

## **ACKNOWLEDGEMENTS**

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Wahida Afroz



## ABSTRACT

This master's thesis is a part of a research project of The Gastroenterology Cell Biology Group investigating molecular responses to gastrin induced gene expression, with focus on how duration and intensity of gastrin signaling is translated into diverse cellular programs like survival, migration and invasion. Previous studies from extensive microarray time series experiments have revealed that the peptide hormone gastrin up-regulates genes involved in various biological process including proliferation, differentiation, migration, endoplasmic reticulum stress (ER stress), and anti-apoptosis/survival. Many of the novel gastrin induced genes are known to be stress effectors in carcinogenesis; and also ATF transcription factor subfamily proteins ATF3 and ATF4 mRNA expression levels are prolonged only in sustained gastrin treated cells. In response to different cellular stresses, phosphorylation of initiation factor eIF2 $\alpha$  (eIF2 $\alpha$ -p) represses global translation coincident with preferential translation of ATF4. ATF4 is known to regulate the expression of genes responsible for amino acid metabolism, cellular redox state and anti-stress responses; including several of the detected sustained gastrin induced genes involved in the balance between apoptosis and survival (e.g., *Grp78 (BiP)*, *Chop*, *Herpud1* and *Mcl1*). Because ATF4 is a common downstream target that integrates signaling from a family of at least four protein kinases that phosphorylates eIF2 $\alpha$ , the eIF2 $\alpha$ -p-ATF4-network is referred to as the integrated stress response

The aim of this master thesis project was to further explore gastrin induced temporal expression and cellular localization of ATF4 and its network proteins in AR42J wild-type cells and ATF4 knockdown (KD) cells. Results obtained by optimized Western blot analysis suggested that gastrin up-regulated the ATF4 protein and phosphorylated the upstream kinase eIF2 $\alpha$  in AR42J cells. We also detected the ATF4-network proteins PERK, GCN2, CHOP and Herpud1, although the gastrin response was less clear for these proteins. Examination of intracellular localization of ATF4, GRP78 (BiP), pPERK, Herpud1 and CHOP using immunocytochemistry and fluorescence microscope confirmed that these proteins were present in both unstimulated and gastrin stimulated wild type AR42J cells. Since ATF4 regulated proteins are correlated with both apoptosis and anti-apoptosis, we investigated the anti-apoptotic effect of gastrin on serum starved wild type (wt) and ATF4 KD cells.

These results suggested that gastrin has anti-apoptotic effects in both wild type and ATF4 knockdown cells; although the anti-apoptosis effect seems even more robust in ATF4 KD cells than wild type cells, indicating that ATF4 is involved in the balance between apoptosis and survival in the gastrin response. The expression and localization of the ATF4 protein were then analyzed in oxyntic mucosa of hypergastrinemic rats; and we found that ATF4 was strongly expressed in ECL cells. Overall, the results presented suggest that ATF4 is an important transcription factor in gastrin mediated regulation of stress responses; and the eIF2 $\alpha$ -p-ATF4-network may be involved in ECL cell hyperplasia.

## ABBREVIATIONS

AP-1	Activator protein-1
ATF6	Activating transcription factor 6
ATF4	Activating transcription factor 4
ATF3	Activating transcription factor 3
Bip	Binding immunoglobulin protein
bzip	Basic leucine zipper
BCL-2	B-cell lymphoma 2
C/EBP	CCAAT/enhancer-binding protein
CCK2R	Gastrin/cholecystokinin-2 receptor
CHX	Cycloheximide
COX2	Cyclooxygenase 2
CREB	cAMP response binding element
CCK2	Cholecystokinin 2
DAG	Diacylglycerol
DR5	Death receptor 5
dsRNA	Double stranded RNA
ECL	Enterochromaffin-like cells
ERK1/2	Extracellular signal-regulated kinase 1/2
Egr-1	Early growth response-1
ER	Endoplasmic reticulum
ERAD	ER-associated protein degradation
eIF2 $\alpha$	eukaryote initiation factor 2
EIF2AK	eIF2-alpha kinases
ERO1- $\alpha$	Endoplasmic reticulum oxidoreductin 1
ECL	Enterochromaffin cell-like
ER stress	Endoplasmic reticulum stress
GADD 153	DNA damage inducible gene 153
GADD34	Growth arrest and DNA damage-inducible Gene 34
Gas	Gastric adenocarcinomas
GCN2	General control non-depressible kinase 2
GRP78	Glucose-regulated protein 78

G-17	Gastrin-17
G-34	Gastrin-34
GRP	Gastrin releasing peptides
HB-EGF	Heparin-binding EGF-like growth factor
HSP5	Heat shock protein 5
HRI	Heme regulated initiation factor
Herpud1	Homocystein-inducible, ER stress-inducible, ubiquitin-like domain 1
HRD1	Hypoxia responsive domain 1
IP3	Inositol triphosphate
IRE1	Inositol-requiring enzyme 1
ISR	Integrated stress response
IP3R	Inositol 1, 4, 5-triphosphate (IP3) receptor
JNKs	c-jun NH <sub>2</sub> -terminal kinase
KI domain	Kinase insert domain
MAPK	Mitogen-activated protein kinase
NFκB	Nuclear factor kappa B
PLC	Phospholipase C
PIP2	Phosphatidylinositol bisphosphate
PERK	Protein kinase-like ER kinase
PPI	Protein pump inhibitor
PKC	Protein kinase C
RANK	Receptor activator nuclear factor-kappa B
RANKL	Receptor activator of nuclear factor-kappa B ligand
SP1	Stimulatory element
STAT	Signal transducers and activators of transcription
TRB3	Tribbles-related protein 3
VEGF	Vascular endothelial growth factor

# TABLE OF CONTENTS

ACKNOWLEDGEMENTS .....	i
ABSTRACT .....	iii
ABBREVIATIONS .....	v
1. INTRODUCTION.....	1
1.1 Gastrin.....	1
1.1.1 Gastrin gene and precursors .....	1
1.1.2 Release of gastrin .....	1
1.1.3 The gastrin receptor and signaling pathways activated by gastrin.....	3
1.1.4 Cellular response to gastrin .....	4
1.2 ER-stress .....	5
1.2.1 GRP78 .....	7
1.3 ATF4.....	8
1.4 The ATF4 network.....	9
1.4.1 P-eIF2 $\alpha$ .....	10
1.4.2 PERK.....	10
1.4.3 GCN2 .....	11
1.4.4 HRI.....	12
1.4.5 PKR .....	12
1.5 ATF4 mediated gene expression.....	12
1.6 Biological function of ATF4 .....	14
1.7 The role of ER-stress and ATF4 in cancer.....	15
1.8 Context and aims of the study.....	15
1.9 Aims and experimental strategy of the master thesis.....	18
2. MATERIALS AND METHODS .....	19
2.1 Cell Model system AR42J.....	19
2.2 Cultivation of Cells .....	20
2.3 Time series experiments and gastrin stimulation .....	20
2.4 Preparation of protein lysates.....	20
2.5 Western blot.....	21
A. Sample preparation and gel eletrophoresis .....	21
B. Blotting of proteins to the membrane .....	22
C. Immunostaining of the blotted membrane .....	23
2.6 Immunocytochemistry.....	27

2.7	Immunohistochemistry.....	28
2.8	Microscopes .....	29
	A. Modern light microscope .....	29
	B. Fluorescence microscope .....	29
2.9	Caspase assay .....	30
2.10	Statistical analysis .....	31
3.	RESULTS .....	33
3.1	Expression of ATF4 protein in response to varying duration of gastrin treatment ...	33
3.2	Gastrin treatment induces phosphorylation of eIF2 $\alpha$ .....	35
3.3	Gastrin mediated expression of proteins in the ATF4 network .....	37
	3.3.1 Total PERK and phospho PERK (pPERK) :.....	37
	3.3.2 Phosphorylation of GCN2 in AR42J cells upon gastrin stimulation:.....	39
	3.3.3 CHOP:.....	41
	3.3.4 Herpud1 is expressed in AR42J Cells.....	42
	3.3.5 Gastrin receptor (CCKBR): .....	43
3.4	Characterization of ATF4 knock down cells generated by retrovirus based RNAi ..	44
	• Cellular morphology of AR42J wt/control cells and ATF4 KD cells:.....	46
3.5	Impact of ATF4 in gastrin mediated survival response: .....	48
	• The gastrin induced anti-apoptotic effect is not abolished by reduced ATF4 protein level .....	48
3.6	Cellular localization of proteins in the ATF4 network:.....	49
	3.6.1 ATF4:.....	50
	3.6.2 GRP78: .....	50
	3.6.3 pPERK: .....	51
	3.6.4 Herpud1: .....	53
	3.6.5 CHOP:.....	53
3.7	The ATF4 protein levels in oxyntic mucosa of hypergastrinemic rats.....	54
4.	DISCUSSION.....	57
5.	REFERENCES .....	63
6.	SUPPLEMENTARY FILE.....	71
	6.1 Expression of ATF4 on AR42J cells in varying duration of gastrin treatment by using fluorescence western blot analysis:.....	71
	6.2 Expression of ATF4 on wild type AR42J cells, ATF4 (sh7) knockdown cells and Firefly control (FFC) cells to different duration of gastrin treatment: .....	72



6.3 Cellular localizations of proteins of ATF4 network on ATF4 (sh7) Knockdown cells:  
73

ATF4:.....	73
GRP78: .....	74
pPERK: .....	75
Herpud1 .....	76
CHOP:.....	77



# **1. INTRODUCTION**

## **1.1 Gastrin**

Gastrin is a peptide hormone which stimulates gastric acid secretion and influences gastric epithelial cell proliferation and maturation [1]. Already in 1905 John Sidney Edkin's postulated the role of the gastric hormone as an acid secretagogue and named it gastrin ("gastric secretion") [1-3].

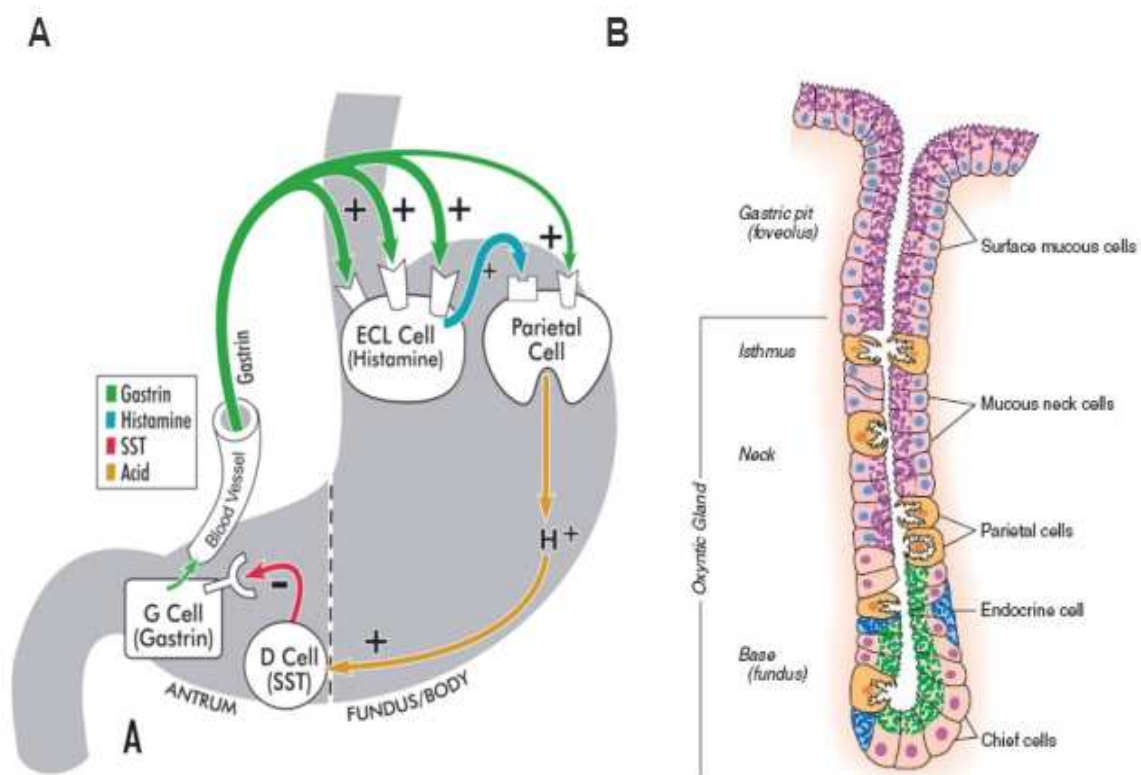
### **1.1.1 Gastrin gene and precursors**

All mammals contain the gastrin gene with three exons that produces a large precursor molecule of 101 (human, mouse) or 104 amino acids (rats) with an N-terminal signal peptide [3, 4]. This large precursor undergoes sequential changes to the C-terminal amidated end products gastrin-17 (G-17) and gastrin-34 (G-3), of which G-17 is the most abundant [5]. The G-cell of the pyloric antral part of the stomach is the primary site for the expression of the gene in adult mammals. In addition, the gene is expressed in developmental pancreatic B-cells and colon, in the pituitary gland and testis and expression has also been described in several types of tumors like colorectal [3], pancreatic and stomach [3, 6, 7] .

### **1.1.2 Release of gastrin**

Gastrin is produced in G-cells of the gastric antrum in response to meal [1]. The neuronal fibers of the stomach released two forms of Gastrin releasing peptides (GRP), a 27-amino acid peptide and a carboxy-10-amino-acid peptide fragment that positively control gastrin release by acting as secretagogues. A GRP homologue, bombesin also stimulated gastrin release [5]. Other than bombesin, gastrin release are stimulated by pituitary adenylate cyclase-activating protein, sucralose, glucose, caffeine, and bacterial lipopolysaccharide [8]. Gastrin secreted from both the luminal and basolateral parts of antral G-cells and released via  $Ca^{2+}$ -dependent regulated pathways. Gastrin released from the secretory vesicles in response to both gastric luminal amino acids and neuronal stimulation and extracellular calcium sensing receptors play an important role in gastrin secretion [7]. Gastrin being secreted from the endocrine G-cells into the circulation through exocytosis and transported to the oxyntic mucosa where it binds to the cholecystinin 2 (CCK2)/gastrin receptors on the enterochromaffin-like (ECL) cells [5, 9]. Gastrin stimulates ECL cells and

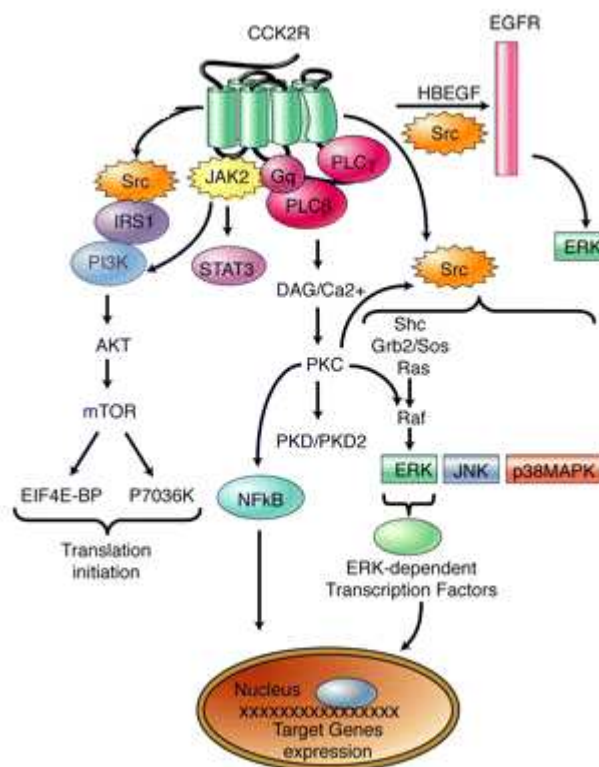
potentiates the release of histamine which stimulates parietal cells to secrete HCl acids. This HCl acid gives negative feedback to the G-cells by releasing the paracrine inhibitor somatostatin to inhibit gastrin secretion (Figure 1A) [3]. The gastric epithelium is being constantly renewed by stem cells which migrate bidirectionally and differentiated into specialized group of cells like mucous-secreting pit and neck cells, pepsinogen-releasing base cells, histamine-releasing ECL cells and acid-secreting parietal cells (Figure 1B). The interactions between these cells types make an appropriate environment for digestion and protection. In addition to acid secretion, gastrin seems to have a central role in regulation of growth and differentiation of both stem cells and the specialized cells [5].



