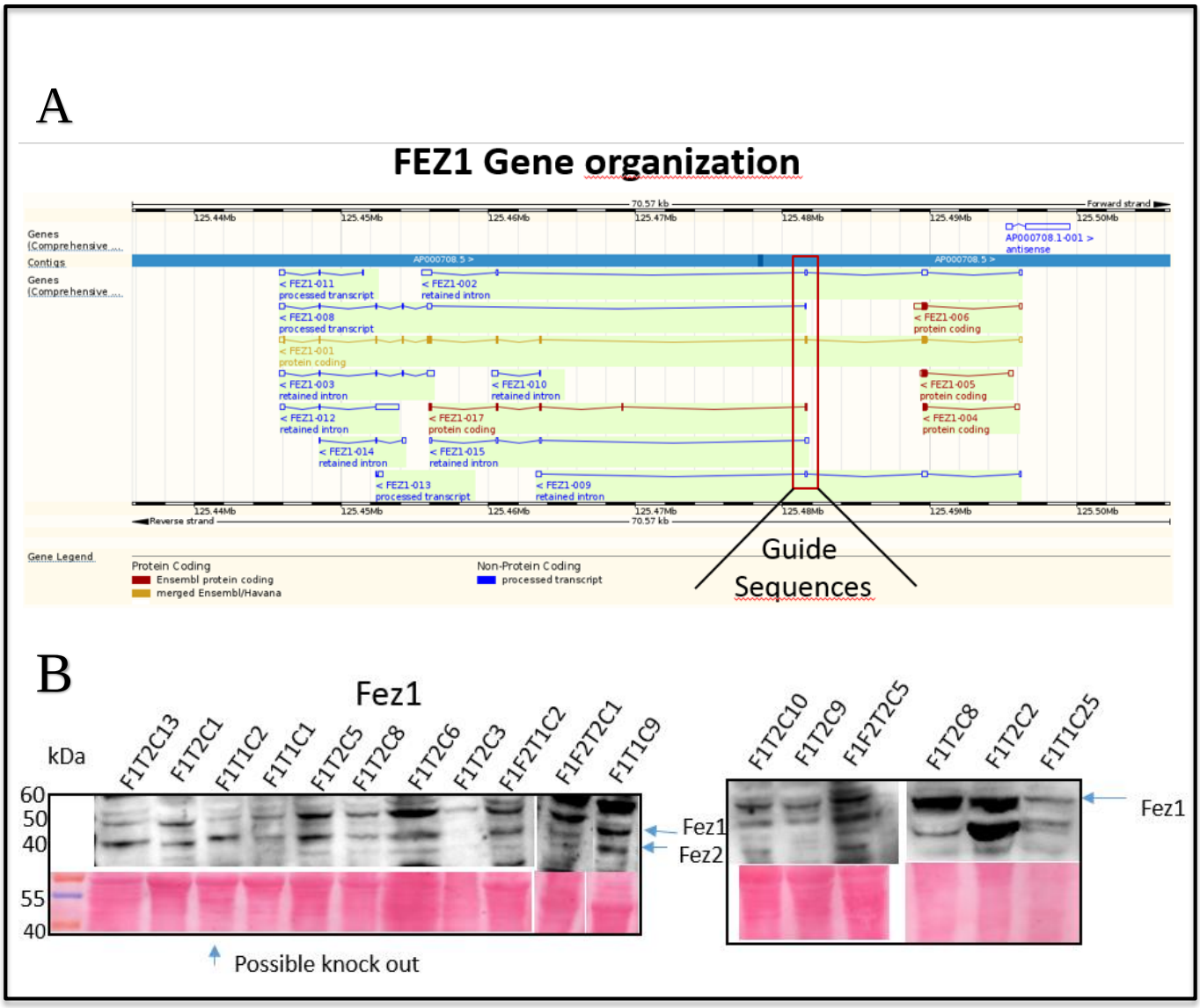

Figure 17. One potential Fez1 knock out cell line is established. A) A figure that shows the exon in Fez1, which the two sets of guide sequences used, had targets within. The guide sequence used were determined by Eva Sjøttem, and the figure adapted from ENSEMBL. **B)** The potential knock out cell lines were tested for Fez1 knock out on western blot. The Fez1 antibodies from cell signaling and Chua et al (2012) were used. The arrows indicate where the Fez1 and Fez2 bands are.