**Figure 1: A) Principle of gastrin release and regulation of gastric acid secretion in the stomach.** The green arrows indicate positive gastrin stimulation and the red arrow indicates the negative stimulation on the G-cells to inhibit gastrin secretion by somatostatin (66). **B) Schematic diagram of an oxyntic gastric gland.** Oxyntic gastric glands are found within the oxyntic mucosa of the corpus part of the stomach and contain mucous neck cells, parietal cells, chief cells, and several types of neuroendocrine cells (67).

### 1.1.3 The gastrin receptor and signaling pathways activated by gastrin

CCK1R and CCK2R are two well-characterized receptors for peptides of the gastrin family [7]. Both receptors are identified to have a prominent role in neuroendocrine tumors in a large number of human cancers [10]. The CCK1R (previously called CCK-A) has low affinity for gastrin but high affinity for the hormone cholecystikinin (CCK), and the CCK2R (previously CCK-B) has high affinity for both gastrin and CCK [11]. In the stomach, gastrin acts on the CCK2R, mainly located on the ECL cells in the acid-producing oxyntic part of the stomach [7]. Increased expression of CCK2R mRNA in colon, pancreatic and gastric cancers are reported in several studies [6, 10]. CCK2R is the part of the seven-transmembrane G-protein coupled receptor super family [7, 10]. An overview of gastrin pathway activated by gastrin by binding with the CCK2R is shown in the Figure 2.

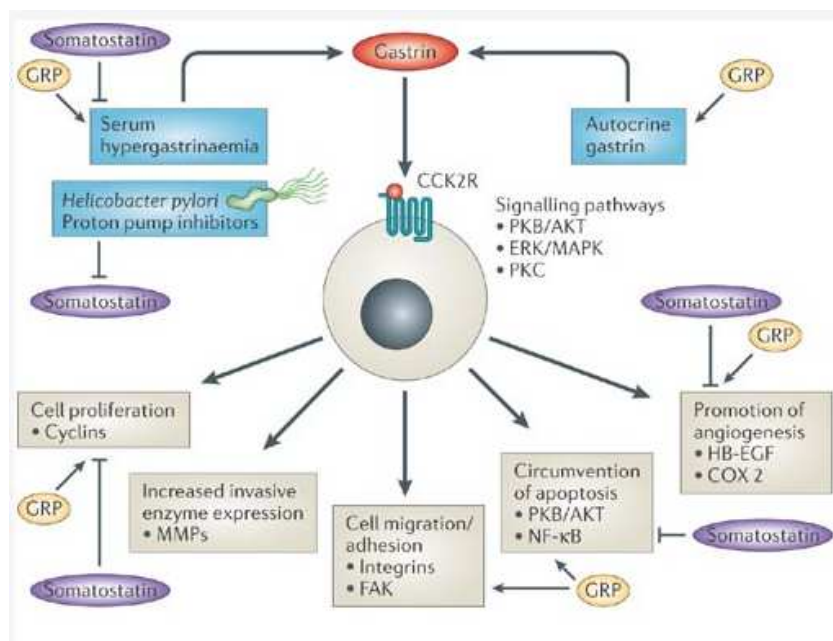


**Figure 2: Signaling pathway activated by gastrin by binding to the CCK2R.** The figure shows the signal transduction of the gastrin receptor (CCK2R) stimulated by gastrin via PKC, ERK1/2, AKT, P<sup>38</sup> MAPK, and JNK pathways to phosphorylate different transcription factors and regulate the translational machinery (68).

Gastrin binds to the G protein of the  $G_q$  family and activates the phospholipase C (PLC) pathway. PLC leads to calcium ( $Ca^{2+}$ ) mobilization and activation of PKC by inducing hydrolysis of phosphatidylinositol bisphosphate (PIP2) to generate inositol triphosphate (IP3) and diacylglycerol (DAG). Gastrin then activates the mitogen-activated protein kinase (MAPK) and also extracellular signal-regulated kinase 1/2 (ERK1/2), c-jun NH<sub>2</sub>-terminal kinase (JNKs), and p<sup>38</sup> MAPKs. Gene expression regulated by gastrin has been shown to be mediated by several transcription factors and co-factors, including stimulatory element (SP1), cAMP response binding element (CREB), early growth response-1(Egr-1), extracellular signal-regulated kinase 1(ERK1),  $\beta$ -catenin, nuclear factor kappa B (NF $\kappa$ B), signal transducers and activators of transcription (STAT3), and activator protein-1(AP-1) [12, 13].

#### **1.1.4 Cellular response to gastrin**

Gastrin binds with the gastrin receptor and can initiate gene expression associated with proliferation, differentiation, migration, invasion, angiogenesis and anti-apoptotic actions (shown in Figure 3) [3, 5, 11]. Gastrin is important in maintaining homeostasis in normal oxyntic mucosa and tumorigenesis [1, 10, 14]. Initially it was thought that gastrin is a potent growth factor for the gastric mucosa and gastrin is not required for the development of gastric mucosa, differentiation of the constituent of the cells and basal proliferation. But further investigation shows that gastrin plays an essential role for the functional maturation of the two primary acid secreting cells, parietal cells and ECL cells. As these two cells contain CCK2Rs, gastrin directly stimulates the maturation of the cells. When gastrin secretion is in physiological range, it is essential for the maintenance of normal epithelial architecture. But increased gastrin secretion together with inflammation may disrupt epithelial cell structure and function leading to gastric hypertrophy [11].



**Figure 3: Gastrin mediated cellular responses.** Gastrin binds the CCK2R and can initiate gene expression associated with cell division, invasion, migration, angiogenesis and anti-apoptosis [5]. PI3K/AKT pathway involves in gastrin mediated proliferation, regulation of migration, protein synthesis as well as has anti-apoptotic effects and regulates the translational machinery [5, 15, 16]. Gastrin induced angiogenesis in human endothelial cell line is mediated through the transcriptional activation of HB-EGF (heparin-binding EGF-like growth factor) and indirectly through the transcriptional up-regulation of cyclooxygenase 2 (COX2) [5].

## 1.2 ER-stress

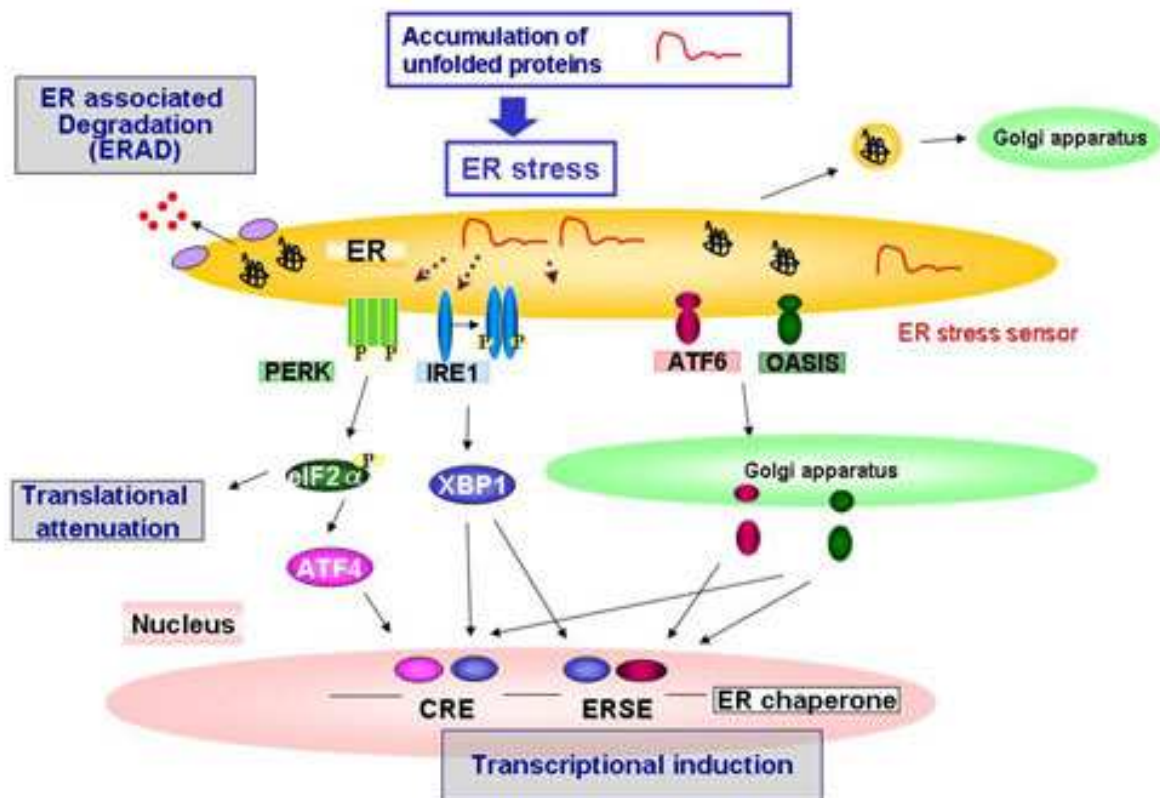
The endoplasmic reticulum (ER) is an organelle in the eukaryotic cells consisting of a continuous network of sacs and tubes [17] [18]. ER plays a crucial role in cell survival and functions [18]. It is the first compartment that regulates the secretory pathways [19], and it is also a primary site for protein folding and assembly, a pool of free  $\text{Ca}^{2+}$  and a site for lipid and sterol biosynthesis [18]. To maintain the homeostasis inside the cell, it is important to sense, respond and relieve stress. Stress can be manifested in the cell in many ways, either endogenously or exogenously including pathogenic infection, chemical insult, genetic mutation, nutrient deprivation and even during normal differentiation. Therefore, stress might occur physiologically or pathologically. The folding capacity of proteins depends on appropriate environment, genetic and metabolic conditions. Inside the ER, co- and post-translational modifications occur, which have importance in folding and oligomeric assembly of proteins [18]. It is anticipated that when protein concentration reaches 100 mg/ml within the ER lumen, protein starts to aggregate. ER has a highly oxidizing

environment that is suitable for protein folding and maturation [20]. Molecular chaperones and quality control factors are present in the ER lumen that assist in folding and trafficking of the newly synthesized polypeptides [21]. The physiological process like aging can also control protein folding [20] where pathological process, includes the onset of diabetes, myocardial ischemia, cardiac hypertrophy, atherosclerosis and heart failure have an influence in aberrant folding of proteins [22]. Under stress, when unfolded or misfolded proteins are accumulated in the ER-lumen, the depletion of ER  $\text{Ca}^{2+}$  storage occurs and the cells initiate a series of protective measures generally called the unfolded protein response (UPR) [23]. The UPR pathway was first described by Sambrook and co-workers in the budding yeast *S. cerevisiae* [24]. This adaptive response has two functions, first detection of misfolded proteins and then correction of protein misfolding [25]. One of the primary functions of the resident molecules are to sense protein misfolding in this organelle and initiate changes in the gene expression which affects the folding capacity of the ER [25].

Proteins that are not folded or unfolded even after the assistance of ER chaperones inside the ER are returned to the cytosol to be degraded and ubiquitinated by the proteasomes through a process known as ER-associated degradation (ERAD) [26].

The duration and degree of the ER stress decides in which direction the cells are going, either activating the survival signals by adaptive response and anti-apoptotic pathways or the death signals by inducing cell death programs. Many endoplasmic reticulum (ER) surface proteins play an important role in relaying the signals determining whether a cell is to differentiate, divide, migrate or die [26]. The three ER membrane-bound protein sensors are pancreatic ER kinase (PKR)-like ER kinase (PERK), activating transcription factors 6 (ATF6), and inositol-requiring enzyme 1 (IRE1) (Figure 4). These ER stress sensors are inactive in the resting cells by binding to the ER chaperone GRP78 (Bip) and may be activated sequentially with PERK being first, rapidly followed by ATF6 and IRE1 [17] [27].





**Figure 4: Principle of the ER-stress pathways.** Proteins are translocated into the ER and enter into the secretory pathways as unfolded polypeptide chains and fold within the lumen of this organelle [26]. Only the properly folded proteins can exit the ER. When unfolded proteins accumulated in the ER, they are bound by the ER chaperone GRP78, instead of the ER sensing molecules PERK, ATF6 and IRE1, which become free and phosphorylated and activate downstream signal transduction. CRE: cAMP response elements, ERSE: ER Stress Response Element (69).

### 1.2.1 GRP78

GRP78 (78 glucose-regulated protein) [17] is also known as the binding immunoglobulin protein (Bip) or heat shock 70 protein 5 (HSP5A), and is a member of the heat shock 70 protein family [20]. GRP78 is the most abundant and best characterized ER chaperones which interacts with the hydrophobic domains of a wide range of proteins [20]. It is also used extensively as a biomarker for the onset of the UPR [23]. During accumulation of unfolded proteins in the ER lumen, GRP78 binds with the unfolded proteins after dissociating from the three ER-stress mediators (PERK, ATF6 and IRE1). It is suggested that co-chaperones might stimulates the release of GRP78. Grp78 acts in a feedback mechanism since ER stress up-regulates the expression of GRP78 by the transcription factors ATF6 and ATF4. Increased GRP78 in the lumen not only binds to the folded proteins but may also

inactivates UPR by binding to PERK, ATF6 and IRE1 [17]. During prolonged ER-stress or if the adaptive or recovery processes by the cells fails, the UPR will initiate apoptotic pathways [18].

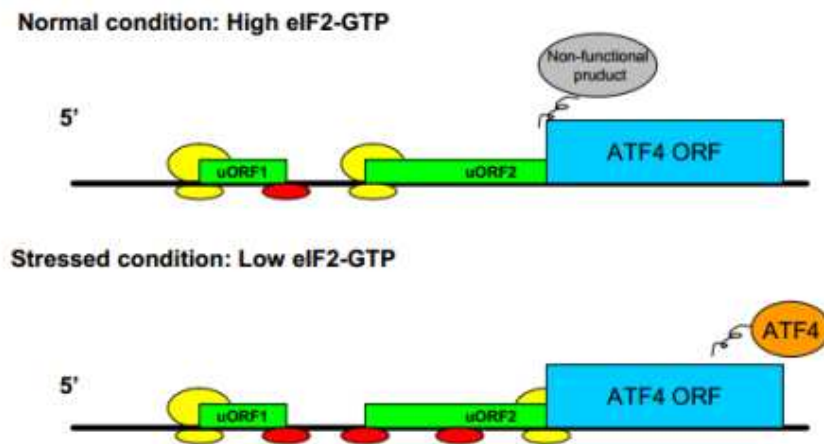
### 1.3 ATF4

Activating transcription factor 4 (ATF4) (also called CREB-2) is a basic leucine zipper (bzip) transcription factor and a member of the ATF/CREB (activating transcription factor/cyclic AMP response element binding protein) family [28, 29]. ATF4 is located at chromosome 22 at cytogenetic band 22q 13.1 and has a genomic size of 2,992 bases (70). The ATF4 protein consists of 351 amino acids (38590 Da) and contains an N-terminal domain-p300 interaction site,  $\beta$ TrCP recognition motif and an acetylation site as indicated. By forming homodimer or heterodimer with other bzip transcription factors, ATF4 contains a strong transcriptional activation domain located at the N-terminal which contains acidic amino acid residues [28]. ATF4 is a stress responsive gene up-regulated by several factors leading to ER stress/UPR, including oxygen deprivation (hypoxia), amino acid and glucose deprivation, calcium alteration, oxidative stress and by the growth factor heregulin [26, 28, 30, 31].

ATF4 plays a central role in mammalian stress response pathways and is an important mediator of the unfolded protein response. It is ubiquitously expressed at low basal level [30]. In mammalian cells, a complex transcriptional program is activated in response to stressors to reduce the effects of the stress and to remedy the cellular homeostasis. Eukaryotic initiation factor 2 $\alpha$  (eIF2 $\alpha$ ) is the central mediator of this translational response and although the activation of eIF2 $\alpha$  causes global reduction of protein translation, it also increases the translation of ATF4 mRNA [28].

The mammalian ATF4 has two upstream open reading frames (uORFs) located in the 5'UTR of the mRNA (Figure 5) preceding the functional coding sequence (ATF4 ORF) [31], and this mediates the poor translation of ATF4 in unstressed cells [30]. Successful translational initiation is dependent on forming the tertiary complex involving among others eIF2-GTP. The abundance of eIF2-GTP in unstressed cells leads to reinitiate translation of the uORF2 coding region which overlaps the ATF4 ORF and produces a non-functional product. Phosphorylation of eIF2 $\alpha$  at Ser-51 by an active eIF2 $\alpha$  kinase during stress inhibits formation of eIF2-GTP and the resulting

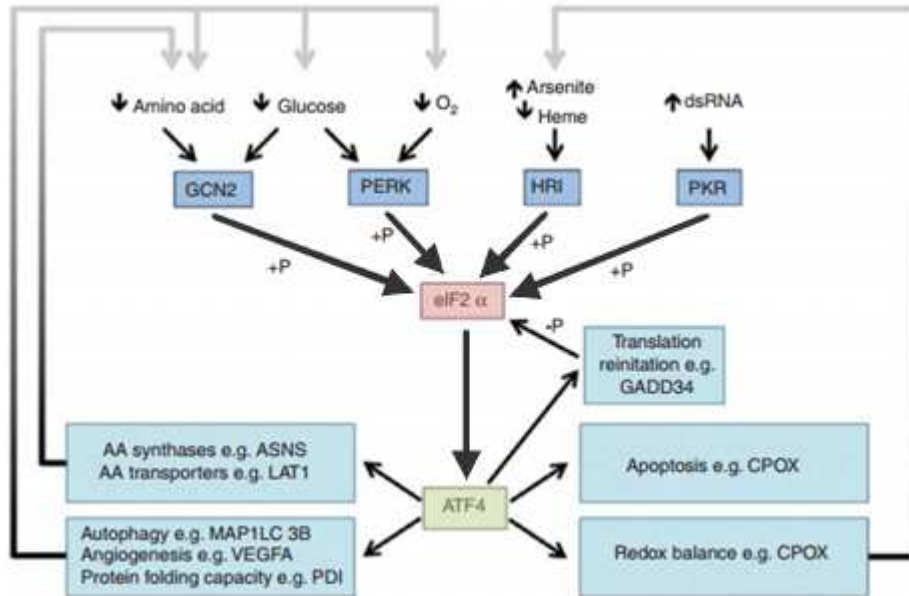
low level slows the translation re-initiation so that uORF2 is bypassed and instead starts translation at the true ATF4 ORF leading to increased ATF4 protein levels [30].



**Figure 5: regulation of ATF4 at translational level.** Black line: ATF4 mRNA; Yellow activated ribosome (with eIF2-GTP); Red: ribosome small subunits (with eIF2-GDP); Green: uORFs; Blue: actual ATF4 ORF [31].

#### 1.4 The ATF4 network

ATF4 is activated by eIF2 $\alpha$  (Figure 4, 6 and 7). In mammalian cells, four eIF2 $\alpha$  kinases are identified: HRI (heme regulated initiation factor 2 alpha kinase, EIF2AK1) is activated during heme deprivation and found in erythroid cells; PKR (interferon-inducible RNA depended protein kinase, EIF2AK2) is activated by double-stranded RNA as part of the interferon anti-viral response; PERK (PKR-like endoplasmic reticulum kinase, EIF2AK3) is activated by unfolded proteins in the ER [4]; and GCN2 (general control non-depressible kinase 2, EIF2Ak4), is activated by uncharged tRNAs during amino acid and glucose deprivation [32] (Figure 6).



**Figure 6: Activation of four eIF2 $\alpha$  kinases under stress and expression of ATF4 by eIF2 $\alpha$ .** eIF2 $\alpha$  has four upstream kinases, GCN2 , PERK, HRI and PKR. These kinases become auto phosphorylated and subsequently promote phosphorylation of eIF2 $\alpha$  activation of the ATF4 network. Based on [33] .

#### 1.4.1 P-eIF2 $\alpha$

eIF2 $\alpha$  is a heterotrimer of subunits  $\alpha$ ,  $\beta$  and  $\gamma$  [34]. Activated PERK leads to translational inhibition [35] by phosphorylating eIF2 at Ser<sup>51</sup> on the  $\alpha$ -subunit, inhibiting the activity of subunit  $\beta$  required for exchange of eIF2-GDP to the active form eIF2-GTP, which is necessary for translation to occur [36]. Phosphorylation of eIF2 $\alpha$  (p-eIF2 $\alpha$ ) can lead to three downstream effects. Firstly, p-eIF2 $\alpha$  reduces general protein translation which decreases the protein folding load in ER and lowers the energy expenditure as both protein synthesis and folding requires ATP [4]. Secondly, it increases clearance of accumulated proteins from the ER by ERAD and finally p-eIF2 $\alpha$  increases expression of pro-survival genes to promote cell survival. This signaling pathway has been termed as an "integrated stress response (ISR)" [22].

#### 1.4.2 PERK

The immediate response during ER-stress in metazoan cells is reversible, transient attenuation of mRNA translation by blocking the influx of newly synthesized polypeptides into the ER-lumen. This translational attenuation is signaled through PERK pathways. PERK is a transmembrane type 1 serine/threonine protein kinase

[21], where the N-terminal domain or luminal domain is bound with GRP78/Bip in unstressed condition and the C-terminal or cytoplasmic domain is responsible for the kinase activity and interacts with eIF2 $\alpha$  [35]. When unfolded proteins are accumulated in the ER lumen, GRP78 releases PERK and binds to the unfolded proteins [35] (Figure 7). Released PERK become activated by dimerization and auto-phosphorylation at several residues in the C-terminal domain [37].

During ER-stress, the translational repression mediated pathway of PERK is activated most rapidly because the phosphorylation of eIF2 $\alpha$  which is a direct substrate of PERK does not depend on nuclear translocation, transcription or translation. So, the inhibition of protein synthesis occurs within 30 minutes following exposure to ER stress. Therefore, the immediate effect is to stop further influx of nascent proteins into an already saturated ER lumen [25].

Even in the absence of PERK, the translational repression is observed through the other eIF2 $\alpha$  kinases. Among them, as HRI is restricted to erythroid cells, GCN2 and PKR are most likely responsible for inhibiting cyclin D1 translation and the subsequent cell cycle arrest [38].

### **1.4.3 GCN2**

GCN2 is a high molecular weight protein kinase containing 1590 amino acids. GCN2 is another proximal cytoplasmic kinase and UPR effectors that can directly or indirectly activate eIF2 $\alpha$  [38].

During amino acid and glucose starvation, it is demonstrated that eIF2 $\alpha$  is activated by the GCN2 kinase and up-regulates ATF4 target genes to maintain homeostasis between amino acid synthesis and transport [39]. GCN2 is also important in regulation of synaptic plasticity and memory, feeding behavior and lipid metabolism. Beside amino acid and glucose starvation, GCN2 also activated by UV radiation and mediates NF $\kappa$ B signaling pathway [39]. Experiments propose that during starvation with any one of the several different amino acids or if there is a defective aminoacyl-tRNA synthetase allele, both cases increase phosphorylation of eIF2 $\alpha$  by GCN2 [40]. During ER stress, like glucose deprivation and hypoxic condition, both GCN2 and PERK are activated and contribute to eIF2 $\alpha$  phosphorylation and ATF4 up-regulation [39].

#### **1.4.4 HRI**

Heme deficiency activates HRI [33]. The HRI protein consists of five domains; The N-terminus domain, C-terminus domain and two kinase regions flanking a central kinase insert (KI) domain. Heme binds to the N-terminus region of HRI and triggers intramolecular auto-phosphorylation of HRI. Stabilization and a HRI-HRI dimer formation occurs that sense the intracellular heme concentration. When heme concentration becomes high, this leads to the phosphorylation which inhibits the HRI activation. During heme deficiency, HRI activated by auto-phosphorylation and leads to phosphorylation of eIF2 $\alpha$  [37] .

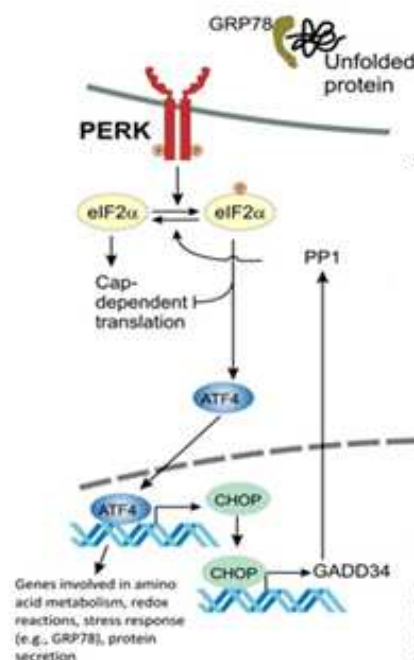
#### **1.4.5 PKR**

During viral infection, PKR is activated by double stranded RNA (dsRNA) [33] and blocks the translation of viral mRNA and promotes apoptosis. The PKR protein is located in the cytosol and in the nucleus. The regulatory N-terminal region contains a dsRNA binding domain, while the C-terminal region has the dimerization interface and carries out catalytic function of the protein. When dsRNA binds to PKR it becomes dimerized. This leads to auto-phosphorylation, stabilization of the dimer and subsequent phosphorylation of eIF2 $\alpha$  [37].

### **1.5 ATF4 mediated gene expression**

Two of the ATF4 target genes, Herpud1 and CHOP, recently shown to be activated by gastrin [41] are describe below. CHOP is a member of C/EBP (CCAAT/enhancer-binding protein) family of transcription factors. CHOP is also known as growth arrest and DNA damage inducible gene 153 (GADD 153) [17]. Initially CHOP was identified as a DNA damage response gene, but has also been shown to play a role in ER-stress induced apoptosis [17]. CHOP protein expression depends on the PERK-eIF2 $\alpha$ -ATF4 pathway of the UPR (Figure 7) and it is regulated at both the transcriptional and translational level. Post-translational phosphorylation on serine residues 78 and 81 by p<sup>53</sup> MAPK increases CHOP activity [17]. CHOP induces cell death by promoting protein synthesis and oxidation during stress in the ER lumen [27]. CHOP acts via three mechanisms to induce apoptosis through its transcriptional activator and repressor functions [37]. First, CHOP alters the transcription of genes involved in apoptotic pathways [17], the BCL-2 family members BCL-2 (B-cell lymphoma 2), Tribbles-related protein 3 (TRB3), and the death receptor 5 (DR5). The

second mechanism involves a feedback loop, by inhibition of protein translation through PERK signaling via up-regulation of GADD34 (growth arrest and DNA damage-inducible gene 153), which dephosphorylates eIF2 $\alpha$  by interacting with protein phosphatase 1 (PP1). And the third mechanism involves oxidation of the ER lumen by ERO1- $\alpha$  (endoplasmic reticulum oxidoreductin 1) [17, 37]. ERO1- $\alpha$  increases the burden of oxidized protein complex in the ER and activates the inositol 1,4,5-triphosphate (IP3) receptor (IP3R), which stimulates ER-calcium release and promotes apoptotic cell death. Thus, regulation of CHOP is an important factor in cell fate either in death or survival. Many publications suggest that the regulation of CHOP expression by activating GCN2 and PERK are not in the same way [37, 42]. Activated GCN2 that phosphorylates eIF2 $\alpha$  is less potent inducer of both ATF4 and CHOP [37]. CHOP can switch from pro-survival to pro-apoptotic signaling by blocking transcription of Bcl-2 [25].



**Figure 7: Activation of ATF4 by PERK.** During ER-stress when unfolded/misfolded proteins are accumulated in the ER-lumen, the PERK transmembrane protein becomes activated after being release from Bip/GRP78. Released PERK becomes activated by dimerization and auto-phosphorylation, which directly phosphorylates eIF2 $\alpha$  at Ser 51 and leads to downstream regulation of translation of ATF4 [31] ( modified from 71).