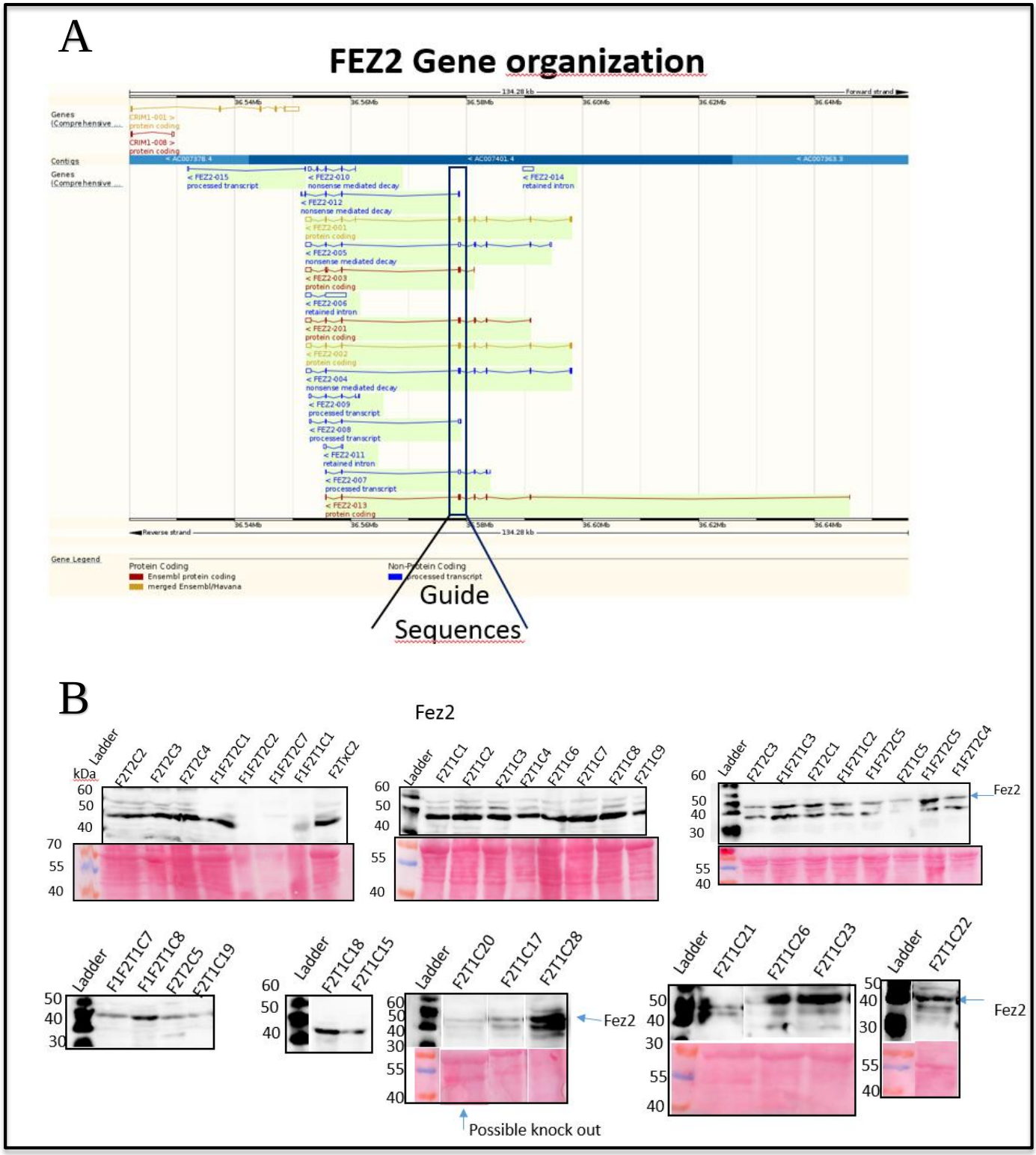

Figure 18. One possible Fez2 knock out cell line is established. A) A figure that shows the exon in Fez2, which the two sets of guide sequences used, had targets within. The guide sequence used were determined by Eva Sjøttem, and the figure adapted from ENSEMBL. **B)** The potential knock out cell lines were tested for Fez2 knock out on western blot. The Fez2 antibody from Sigma Aldrich was used. The arrows indicate where the Fez2 bands are.