The transcriptional up-regulation of Herpud1 is promoted in ERAD by the PERK-ATF4 pathway [33]. Herpud1 (Homocystein-inducible, endoplasmic reticular stress-inducible, ubiquitin-like domain 1) is an ER-membrane protein with an N-terminal ubiquitin-like (UBL) domain projecting into the cytosol. The role of HERPUD1 in the ER-homeostasis is unclear. During ER-stress, Herpud1 may stabilize ER  $\text{Ca}^{2+}$  homeostasis and mitochondrial function in neural cells [43]. The ERAD pathway depends mainly on ubiquitin and proteasome-dependent degradation to remove the misfolded proteins [44]. Before proteasomal degradation the ERAD substrates are retro-translocated from the ER to the cytoplasm. In the ER membrane, ubiquitin-protein ligases, like HRD1 (hypoxia responsive domain 1) and gp78 and the action of p97 are responsible for the ubiquitination of the ERAD substrates for their retro-translocation to the cytoplasm [44]. Recent studies showed that Herpud1 plays an essential role in the ERAD pathways. Herpud1 binds directly to HRD1 and forms a complex with the ERAD machinery [45].

## 1.6 Biological function of ATF4

ATF4 has many biological functions. It can act as a transcriptional activator as well as a repressor, and has a main role as a protective gene during ER and oxidative stress. It also has a role in the development of the skeleton and the eye, and during hematopoiesis [28, 33]. At first ATF4 was described as a transcriptional repressor that has an inhibitory effect on transcription via *cAMP response element* (CRE) of the human encephalin promoter and other genes. This negative regulatory effect of ATF4 was seen in long-term memory storage. On the other hand, ATF4 also act as a transcriptional activator, inducing genes like receptor activator of nuclear factor-kappa B (RANK) ligand (RANKL), Osteocalcin, E-selectin, VEGF (vascular endothelial growth factor), Gadd153 (growth arrest- and DNA damage-inducible gene 153), Gadd34 (growth arrest- and DNA damage-inducible gene 34), asparagine synthetase and several genes involved in mitochondrial function, amino acid metabolism and redox chemistry [28] [29] .

Various stress signals like metabolic stress (such as glucose and amino acid deprivation), oxidative stress and ER-stress are incorporated in the ATF4 pathway and increases translation of ATF4. During metabolic and ER stress, ATF4 can initiates a regulatory feedback mechanism by GADD34 to ensure inhibition of protein



synthesis via PERK pathway. GADD34 is a component of the phosphatase complex which can dephosphorylates eIF2 $\alpha$ . This may restart the translation and may ensure the transient nature of protein synthesis inhibition [28].

### **1.7 The role of ER-stress and ATF4 in cancer**

During ER-stress, adding up with the cellular mechanism by which the stress is sensed, there has also been focus on the consequences of the altered homeostasis that affects diverse areas of cellular functions such as gene expression, metabolism, cell signaling and apoptosis [25]. The downstream of these pathways including angiogenesis, invasion, metastasis, and genomic instability have been associated with tumor progression and tumoral conditions. The stress response pathways that are an important target for the cancer therapy are activated here [33]. Higher levels of ATF4 protein is observed in primary human tumors than in normal tissue, and this also facilitate tumor growth in xenograft models [46]. VEGF and E-selectin which are associated with tumor metastasis are induced by ATF4. In ER stress, ATF4 can induce activating transcription factor 3 (ATF3) which inhibits the function of P<sup>53</sup> that regulate mitochondrial respiration [28].

### **1.8 Context and aims of the study**

As described in the introduction, the peptide hormone gastrin is the central regulator of gastric acid secretion. Gastrin plays a prominent role in regulation of growth and differentiation of gastric mucosa [5, 47]. In normal physiological conditions, gastrin levels are transiently increased in response to a meal. Hypergastrinemia can occur as a result of atrophic gastritis or pharmacologically inhibition of gastric acid secretion, which interrupts negative feedback mechanisms on gastrin producing G-cells residing in the gastric mucosa [48]. Since transiently increased gastrin levels have important physiological functions in the gastrointestinal, while sustained high gastrin levels (hypergastrinemia) are related to pathophysiological processes [5, 47, 48], it is of interest to examine how the duration of gastrin treatment affects gene expression and molecular responses.

The Gastroenterology Cell Biology Group have performed multidimensional analysis (mRNA, microRNA, protein synthesis perturbation with cycloheximide, CHX) of

temporal patterns of the transcriptome in adenocarcinoma cells to identify molecular networks in gastrin-regulated gene expression [41, 49, 50] (Table 1).

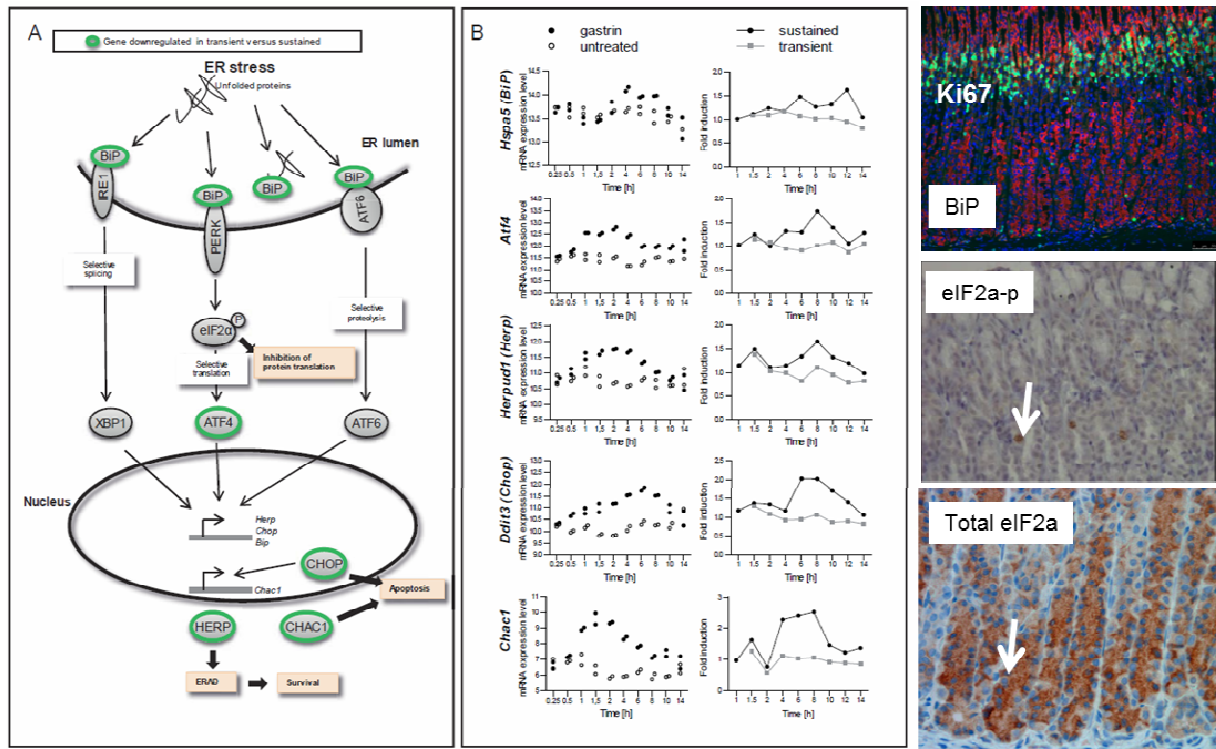
**Table 1: Genome-wide gene expression time series experiments in AR42J cells**

	Treatment gastrin (G17 10nM)	samples	Platform	Accession nr.
1	Sustained gastrin (24 h); transient gastrin (2 h); controls (24 h)	156	cDNA**	E-MTAB-123
2	Sustained gastrin (14 h); controls (14 h)	48	Illumina**	GSE3286
3	Sustained gastrin (14 h); transient gastrin (1 h), controls (14 h)	60	Illumina**	GSE3286
4	Sustained gastrin (10 h) +/- CHX; controls (10h) +/- CHX	32	Illumina**	E-MTAB-1268
5	Sustained gastrin (14 h); controls (14 h)	48	EXIQON***	

\*cDNA two colour microarrays; \*\*Illumina ExpressionBead Chip; \*\* CHC: cycloheximide (protein synthesis inhibitor) added 30 min before gastrin treatment; \*\*\* miRCURY LNA Array microRNA Profiling Services. The time series experiments comprise 7-11 time points; 2-3 biological replicates. With the permission of Dr.Torunn Bruland.

Data sets from these extensive microarray time series experiments have revealed that the peptide hormone gastrin up-regulate genes involved in several biological processes which may contribute to carcinogenesis, including proliferation, endoplasmic reticulum stress (ER stress), survival, anti-apoptosis, differentiation and migration [5, 41, 49, 50].

ER stress is increasingly documented to play a role in carcinogenesis as well as in cell homeostasis and inflammation [27, 51-53], and the research group found that ER stress related genes, the pro-survival factor clusterin and the cellular anti-apoptotic effect of gastrin are dependent on sustained gastrin treatment [41, 49] (selected genes shown in Figure 8 A-B). Since increased protein levels are determined by transcriptional as well as post-translational processes (i.e., control of translational rate and protein degradation), it is of interest to further explore the correlation between gastrin regulated mRNA levels and the expressed proteome. The aim is to elucidate the cellular and biological foundation of e.g., sustained gastrin responsive ER stress genes expressed in relation to ECL hyperplasia and growth of oxyntic mucosa (Figure 8C).



**Figure 8: Sustained gastrin induced genes involved in ER stress/ UPR**

**A:** Schematic presentation of the three signalling pathways initiated by the stress sensors IRE1, PERK and ATF6 in the UPR. The green circles indicate genes differentially expressed in transiently (gastrin removed after 1 hour) versus sustained (gastrin present for 14 hours) treated cells [41]. **B:** Data from two independent time series microarray experiments showing time profiles for UPR genes differentially expressed in transiently versus sustained treated cells [41]. **C:** Selected pictures showing immunohistochemical expression of ER stress/UPR related proteins in mucosa from rats with pharmacological elevated gastrin levels (work in progress). With the permission of Dr. Torunn Bruland and Dr. Ingunn Bakke.

Previous results indicate that the distinct mRNA expression patterns in AR42J cells treated in transient and sustained modes are, at least in part, linked to the duration of ERK1/2 activation and expression of the AP-1 components like JUNB as well as the ATF subfamily proteins ATF4 and ATF3 for which the mRNA expression levels are prolonged only in the sustained mode [41]. Activating transcription factor-4 (ATF4) is known to mediate several of the sustained gastrin regulated genes involved in cellular responses related to apoptosis or survival, including *Chop*, *Herpud1* and *Mcl1* [5, 41, 49, 50]. In this thesis, we further explore the dynamics and cellular responses to gastrin induced gene expression with focus on proteins in the ATF4 network.

## **1.9 Aims and experimental strategy of the master thesis**

- Characterize gastrin induced temporal protein expression of ATF4 in AR42J cells by Western Blot semi-quantitative analysis.
- Characterize expression and cellular localization of proteins in the ATF4 network regulated by gastrin in AR42J wild-type and ATF4 knock down cells.
- Study survival responses in AR42J wild-type and ATF4 knock down cells.
- Examine expression of ATF4 protein in normal and hypergastrinemic rats.

## 2. MATERIALS AND METHODS

### 2.1 Cell Model system AR42J

The rat pancreatic acinar cell derived cell line AR42J (American Type Culture Collection (ATCC), Rockville, MD) [54] express gastrin/CCK2 receptors endogenously and may therefore be used as a model system to study gastrin responses like proliferation [14], differentiation [55] and apoptosis [56-59]. As described earlier, the Gastroenterology Cell Biology Group has performed several large genome-wide microarray time-series experiments to identify molecular networks in gastrin-regulated gene expression (Table 1). In the present master thesis temporal protein expressions were analyzed in AR42J wild type cells (wt) (by Western Blot analysis and immunocytochemistry (ICC) and the impact of reduced protein expression of ATF4 was analyzed in cells where ATF4 was partly knocked down (KD) by retrovirus-based RNAi (ATF4 KD). Cells stably transfected with retrovirus-based RNAi against Firefly (FF KD) or empty vector (EV) were used as controls in the KD experiments (Table 2).

**Table 2: Time series experiments used for protein detection**

	Treatment gastrin (G17 10nM)	Samples*	Performed by**	References
<b>1</b>	AR42J wt cells: Sustained gastrin (24 h); transient gastrin (1 h), controls (24 h) T0, 1h, 1h 5min, 1h 15 min, 2h,6h, 8h,10h,12h,14h,24h	3 X 33 BRI BRII BRIII	LKMS, CSF,TB 2010	[41]
<b>2</b>	AR42J wt AR42J ATF4 KD cells AR42J FF KD cells AR42J EV cells +/- gastrin at 4 h	2 X 4 BRI BRII	CSF, TB 2012-2013	<b>Present work</b>
<b>3</b>	AR42J wt AR42J ATF4 KD cells AR42J FF KD cells AR42J EV cells +/- gastrin at 10h, 24h, 72h	1 X 4BRI	<b>WA</b> <b>2013</b>	<b>Present work</b>

\* BRI, BRII and BRIII: Biological replicates 1-3 respectively. LKS: Linn-Karina Myrland Selvik, CSF: Christina Sæten Fjeldbo, TB: Torunn Bruland, WA: Wahida Afroz.

## **2.2 Cultivation of Cells**

All the different AR42J cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) containing 4.5 g/l glucose and supplemented with 0.1 mg/ml penicillin-streptomycin, 0.1 mg/ml L-glutamine, 1µg/ml fungizone, 1 nM sodium pyruvate (all from Invitrogen) and 15% fetal bovine serum (FBS) (Lonza BioWhittaker, Basel, Switzerland). The cells were cultured in a modified environment at 37°C in 5% CO<sub>2</sub> atmosphere and subcultured every 4-5 days.

## **2.3 Time series experiments and gastrin stimulation**

Before the time series experiments cells were grown in 6-well plates (3 × 10<sup>5</sup> cells / well) for 72 hours. Then the growth medium was replenished with 2 ml serum-free DMEM and the cells serum starved for 20-24 hours before adding 10 nM gastrin (G-17, Sigma-Aldrich, St. Louis, MO). Treated and untreated cells were grown in parallel and harvested for protein lysates (pool of 2-3 technical replicates) at several time points. The samples analyzed in this thesis are summarized in Table 2.

## **2.4 Preparation of protein lysates**

To run a western blot the cells have to be lysed in order to release the proteins of interest. The cultured cells were washed twice with cold PBS and added 350 µl of lysis buffer per well containing 1ml RIPA buffer (Thermo Scientific, Rockford, IL) with 10µl protease inhibitors and 10µl phosphatase inhibitors (Halt Protease / Phosphatase Inhibitor Single-Use Cocktail (100x) (Thermo Scientific) and 20µl complete EDTA-free protease inhibitor cocktail. Homogenization was done using syringe and needle, cell debris removed by centrifugation (15000 g, 15 minutes at 4°C) and the supernatant stored at -80°C. Protein concentrations were calculated using the Pierce® BCA Protein Assay Kit (Thermo Scientific) according to the manufacturer's instructions.

In the present thesis, protein lysates from the gastric oxyntic mucosa of hypergastrinemic rats were also analyzed. These were prepared as describe [58]. In short: Hypergastrinemia were induced by daily gavage of the proton pump inhibitor pantoprazole (Nycomed, Konstanz, Germany) (400 µmol/kg body weight) for 4 weeks. Tissue samples of the gastric oxyntic mucosa were taken from the greater curvature and homogenized in 350 µl RIPA buffer with protease and phosphatase

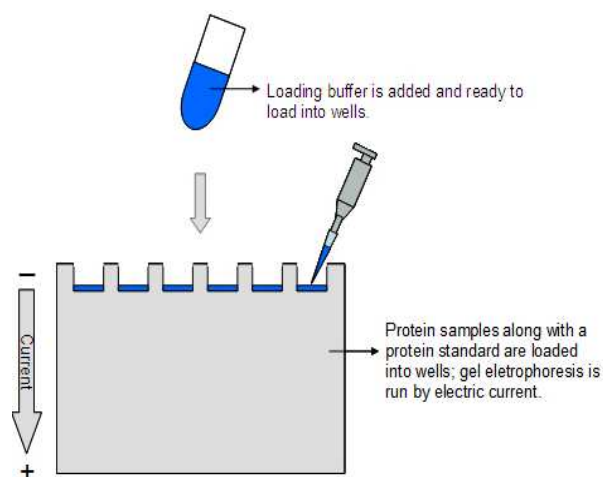
inhibitors using Ultra-Turrax rotating knife homogenizer, centrifugated and the supernatant stored at -80°C.

## **2.5 Western blot**

Western blotting, also known as protein blotting or immunoblotting, is an analytical technique used to detect specific proteins. The technique is based on three main principles: the separations of protein mixtures by size using gel electrophoresis, the efficient transfer of separated proteins to a blotting membrane and specific detection of a target. The detected target proteins will be visualized as a band on the membrane, and the level of proteins can be semi-quantified using simultaneous detection of a "housekeeping protein" as loading control. Western blot is done in three steps and each step was optimized and performed as describe below:

### **A. Sample preparation and gel eletrophoresis**

Usually the protein of interest that is to be recognized by the antibody consitute only a small portion of the total protein in the sample. NuPAGE LDS sample buffer (4x) and NuPAGE reducing agent (10x) were added according to the manufacturer's instructions. Equal amount of the samples were loaded to each well in the gel (Figure 9). A standard ladder consisting of 1 µl Magic mark XP western standard (Invitrogen) and 5 µl kaleidoscope (Bio-Red Laboratories, Hercules, CA) was added in one well as a molecular marker, which provides information about the size of the detected proteins. The gel electrophoresis was performed using the NuPAGE system and NuPAGE MOPS SDS Running buffer (1x) prepared as instructed by the manufacture. In this thesis the protocol was optimized for each combination of protein and sample type as shown in Table 3.



**Figure 9: Schematic diagram of protein samples loaded for gel electrophoresis.** Protein samples together with NuPAGE LDS sample buffer and NuPAGE reducing agent are added into each wells and gel electrophoresis are run (72).

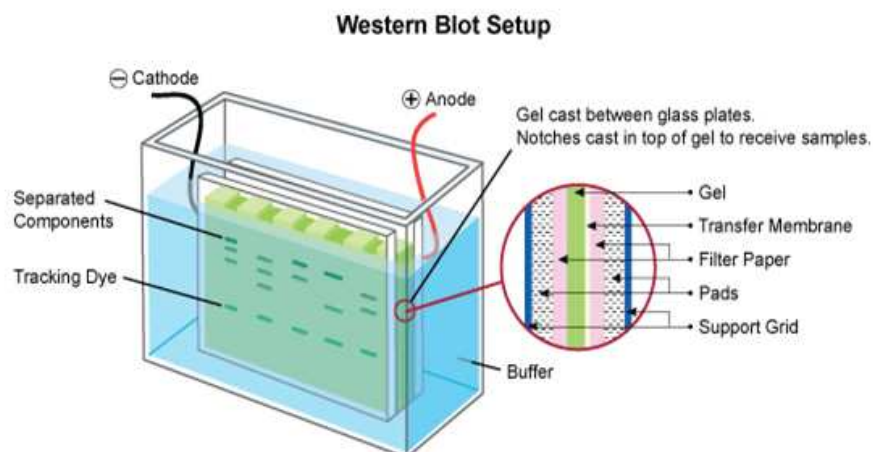
**Table 3: Optimized gel electrophoresis and blotting protocols**

Protein type	Gel electrophoresis	Running time	blotting	Blotting time & conditions
<b>ATF4</b>	200 volt	1 hour	30 volt	1 hour at RT
<b>PERK &amp; pPERK</b>	200 volt	2 hours	30 volt	2 hours on Ice
<b>pGCN2</b>	200 volt	2 hours	30 volt	2 hours on Ice
<b>peIF2<math>\alpha</math></b>	200 volt	1 hour	30 volt	1 hour at RT
<b>CCK2R</b>	200 volt	1 hour	30 volt	1 hour at RT
<b>Herpud1</b>	200 volt	1 hour 30 minutes	30 volt	1 hour at RT
<b>CHOP</b>	200 volt	1 hour 15 minutes	30 volt	1 hour at RT
<b>GRP78</b>	200 volt	1 hour 15 minutes	30 volt	1 hour at RT
<b>Rat tissue lysate</b>	200 volt	1 hour	30 volt	1 hour at RT

## B. Blotting of proteins to the membrane

The proteins were transferred onto a 0.45  $\mu\text{m}$  Polyvinylidene Difluoride Transfer (PVDF) Membrane (Amersham hybond-P, GE Healthcare, Buckinghamshire, UK) using NuPAGE Transfer Buffer (1x) (Invitrogen) (Figure 10) according to the manufacturer's protocols. Blotting time was also optimized to get a better transfer from 1 hour to two hours at 30 volt constant current (Table 3).





**Figure 10: Principle of protein transfer to PVDF membrane.** Principles of the western blot apparatus and transfer of sample from the gel to the PVDF membrane (73).

### C. Immunostaining of the blotted membrane

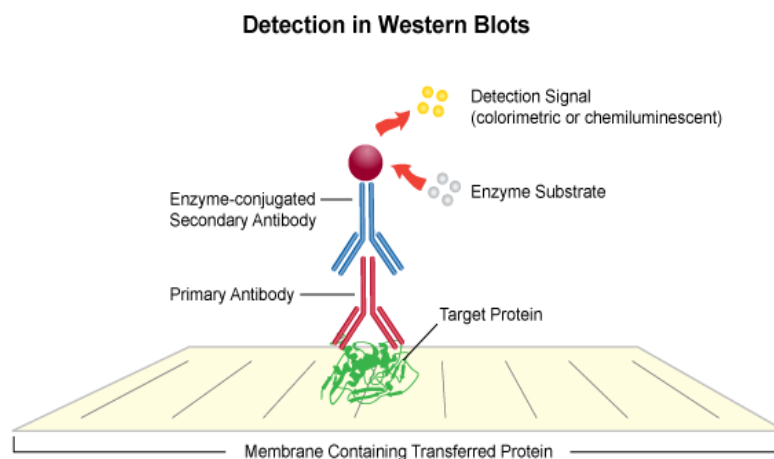
This step consists of blocking and incubation with primary and secondary antibodies.

**Blocking:** The membranes were blocked in TBST (50mM Tris.HCl pH 7.5, 150 mM NaCl, 0.1% Tween 20) with non-fat dry milk or 5 % Bovine Serum Albumin (BSA) (Sigma-Aldrich) for 1hour at room temperature (RT) or overnight at 4°C with gentle shaking. The blocking conditions were optimized for each of the protein targets to get less unspecific bands (Table 4).

**Table 4: Optimized blocking protocols**

Protein type	Blocking reagent	Time
ATF4	BSA & Milk	Overnight at 4°C
PERK & pPERK	BSA	1 hour at RT
pGCN2	BSA & Milk	1 hour at RT
peIF2α	BSA	1 hour at RT
CCK2R	BSA & Milk	1 hour at RT
Herpud1	BSA & Milk	1 hour at RT
CHOP	BSA	1 hour at RT
GRP78	BSA & Milk	1 hour at RT

**Incubation with antibody:** The antibodies were diluted in TBST with 1 % BSA or 5% milk and incubated overnight at 4°C (or for 2 hours at room temperature for ATF4 on rat tissue extract) with gentle shaking. All primary antibodies are listed in Table 5. As secondary antibody horseradish peroxidase (HRP) conjugated goat anti rabbit IgG (Cell-Signaling, cat no 7074, MA) was used. It was diluted 1:8000 in TBST with 1 % BSA or 5 % milk and incubated at room temperature for one hour. Between the incubations the membranes were washed with TBST 5 x 5 minutes. The principles of primary and secondary antibodies binding are demonstrated in Figure 11 .Binding of the secondary antibody was visualized using a SuperSignal West Femto Maximum sensitivity substrate (Thermo Scientific) and Kodak Image station 200R (Kodak, Pittsburgh, PA). The SuperSignal substrate consists of luminal/enhancer solution and peroxide buffer. The exposure time of the Kodak image varied from 5 seconds to 10 minutes according to the intensities of the protein signals. After detection, the membranes were washed with TBST 4 x 10 minutes to completely clean the membranes of the detection solution.



**Figure 11: Principle of antibody detection of proteins on the membrane.** Both primary and secondary antibodies bind with their target protein. The enzyme conjugated secondary antibody enables visualization of the target protein by adding enzyme substrate (74).

**Table 5: Primary antibodies used for western blot**

<b>Antibody host</b>	<b>Dilution</b>	<b>Dilution reagents</b>	<b>Molecular weight</b>	<b>Producer</b>
<b>ATF4 Rabbit polyclonal</b>	1:800	Milk & BSA	50-60 kDa (calculated 39 kDa)	10835-1-AP Proteintech
<b>ATF4 Rabbit polyclonal</b>	1:800	Milk & BSA	38 kDa Observed: 40-50 kDa	Sc-200 Santa Cruz
<b>Herpud1 Rabbit polyclonal</b>	1:400	BSA	44 kDa	Ab-73669 Abcam
<b>PERK Rabbit monoclonal</b>	1:1000	BSA	140 kDa	C33E10 Cell Signaling
<b>pPERK Rabbit monoclonal</b>	1:1000	BSA	170 kDa	16F8 Cell Signaling
<b>peIF2<math>\alpha</math> Rabbit monoclonal</b>	1:1000	BSA	38 kDa	3597 Cell signaling
<b>GRP78 Rabbit polyclonal</b>	1:1000	Milk & BSA	78 kDa	105019 Abcam
<b>CHOP Rabbit monoclonal</b>	1:1000	BSA	19 kDa Observed: 31 kDa	11419 Abcam
<b>CCK2R Rabbit polyclonal</b>	1:1000	Milk & BSA	75 kDa	250659 Abbiotec
<b>pGCN2 Rabbit polyclonal</b>	1:500/10000		181 kDa Observed: 190 kDa	bs-3156R Bioss

For detection of housekeeping genes (loading control), blocking, washing, and antibody incubation (Table 6) were performed with SNAP i.d. Protein Detection System (Millipore, Billerica, MA) (Figure 12) according to the manufacturer's instructions, using TBST with 1% BSA for blocking and antibody dilution. As secondary antibody HRP-conjugated goat anti mouse IgG (Dako, cat no P0447) was used and the binding was visualized by use of the SuperSignal West Femto Maximum Sensitive Substrate (Thermo Scientific) and Kodak Image Station 2000R (Kodak, Pittsburgh, PA).



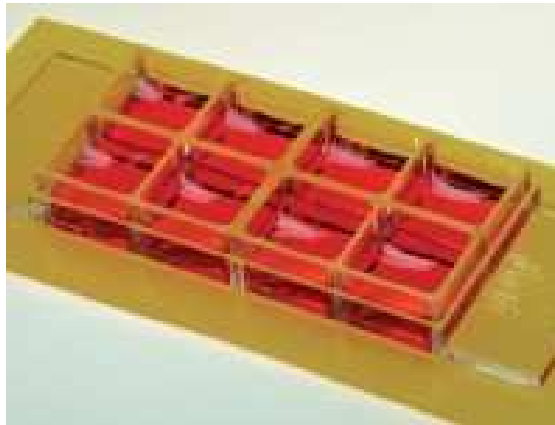
**Figure 12: The SNAP id system with the blot holder.** This system also consists of a vacuum and a container where all the liquids are collected. This SNAP id is used to incubate the membrane with housekeeping antibodies which takes only 30 minutes (75).

**Table 6: Primary antibodies used for western blot housekeeping proteins**

	Antibody host	Target	Dilution	Mol. weight	Producers
<b>Primary antibody</b>	mouse monoclonal	$\alpha$ -tubulin	1:100-10000	50 kDa	sc-5286 Santa Cruz
	mouse monoclonal	$\beta$ -actin	1:500-10000	42 kDa	ab-8226 Abcam
	mouse monoclonal	GAPDH	1:5000	38kDa	Ab-9484 Abcam
	Rabbit Monoclonal antibody	Histon H4	1:10000	10 kDa	05-858 Milipore

## 2.6 Immunocytochemistry

For immunocytochemistry (ICC) on cultured cells, AR42J wild type and ATF4 KD cells were seeded into silicone wells (2/4/8) attached to Amino-Silane coated slides (25x75 mm) (Corning® Ultra GAPS™ Slides, NY) (Figure 13) printed with transfection reagents.



**Figure 13: Cell culture in silicone wells for immunocytochemistry (76).**

The cells were serum starved for 24 hours before adding 10 nM G-17 and the cells were stimulated in parallel with untreated cells for 6-20 hours. The stimulation was stopped and the cells were fixed onto the slide by incubation with fixation solution (3.7% Paraformaldehyde + 4% sucrose in PBS, pH ~ 7). After permeabilization (0.1% Triton X-100) and blocking (1 % BSA in PBS), the cells were incubated with the primary antibody (Table 7) diluted in 0.5 % BSA in PBS (Table 7), followed by the secondary antibody conjugated with green fluorescence (goat anti-rabbit IgG Alexa 488, cat no A-11006, Invitrogen). The silicone wells were removed, the slides dried and nuclear staining added by using mounting medium with DAPI (Vectashield Hard set, Vector, Burlingame, USA). The slides were stored at 4°C for at least overnight before the cells were visualized using a fluorescence microscope.