Discussion

The putative NLS in Fez1 at position 286 is functional

The putative NLS of Fez1 had never been tested experimentally. To prove that a NLS really is a NLS one needs to test the following (Lange et. al. 2007):

1. Putting the NLS into another protein without an NLS, that normally doesn't enter the nucleus, should be sufficient for that protein to enter the nucleus.
2. Nucleocytoplasmic transport of the protein into the nucleus should be reduced very much when the NLS is altered (or deleted).
3. There must be an import receptor that interacts with the NLS.
4. Disrupting this import receptor should disrupt the import of the protein.

Confocal fluorescent microscopy images of NLS-EGFP-βgal and EGFP-βgal show that NLS-EGFP-βgal was more accumulated in the nucleus than EGFP-βgal after LMB treatment. This alone does not prove that the NLS is functional, but it suggests it. The cut of score of 6, given by the online NLS predictor, suggests that the NLS is a weak NLS. It would have been ideal to put the NLS into a protein that does not enter the nucleus. Originally we did believe that the βgal protein would not enter the nucleus. However, the βgal protein without the Fez1 NLS was partially translocated to the nucleus after LMB treatment. The fact that βgal was not accumulated in the nucleus without LMB treatment shows that it most likely also has a NES. This probably affected the localization of NLS-EGFP-βgal without LMB treatment. A way to test the NLS further, is to mutate the NLS sequence in the Fez1 131-392 deletion construct.

Our results show that Fez1 has two NLS

Proteins bigger than 40 kDa cannot enter the nucleus by passive diffusion (Pankiv et. al. 2010). The Fez1 deletion constructs EGFP Fez1 1-130 and EGFP Fez1 2-270 were accumulated in the nucleus after LMB treatment. Because these are bigger than 40 kDa (Table 20) this suggests that Fez1 has a second NLS in the 2-130 region. Under starved and normal conditions full length EGFP Fez1, EGFP Fez1 1-130 and EGFP 2-270 were not accumulated in the nucleus, while EGFP Fez1 131-392 and EGFP 309-392 were accumulated in the nucleus. This suggests that EGFP Fez1 131-392 does not have a NES, and that the NES is likely to be in the 2-130

region. The mapped LIRs of Fez1 are inside the 2-130 region (Alemu, unpublished) and these LIRs were mutated and imaged by confocal fluorescent microscopy. This did not seem to affect the Fez1 NES as the Fez1 mutants were not accumulated in the nucleus.

Alborghetti et al (2011), suggest that Fez1 and Fez2 genes originated from the ancestral Unc-76 gene. A study revealed that the Fez1 NLS KKRRK 290-294, which is the NLS at position 286, is evolutionary quite conserved in all Fez1 genes that resulted from the putative unc-76 gene duplication (Alborghetti et al, 2011). Fez2 has a conserved polybasic region (KKKKK) corresponding to the Fez1 NLS (KKRRK). It would have been interesting to see if Fez2 also goes into the nucleus by LMB treatment.

10 cell lines with inducible expression of EGFP-Fez1 mutants or EGFP- Fez2 were established

Flp-In cell lines, (except LIRm(1,F56A,3)), in all established cell lines were expressed at detectable levels. However, there was also expression in non-induced cells. We expected to see a strong difference between induced and not induced samples on the western blots. The not induced cell lines should have almost no expression of the EGFP-fusion proteins due to the tet-operators in the CMV promoter (T-rex system). Instead the western blots revealed expression also in not induced cells, and there were some green cells in non-induced cell images. One possible explanation could be that the FBS contains tetracycline because some cows have tetracycline in their diet. This could cause induction in the samples that were not exposed to tetracycline for 12 hours by us. It could also be leakage from the promoter. The ideal thing to do would have been repeat the experiments with cells growing in FBS without tetracycline.

Cells can auto-fluorescence, which means that components inside cells can fluorescence on their own. Mitochondria and lysosomes, for example do this (Monici, M. 2005). In confocal fluorescent microscopy proteins that are studied are made visible by tagging them with a fluorophore such as enhanced green fluorescent protein (EGFP) or antibodies that fluorescence (immunofluorescence). Due to autofluorescence both transfected and not transfected cells can appear, for example green in the confocal fluorescent microscope. Cells expressing EGFP tagged gene of interest are expected to be greener than auto-fluorescent cells. Therefore we tried to only image the cells with strong fluorescence, to be certain that the cells expressed the

gene of interest. This probably selected for overexpressing cells, which can have affected localization of the gene of interest. Still this is better than imaging cells that don't contain the gene of interest at all. When imaging the stable cell lines, the practice of only imaging the greenest cells was continued. This ensured that the cells imaged were expressing the gene of interest.

Fez1 was co-localized in a perinuclear dot with Gabarap.

It has recently been discovered that Gabarap traffics from the pericentriolar matrix (a part of the centriole which is a component of the centrosome) to forming autophagosomes (Joachim et al, 2015). Fez1 might have centrosomal functions (Lanza et al, 2010; Lanza et al 2008). The centrosome is a major part of the microtubule organizing center (MTOC). We therefore hypothesize that the co-localized dots represent the MTOC.

No co-localized dots were detected in Fez1 mutant S58A and LIRm(1,r2,3). We do not have an image of LIRm(1,r2,3) with the perinuclear dot and Gabarap staining. Therefore more images are needed. Co-localization was detected in S58E, LIRm(1,3) and LIRm(1). This indicates that LIR1 and LIR3 are not important for co-localization of Fez1 and Gabarap in this perinuclear dot. The reverse LIR2 may however be important for Gabarap co-localization. Furthermore LIR2 is close to S58. It could be that phosphorylation of S58 regulates LIR2 interactions with Atg8 proteins. Alternatively Fez1 may need transport with kinesin-1 to reach Gabarap. Fez1 S58A mimics the un-phosphorylated S58 form. It is also possible that co-localization of Fez1 and Gabarap in this perinuclear dot is independent of all the Fez1 LIRs.

Fez1 is believed to negatively regulate autophagy by forming a complex with SCOC (Behrens et al, 2013). Gabarap induces autophagy when it activates ULK1 (Joachim et al, 2016). Fez1 and Gabarap being co-localized might affect autophagy and be involved in the regulation of autophagy. When Fez1 and Gabarap are bound, an autophagy promoter and a protein believed to be an autophagy inhibitor are in complex. Fez1 and maybe also Fez2 may recruit Gabarap to MTOC and inhibit its movement to the autophagosomes. This would inhibit autophagy.

We are the first to discover that Fez2 co-localizes with Gabarap

Fez2 was co-localized with Gabarap in big perinuclear dots. The dots were larger than the Fez1-Gabarap dots.

Fez1 and Fez2 show an identity of 41% and a similarity of 68% overall (Assmann et al, 2006). In the C-terminal region there is 60% amino acid sequence identity and 76% similarity (Assmann et al, 2006). While the reverse LIR, r2, is not conserved in Fez2, LIR3 is conserved (Alborghetti et al, 2011; Image 5, introduction). Fez2 co-localized with Gabarap might also represent the MTOC as we hypothesized for Fez1 Gabarap co-localization.

Fez1 and Fez2 were both co-localized with LC3B in dots or aggregates

We wanted to see if there would be more or less Fez1 and Fez2 close to LC3B and whether or not there would be dots and if these would be co-localized. LC3 is often used as a marker of autophagosomes, although higher LC3 levels can be caused by other events. Because it is believed that Fez1 negatively regulates autophagy when it forms a complex with SCOC (Behrens et al, 2013), we were expecting less autophagy where there was a lot of Fez1.

The results show that Fez1 and LC3B are co-localized. The dots do not seem to be the same dots observed in Fez1-Gabarap or Fez2-Gabarap images.

Fez1 bind to kinesin-1 together with JIPI and are together sufficient to activate kinesin-1 (Blasius et al, 2007). JIPI also binds to dynein (Fu et al, 2014) and has a LIR which it can use to interact with LC3 (Fu et al, 2014). This is thought to be important in the regulation of dynein-driven transport of autophagosomes (Fu et al, 2014). We therefore hypothesize that co-localized Fez1 and LC3B could be related to dynein-driven transport of autophagosomes. It would be very interesting if this were true, also in relation to HIV type 1. Fez1 binds HIV-1 capsids and is involved in their transport (Malikov et al, 2015).