**Table 7: Primary antibodies used for immunocytochemistry**

Antibody host	Dilution	Producers
<b>ATF4</b> Rabbit polyclonal	1:50/75	10835-1-AP Proteintech
<b>ATF4</b> Rabbit polyclonal	1:50/75	Sc-200 Santa Cruz
<b>Herpud1</b> Rabbit polyclonal	1:400	Ab-73669 Abcam
<b>pPERK</b> Rabbit monoclonal	1:50	Thr980 Cell Signaling
<b>peIF2<math>\alpha</math></b> Rabbit monoclonal	1:50	3597 Cell signaling
<b>GRP78</b> Rabbit polyclonal	1:100	Ab-105019 Abcam
<b>CHOP</b> Rabbit monoclonal	1:100	Ab-11419 Abcam

## 2.7 Immunohistochemistry

Immunohistochemistry (IHC) is a method for detecting antigens (proteins) in a tissue section. Tissue sections of gastric oxyntic mucosa from normal and hypergastrinemic rats were dewaxed and rehydrated in graded solutions of alcohol, incubated in 3% H<sub>2</sub>O<sub>2</sub> to quench endogenous peroxidase activity and microwaved in citrate buffer (pH 6) for 15 minutes to retrieve the antigens. Washing was done using TBS with 0.05 % Tween-20 (Merck, Germany). The slides were incubated with anti-ATF4 (ProteinTech) diluted 1:250 in TBS with 0.025% Tween-20 and 1% BSA for overnight at 4°C, and the immunoreaction was visualized using the rabbit/mouse EnVision-HRP Immuno Detector DAB (3'3'diaminobenzidine) Substrate-Chromogen kit (Dako, Denmark). Counterstaining was done with hematoxylin and mounting with Glycergel (DAKO).

## 2.8 Microscopes

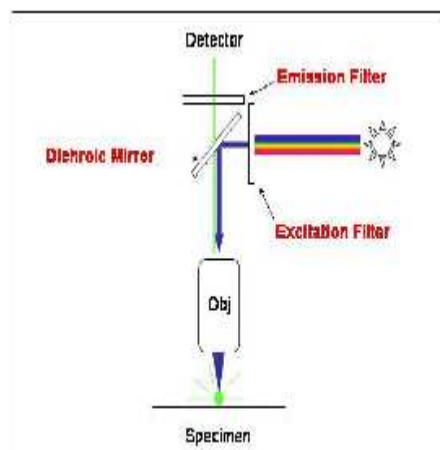
The most common type of microscope is the optical microscope or modern light microscope which emerges light to see the object. In this thesis, modern light microscope and immunofluorescence microscope were used to study the cell morphology, cell growth, cultured conditions, cell density, cell count, cellular localization and expression of different proteins.

### A. Modern light microscope



**Figure 14: Inverted light microscope.** This microscope uses visible light and a system of lenses to magnify images of small objects (77). The light microscope is so called as it emerges light to the object to detect small objects. This is the most popular, most well-known and well-used research tool in biology.

### B. Fluorescence microscope

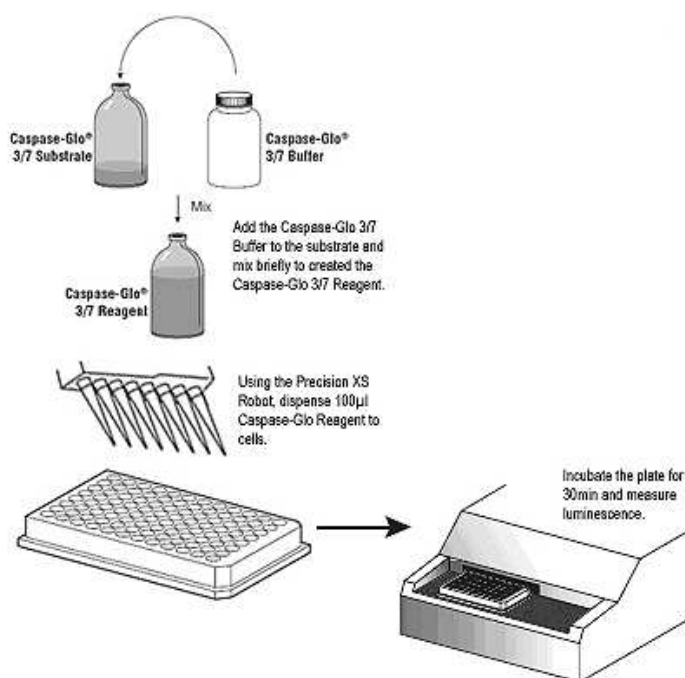


**Figure 15 : Fluorescence microscope and different parts.** On the left, a picture of fluorescence microscope with mercury-vapor lamp (78). On the right, schematic diagram of the fluorescence microscope with different parts (79).

A fluorescence microscope is a special microscope where fluorochromes are used to label or tag biological samples (Figure 15). The fluorochromes give light of a given wave length when excited by incident light of a different (shorter) wave length. In this thesis the immunocytochemically stained cells were visualized using the 1X71 Olympus fluorescence microscope and excitation / emission filter no 5. Pictures were made using the monochrome camera of the P<sup>^</sup>cell software to add the nuclear color (blue or purple) and the color (green) showing the specific antibody binding.

## 2.9 Caspase assay

To examine apoptosis in cultured cells, the caspase-3 and caspase-7 activities were measured using the luminescent Caspase-Glo<sup>®</sup> 3/7 assay (Promega, Madison, WI) and VICTOR<sup>3™</sup> 1420c plate reader (Perkin Elmer) (Figure 16) according to the manufacturer's instructions. Caspase 3/7 are the members of highly conserved, cysteine-dependent aspartate-specific proteases, which play a central role in the execution of apoptosis by proteolytically degradation of intracellular proteins. The caspase activity was calculated by measuring the luminescence of each sample via Wallac 1420 Victor3 plate reader (Perkin Elmer).



**Figure 16: Principles of the 3/7 caspase assay.** The caspase assay solutions are mixed and added to the cells before incubation with substrate and reading of the resulting luminescence from each sample (80).



## **2.10 Statistical analysis**

The data were plotted as means  $\pm$ SD as error bars. A two-tailed student's t-test which determines whether two samples have the same mean or not, was used to evaluate statistical significance.  $P < 0.05$  was considered as significant. In the analysis of the apoptosis assay, the data were log transformed before statistical analysis and relative intensities for each technical replicate were calculated relative to the median of the untreated firefly control (FFC) in each independent biological experiments, as described in [60] for cells. For presentation, the data were transformed back to the original scale and plotted as mean with 95% confidence interval (CI) as error bars. Student's t-test was used to evaluate statistical significance.

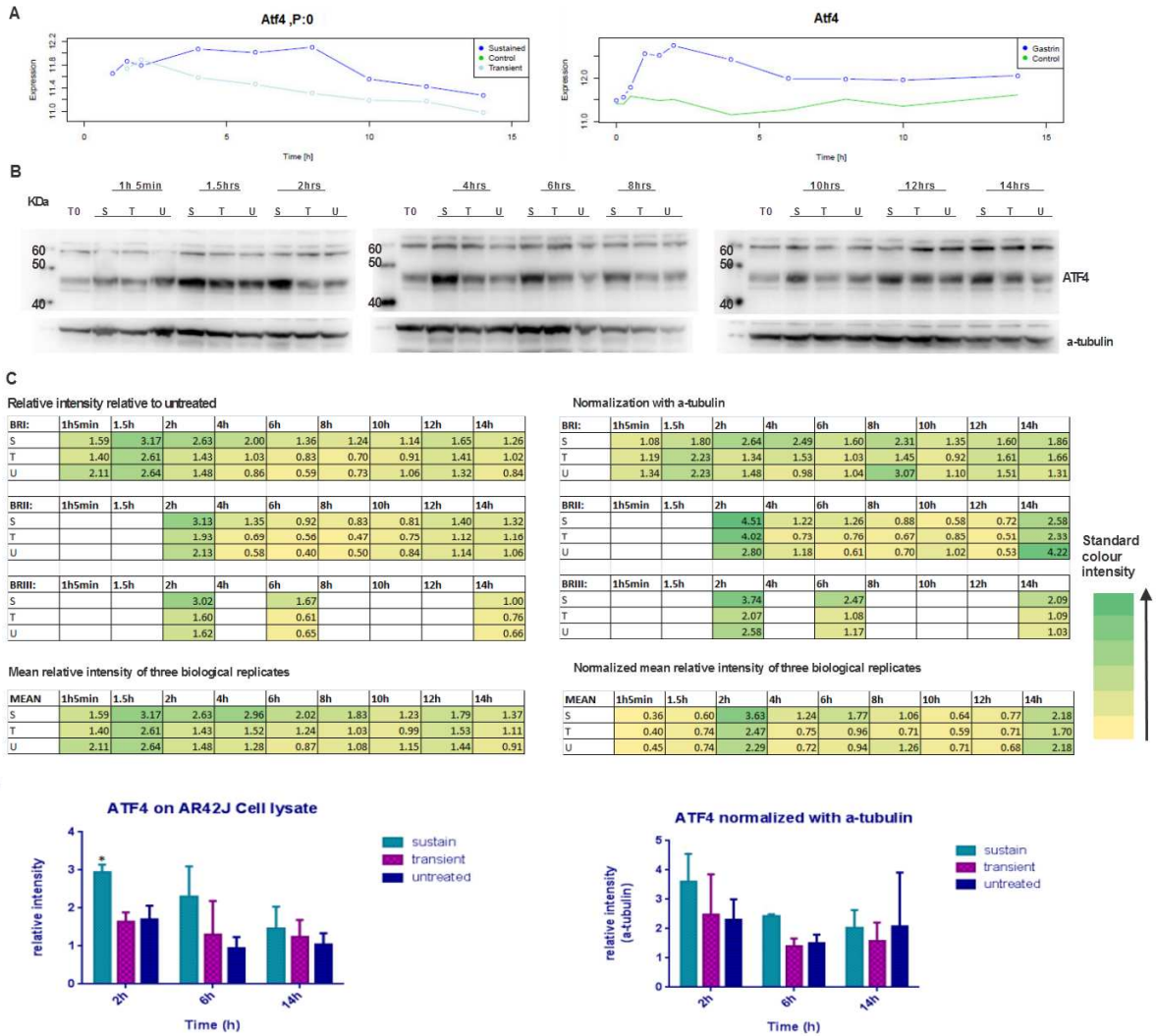


### 3. RESULTS

#### 3.1 Expression of ATF4 protein in response to varying duration of gastrin treatment

Genom-wide microarray time series experiments in adenocarcinoma cell line AR42J cells were performed by researchers in the Gastroenterology cell Biology Group in collaboration with at the NTNU Genomic Core Facility (GCF). The experiments showed that the transcriptional program in sustained gastrin treated cells was significantly different from the transcriptional program in transiently gastrin treated cells. The mRNA expression profile for ATF4 (Figure 17A) shows that there is a significant difference in mRNA levels in sustained *versus* transient (left panel A) as well as in sustained treated *versus* untreated cells (right panel A). Based on this finding, we wanted to explore how the ATF4 protein is expressed in sustained gastrin treated cells compare to transiently treated and untreated cells. Therefore, western blot analysis of ATF4 protein was performed at all-time points starting from 1 hour 5 minutes up to 24 hours in order to see how long the ATF4 protein levels were up-regulated. To support this result, we also performed western blot analysis using fluorescence at 6, 14 and 24 hours (shown in Supplementary Figure S1).

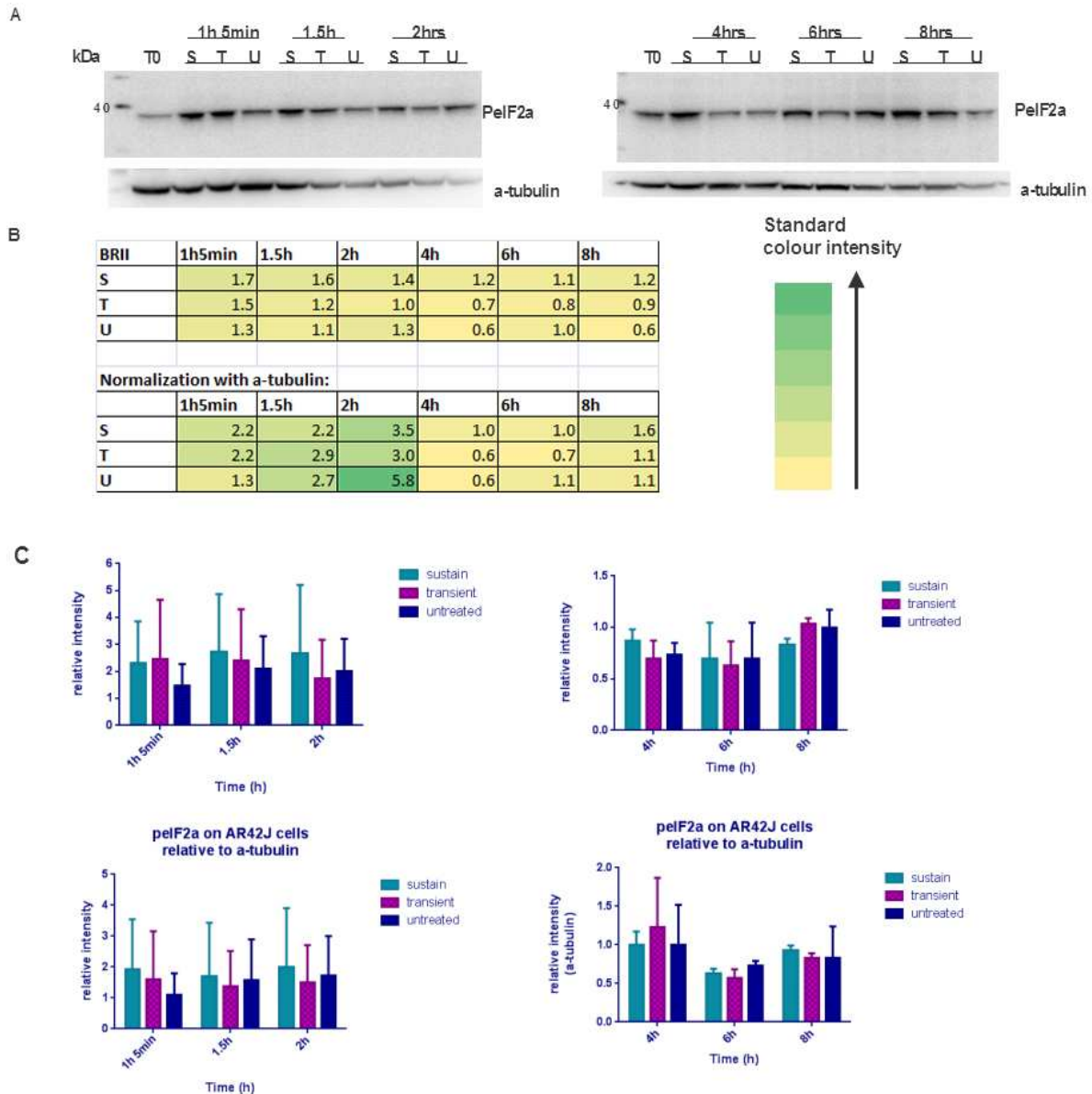
The molecular weight of ATF4 protein is 48 kDa. In this experiment, the band was found at 48 kDa (Figure17, panel B). The ATF4 protein seems to be up-regulated in sustained gastrin treated cells after 1.5 hour and remain up-regulated up to 10 hours compared to transiently and untreated cells; which matches the temporal mRNA profiles. The relative intensity of the three biological replicates are shown in the heat map together with the relative intensity normalized with  $\alpha$ -tubulin (Figure 17, panel C). The relative intensities (mean $\pm$  SD) are shown in Figure 17D. Overall, the results show that sustained gastrin treatment induces prolonged expression of the ATF4 protein.