We ended up with only two potential knock out cell lines after testing 54 cell lines

The CRISPR/Cas9 set up ended up with 17 cell clones that were tested for Fez1 knock out and 37 cell clones that were tested for Fez2 knock out. The BD FACSARIA iii cell sorter, sorted out a single transfected cell into each 96-well plate and we used 6 96-well plates. 96 wells*6 plates = 576 cells. Of these only 54 clones grew. Among the clones that grew there were only two potential knock outs, and these two need further testing to determine if they truly are knock outs.

This low number of clones could mean that Fez1 and Fez2 are important for cell survival. Clones with Fez1 or Fez2 knock out could simply have died. However, Fez1 is mainly expressed in brain cells so if cells outside of the brain don't normally express Fez1 then HEK293 cells should be able to survive without Fez1. Another explanation is that cells generally don't like to be alone because they need growth factors from other cells. As media for the cells in the 96-well plates we used new media with 20% FBS. The cells could potentially have grown quicker and maybe more cells would have survived if we had used 50% old/used media in the 96-well dish. By old/used media I mean the media that the same cells were grown in before they became separated by the cell sorter. Old/used media contains growth factors.

The HEK293 Flp-In cells genome structure and sequence can have changed from the normal human genome after being cultivated in dishes for decades. In other words the area coding for the Fez1 and Fez2 genes may have been changed in HEK293 Flp-In cells. HeLa cells for instance have a changed number of chromosomes. HeLa cells have 76-80 chromosomes according to Landry et al (2013) rather than 46. Of these chromosomes 22-25 are abnormal (Landry et al, 2013). If the HEK293 Flp-In genome is changed in the Fez1 and Fez2 region the Cas9 endonuclease would not have been able to make the correct single strand nicks required for knock out of Fez1 and Fez2.

Conclusion

In this study we have found that the predicted Fez1 NLS at position 286 is functional. Furthermore, our data suggests that Fez1 has a second NLS in the Fez 2-130 region. We also mapped a functional NES in the 2-130 region. We established stable cell lines with inducible expression of various EGFP-Fez1 LIR and S58 mutants, in addition to EGFP-Fez2. Imaging showed that Fez1 is mainly cytoplasmic localized with enrichment in some perinuclear dots. Interestingly, the autophagic protein Gabarap co-localized with Fez1 in some of these dots. This co-localization seems to be independent of Fez1 LIR1 and LIR3. More images are needed to determine if Fez1 LIR2 is important. Also Fez2 is mainly cytoplasmic. Fez2 co-localizes with Gabarap in one large perinuclear dot. The Fez1 LIR2 is not conserved in Fez2. These dots may represent the MTOC center, which is reported to recruit Gabarap. Fez1 and Fez2 both co-localized with LC3B in certain dots or aggregates. These dots or aggregates appear to be different from the Fez1-Gabarap and Fez2-Gabarap dots.

Unpublished data from our group has shown that Fez1 interacts directly with Atg8 proteins and prefers Gabarap (Alemu, unpublished). LIRs are used to interact with LDS on Atg8 proteins. Therefore the cell lines expressing Fez1 LIR mutants can give useful information on how this interaction occurs, regardless of how it occurs. The LIR mutants may also provide information on how the interaction affects autophagic activity.

Two cell lines expressing EGFP-Fez1 S58 mutations were established. One of these is a phosphorylation mimicking mutant of Fez1 (S58E). The other is mimicking the un-phosphorylated form (S58A). S58 is close to LIR2, and hence may possibly regulate Atg8 interactions of the reverse LIR2. The phosphorylation mimicking mutant co-localized with Gabarap, while the un-phosphorylated mimicking mutant did not. This observation is based on few images so the experiment has to be repeated in future studies.

The CRISPR/Cas9n knock-out experiments resulted in only two promising knock-out clones. These have to be verified further by western blots and genome sequencing. The low efficiency of the knock-out strategy may indicate that the Fez proteins are essential for cell growth and survival. It could also be that one or more of the target sequences has changed and therefore was not recognized and cleaved by Cas9 endonuclease. Establishment of Fez1 and/or Fez2 knock out cell lines may provide useful information on their roles as regulators of autophagy. In addition information on the general cellular functions of Fez1 and Fez2 may be obtained.

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