**Figure 17: Expression of ATF4 in response to different durations of gastrin treatment. A) Left panel: Temporal expression of ATF4 mRNA in AR42J cells treated with gastrin in a sustained (S) versus transient (T) mode (From Exp. 3 in Table 1). Right panel: Temporal expression of ATF4 mRNA in sustained gastrin treated versus untreated cells (From Exp. 2 in Table 1). **B)** Western blot image of ATF4 protein in sustained (S), transiently (T) and untreated (U) cells at 1-14 hours. Molecular weight of the ATF4 protein is 48 kDa (shown in the gel picture). Housekeeping gene ( $\alpha$ -tubulin) is shown at different time points in the lower images. Results of one of the three biological replicates are shown. T0: time point zero. **C) Left panels: Heat maps showing relative intensity compare to time point zero (T0) of all three biological replicates (BRI, BRII and BRIII) at different time points. The right panels show the relative intensity of ATF4 protein normalized with housekeeping gene ( $\alpha$ -tubulin) at all-time points in three biological replicates and below is the mean intensity of the ATF4 normalized with  $\alpha$ -tubulin. A schematic diagram of standard color intensity for the heat is shown in the right most panels. **D) Lower left panel: Relative intensity (mean  $\pm$  SD) of the three biological replicates at 2, 6 and 14 hours, lower right panel: Relative intensity (mean  $\pm$  SD) at same time points normalized with housekeeping gene ( $\alpha$ -tubulin). Error bar represents SD. \* P value is significant ( $P < 0.05$ ).******

### **3.2 Gastrin treatment induces phosphorylation of eIF2 $\alpha$**

Since ATF4 may be activated by many cellular stresses that are sensed by the upstream eIF2 $\alpha$  kinase, we wanted to examine whether this kinase was phosphorylated upon gastrin treatment in AR42J cells. The eIF2 $\alpha$  protein were detected at 38 kDa (Figure 18 A) and were up- regulated in sustained and transiently gastrin treated cells starting from 1 hour 5 minutes of gastrin treatment and remained higher expressed up to 8 hours compared to untreated cells. Similar results were observed in the experiments done in collaboration with Christina Sæten Fjeldbo for all time points in all three biological replicates. The intensities of the bands were quantified in relation to time point zero (T0) (Figure 18, panel B). The mean of all three biological replicates (Figure 4, panel C) were calculated and statistical analysis (mean +/- SD) were done with the quantified relative intensity relative to time point zero (T0). Although there were some differences between the biological replicates as illustrated by the large SD values in Figure 20C, the increased expression of phosphorylated eIF2 $\alpha$  in sustained gastrin treated cells in comparisons with transiently and untreated cells were reproducibly detected in all three replicates. Thus, we concluded that different treatments of gastrin have different impacts on the phosphorylation and activation of eIF2 $\alpha$ ; and the p-eIF2 $\alpha$ -ATF4 network is probably activated for a prolonged time in sustained gastrin treated cells.



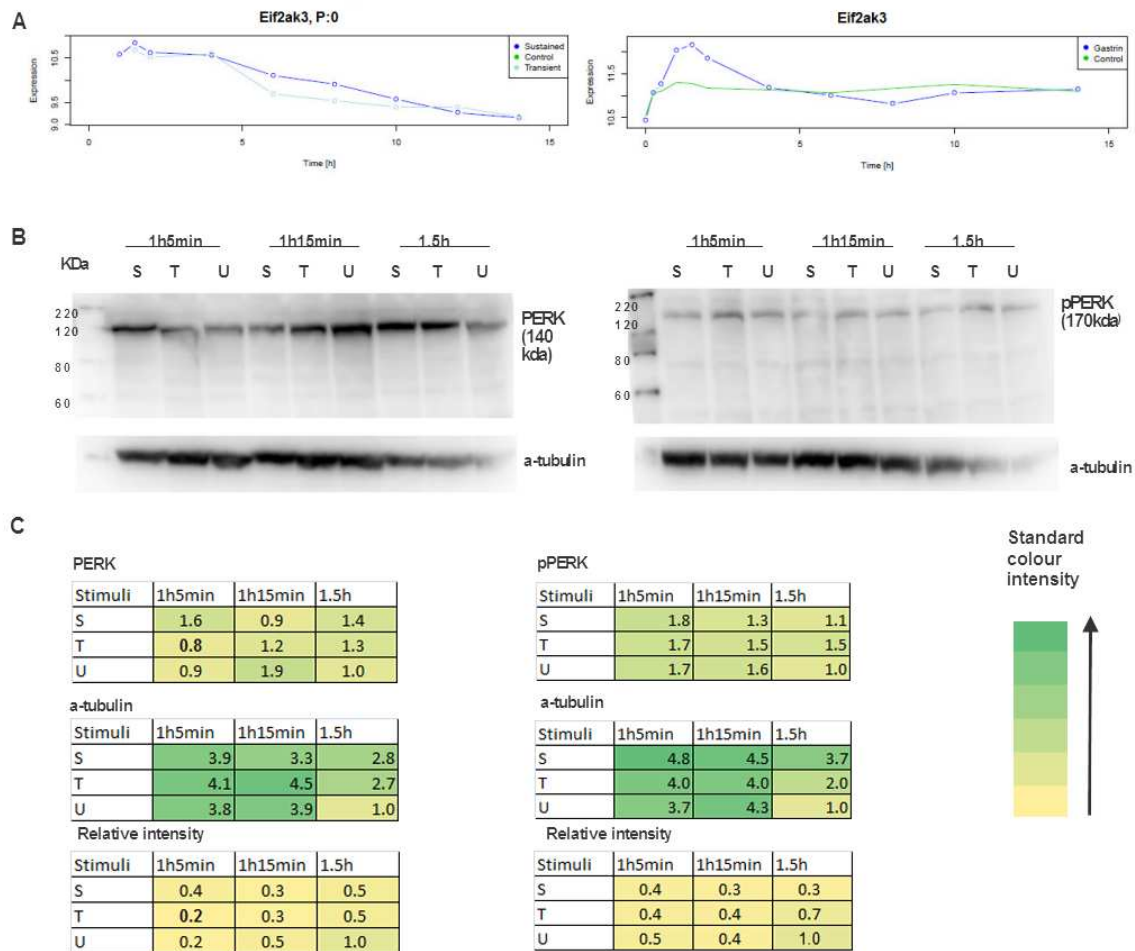
**Figure 18: Increased pelf2 $\alpha$  expression is prolonged in stimulated cells with gastrin.** The panels show western blot analysis of the pelf2 $\alpha$  protein in sustained (S); transiently (T) gastrin treated and untreated (U) cells. **A)** Western blot image of pelf2 $\alpha$  at different time points together with housekeeping gene ( $\alpha$ -tubulin) are shown below. The molecular weight of the pelf2 $\alpha$  is 38 kDa. **B)** The middle panels show heat map of pelf2 $\alpha$  protein calculated from relative intensity to the time point zero (T0) and also the relative values normalized with  $\alpha$ -tubulin below. A schematic diagram of standard color intensity for the heat map is shown in the right most panels. **C)** In the lowest panels, Relative intensities (mean  $\pm$  SD) with the quantified relative intensities to time point zero (T0) in sustained, transiently and untreated cells at different time points for all three biological replicates.

### 3.3 Gastrin mediated expression of proteins in the ATF4 network

In this thesis, we have focussed on how the ATF4 protein is expressed under gastrin stimulation and how other ATF4 network proteins are regulated during gastrin treatment. So, we have performed western blot analysis of total PERK, pPERK, PGCN2 and two ATF4 target gene CHOP and Herpud1.

#### 3.3.1 Total PERK and phospho PERK (pPERK) :

We first analyzed the expression of the ER-stress activated protein kinase PERK. The genome-wide expression study indicated that the mRNA level of total PERK is slightly higher at early time points in sustained *versus* transiently treated cells, (Figure 19, panel A). Although we found the expected band at 140kda, there is no clear difference seen between sustained, transiently and untreated cells at 65, 75 and 90 minutes (Figure 19 B, left panel). The band for the pPERK is found at 170 kDa, with no difference between sustained, transiently and untreated cells (Figure 19B, right panel). The relative intensities for the total PERK were slightly higher in sustained gastrin treated cells but when normalized with relative intensities of the housekeeping gene ( $\alpha$ -tubulin) there is no difference (Figure 19, left panel C). Similar result is also seen for the pPERK (Figure 19, right panel C). Sustained gastrin treated cells may express slightly higher levels of PERK at 65 minutes compared to untreated cells at that time. For pPERK it is difficult to say if there is any difference between sustained, transiently or untreated cells. Thus, from the present results, it is difficult to conclude if gastrin has an effect on PERK phosphorylation for downstream signal transduction. However, since PERK and pPERK proteins are expressed in AR42J cells, PERK might be phosphorylates at earlier time points and have an gastrin effect; which should be explore further.

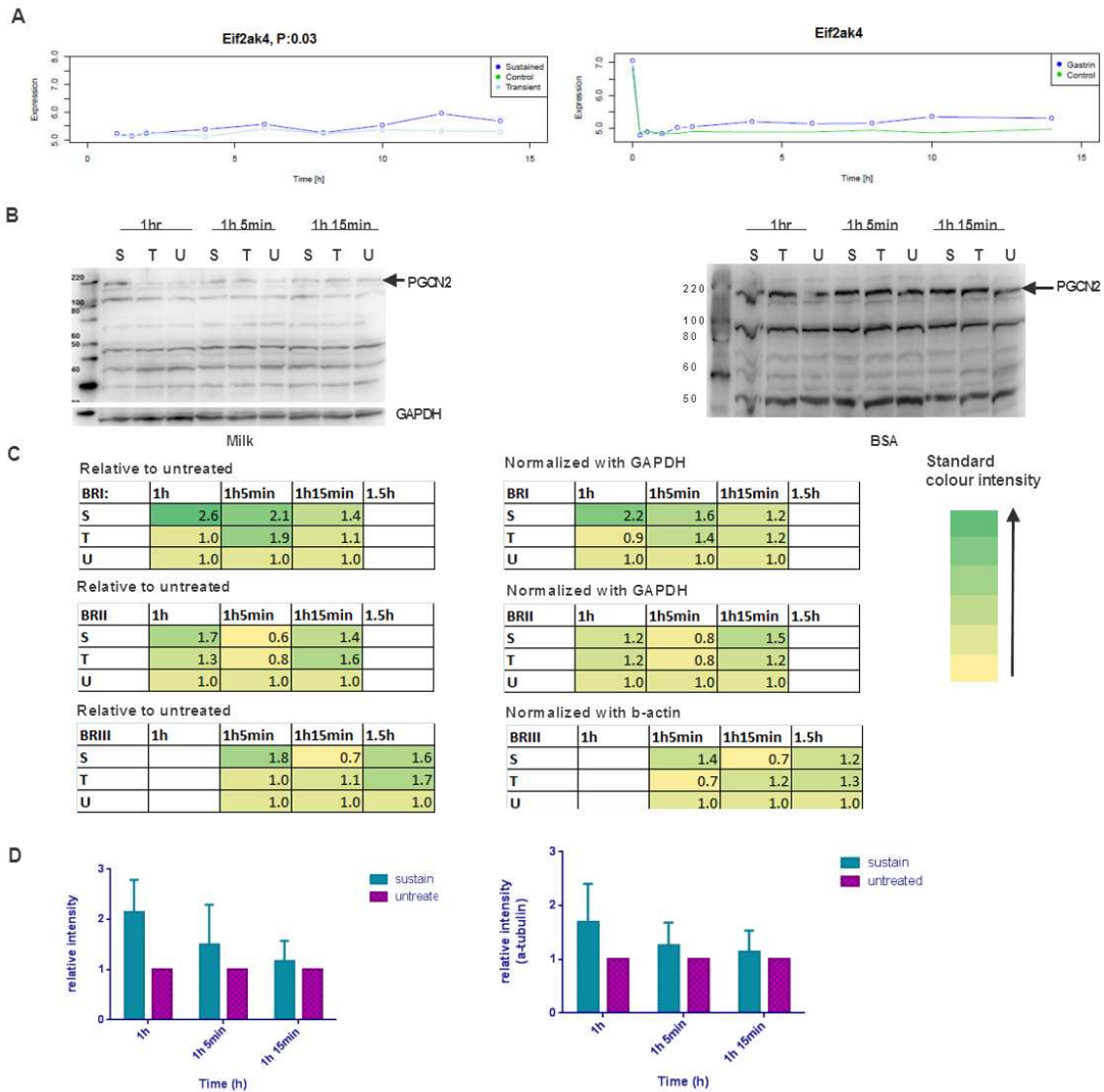


**Figure 19: Expression of total PERK and pPERK in AR42J cells upon gastrin stimulation.** **A)** Expression of total PERK (Eif2ak3) in response to different durations of gastrin treatment. **A) Left panel:** Temporal expression of total PERK (Eif2ak3) mRNA in AR42J cells treated with gastrin in a sustained (S) *versus* transient (T) mode (From Exp. 3 in Table1). **Right panel:** Temporal expression of total PERK (Eif2ak3) mRNA in sustained gastrin treated *versus* untreated cells (From Exp. 2 in Table 1). **B)** Western blot image of total PERK and pPERK in untreated (U), sustained (S) and transiently (T) gastrin treated cells at 1 hour 5 minutes, 1 hour 15 minutes and 1.5 hours (i.e., 65, 75 and 90 minutes after gastrin treatment in the sustained mode). **Left images:** Western blot of total PERK (140 kDa) and house-keeping gene ( $\alpha$ -tubulin), **Right images:** Western blot of pPERK (170 kDa) together with  $\alpha$ -tubulin. **C)** Heat-maps of quantification of total PERK and pPERK. The relative intensities of total PERK and pPERK were calculated relative to untreated control for 1.5 hours. Normalized value relative to  $\alpha$ -tubulin is shown in the lower panels. A schematic diagram of standard color intensity for the heat map is shown in the right most panels.



### **3.3.2 Phosphorylation of GCN2 in AR42J cells upon gastrin stimulation:**

ATF4 may also be activated by the GCN2 eIF2alpha kinase, a sensor of e.g., amino acid deficiency [39]. Data from the genome wide time series experiments show that there is a small increase in GCN2 mRNA level upon gastrin stimulation compared to control cells (Figure 20A). We therefore wanted to examine if gastrin induces phosphorylation of GCN2 (PGCN2) in AR42J cells, and performed western blot analysis at 60, 65 and 75 minutes post sustained gastrin treatment. The bands for the PGCN2 were found at 181 kDa (Figure 20B) and there were an increased expression of PGCN2 in sustained gastrin treated cells at 60, 65 and 75 minutes compared to untreated cells, as shown in the representative heat map in Figure 20C. The differences were reproduced in three biological replicates (Figure 12D). Thus, from this western blot analysis, we can state that the GCN2 protein is expressed in AR42J cells. However, although sustained gastrin treated cells show slightly higher expression of PGCN2 compared to untreated cells, we need to further explore if phosphorylation of GCN2 is regulated by gastrin or not.

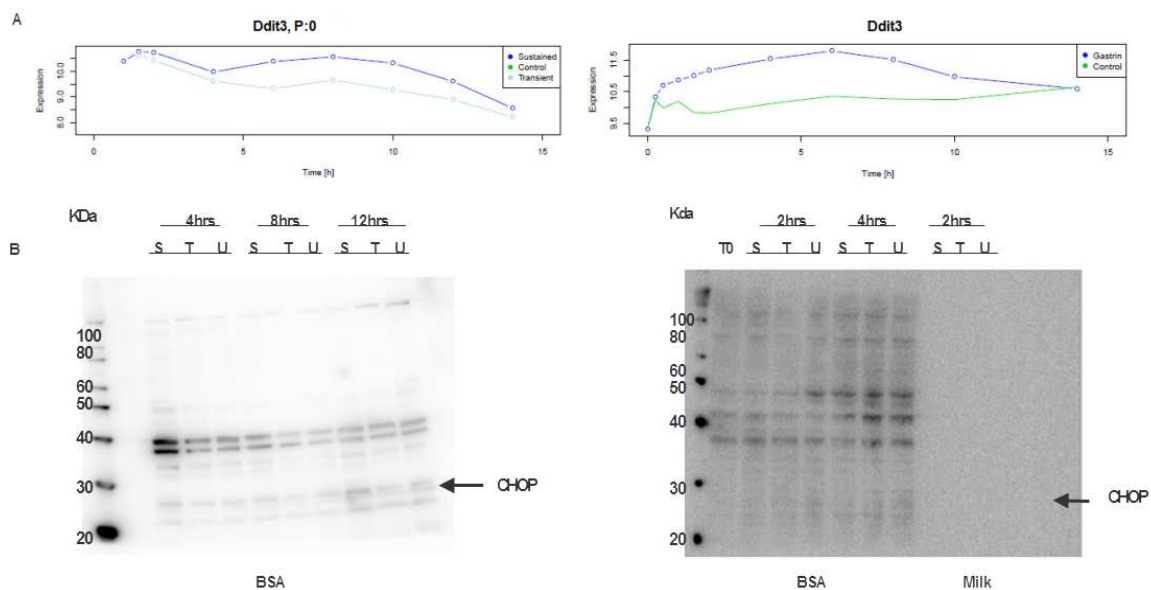


**Figure 20: Expression of PGCN2 upon gastrin treatment.** **A) Left panel:** Temporal expression of GCN2 (Eif2ak4) mRNA in AR42J cells treated with gastrin in a sustained (S) versus transient (T) mode (From Exp. 3 in Table1). **Right panel:** Temporal expression of GCN2 (Eif2ak4) mRNA in sustained gastrin treated versus untreated cells (From Exp. 2 in Table 1). **B)** Western blot image of PGCN2 at early time points (1 hour, 1 hour 5 minutes and 1 hour 15 minutes; i.e., 60-75 minutes) in sustained (S) and transiently (T) gastrin treated and untreated cells. A representative image of biological replicates (BRI) showing PGCN2 bands from three independent experiments are shown here. The protocol was optimized for blocking conditions (% BSA or 5% milk), as well as antibody dilution. Molecular weight of the PGCN2 is 181 kDa. **C) Left panels:** the heat-maps of quantified intensities of all three biological replicates (BRI, BRII and BRIII) relative to untreated cells at the time points indicated. **Right panels:** Relative intensities normalization with house-keeping gene (GAPDH). A schematic diagram of standard color intensity for the heat map is shown in the right most panels. **D) Lower right panel:** Relative intensity (mean +/- SD) of the three biological replicates at 60 minutes, 65 minutes and 75 minutes between sustained gastrin treated and untreated cells. **Left panel:** relative intensity of the three biological replicates. The right panel shows normalization with housekeeping gene ( $\alpha$ -tubulin).

## Downstream target genes of eIF2 $\alpha$ at ATF4 pathway:

### 3.3.3 CHOP:

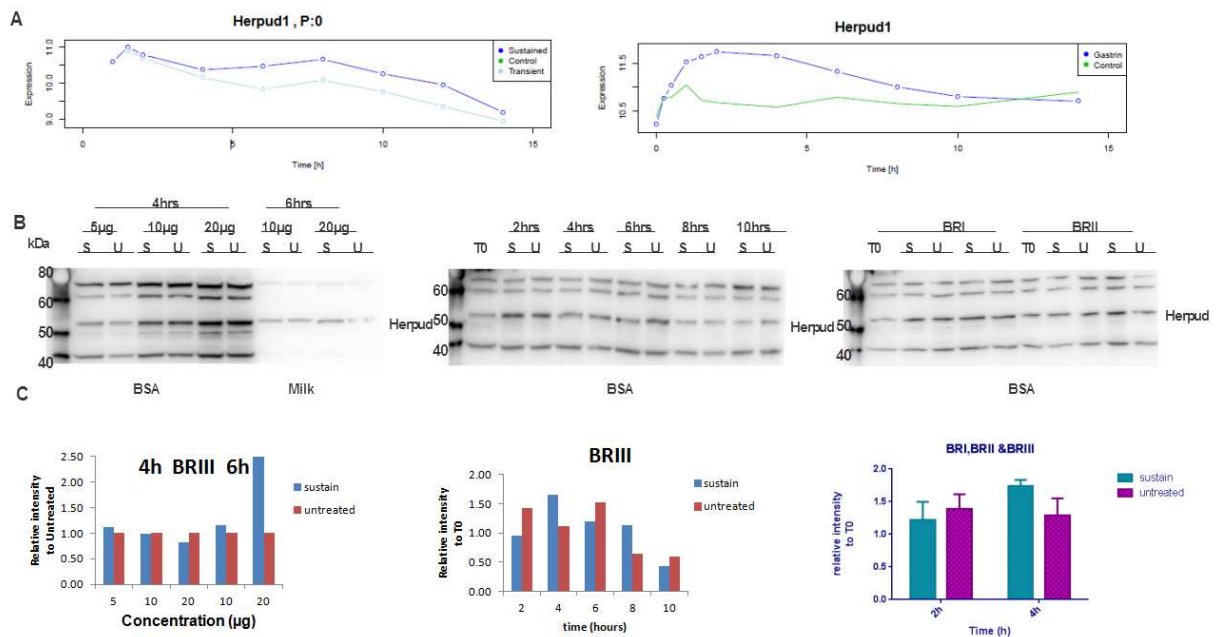
CHOP is an important stress marker that can switch from pro-survival to anti-apoptotic pathway and key downstream target of ATF4 [37]. Time series microarray experiments showed that the mRNA level of CHOP was significantly higher in cells treated with gastrin in a sustained mode compared to transiently as well as untreated cells (Figure 21, A left and right panels respectively). Thus, it was of interest to examine if the CHOP protein is regulated differently upon gastrin stimulation in AR42J cells. We analyzed the protein level by Western blot at 2, 4, 8 and 12 hours after adding gastrin the sustained mode. Although a significant difference had been shown in mRNA level of CHOP, it was difficult to see any difference between sustained, transiently and untreated AR42J cells at the protein level (Figure 21B). The bands were found at 29 kDa but the bands were too weak to see any gastrin effect. Thus, the protein level of CHOP in AR42J cells upon gastrin stimulation cells seems to be relatively low.



**Figure 21: Expression of CHOP protein in AR42J cells upon gastrin stimulation. A)** CHOP mRNA measured by microarray gene expression analysis. Left panel: Temporal expression of CHOP (Ddit3) mRNA in AR42J cells treated with gastrin in a sustained (S) *versus* transient (T) mode (From Exp. 3 in Table1). Right panel: Temporal expression of CHOP (Ddit3) mRNA in sustained gastrin treated *versus* untreated cells (From Exp. 2 in Table 1). **B)** Western blot image of CHOP protein in sustained (S), transiently (T) gastrin treated and untreated (U) cells at different time points. The protocol is optimized by using 5% BSA or 5% Milk for blocking. Molecular weight of CHOP is 19kda, but we observed bands between 29-31 kDa.

### **3.3.4 Herpud1 is expressed in AR42J Cells**

From the time series experiments of mRNA level of Herpud1, a clear difference between gastrin treated and control cells were seen (Figure 22A). So, in order to examine if similar difference could be observed at the protein level we performed western blot analysis using protein lysate from sustained gastrin treated and untreated cells. Experiments were done using different concentrations of protein (Figure 22 B, left panel) and we observed that the band is very strong above 50 kDa. Therefore, we can conclude that Herpud1 protein is highly expressed in AR42J cells. We have optimized the protocol using both BSA and milk; and in the milk only one specific band was found that verifies the location of the correct band for Herpud1. To detect any effect of gastrin, we used 5 µg of protein lysate at different time points (Figure 22 B, middle panel). Herpud1 were up regulated in sustained gastrin treated cells at 4 and 8 hours compared to untreated cells (Figure 22B-C; biological replicate BRIII). To compare the results we performed western blot analysis on protein lysate from two other biological replicates (BRI and BRII) at 2 and 4 hours (Figure 22, right panels B and C) and found that Herpud1 is also up regulated in biological replicates (BRII) at 4 hours in sustained gastrin treated cells. From these observations we conclude that Herpud1 is highly expressed in AR42J cells and up regulated in sustained gastrin treated cells at 4 hours compare to untreated cells in two of the three biological replicates.

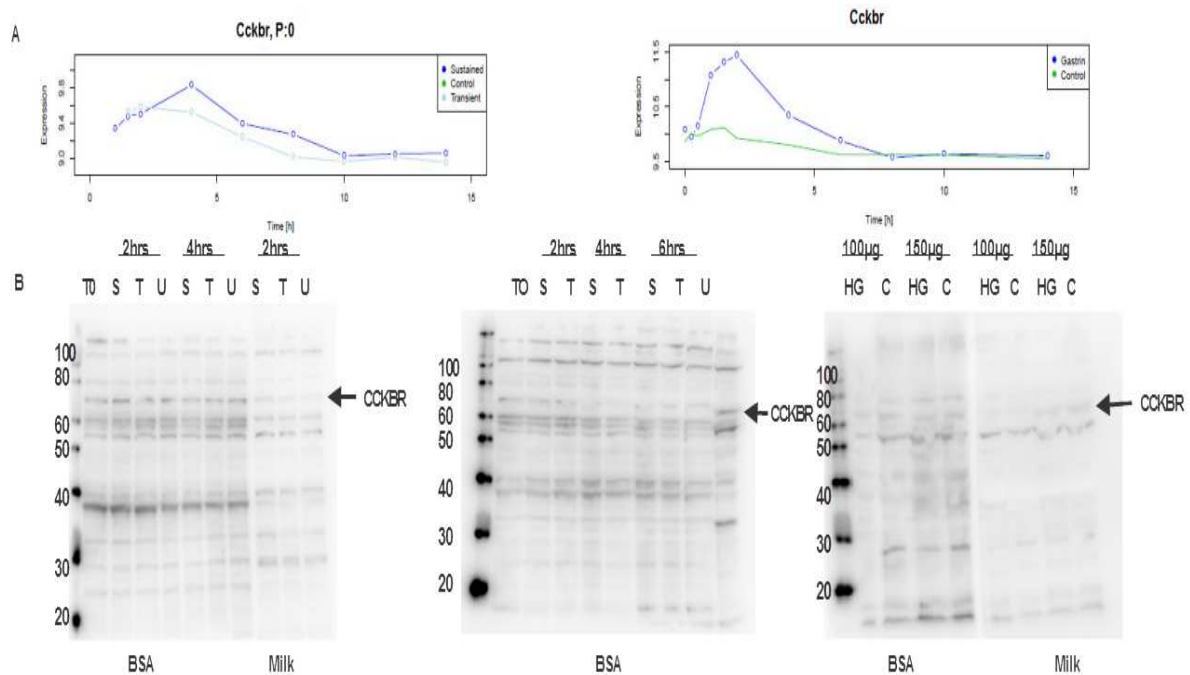


**Figure 22: Expression of Herpud1 in AR42J cells upon gastrin treatment.** **A) Left panel:** Temporal expression of Herpud1 mRNA in AR42J cells treated with gastrin in a sustained (S) *versus* transient (T) mode (From Exp. 3 in Table1). **Right panel:** Temporal expression of Herpud1 mRNA in sustained gastrin treated *versus* untreated cells (From Exp. 2 in Table 1). **B)** Detection and expression of Herpud1 protein upon sustained gastrin treatment. In the left panel (B), different concentration of protein lysates are measured at 4 and 6 hours with 5% BSA and 5% Milk as blocking solutions, respectively. Molecular weight of the Herpud1 is 44 kDa and lower panel (B) shows the graph of the quantified relative intensity compared to untreated cells. The middle panel (B) shows detection of Herpud1 at different time points using 5 µg protein lysate in 5% BSA. The right panel shows results from two other biological replicates (BRI and BRII) at 2 and 4 hours sustained gastrin treatment compared to untreated cells. **C)** Quantification relative to the untreated cells. Left panel(C) shows the quantification of the different concentration of proteins at 4 and 6 hours from biological replicate three (BRIII), middle panel(C) shows relative intensity to time point zero (T0) in sustained and untreated cells at the different time points; and the right panel(C) shows the relative intensity (mean +/- SD) of all the three biological replicates at 2 and 4 hours.

### 3.3.5 Gastrin receptor (CCKBR):

In the genome-wide time series experiments, we observe differences in the mRNA level of the gastrin receptor CCK2R in sustained gastrin treated *versus* transiently treated and control cells, especially at early time points (Figure 23, panel A). Based on these results, we wanted to examine if gastrin has an effect on the protein expression of its own receptor and performed western blot analysis on lysate from AR42J cells and tissues from hypergastrinemic rats. The band for the gastrin receptor on the western blot gel picture is found on 75 kDa but the bands were too weak to see a clear difference between sustained, transiently and untreated cells (Figure 23 B, left and middle panels). To examine the gastrin response on the

receptor in rat tissue, western blot analysis of hypergastrinemic and control rat oxyntic tissue were performed using different concentrations of protein lysate (Figure 23 B, right panel). Still we found the gastrin receptor band on 75kda, but the bands were too weak to detect a difference. From the above study, we can conclude that the gastrin receptor (CCK2R) is present in both cell and tissue lysate; but it is difficult to see any gastrin effect on the CCK2R protein expression.

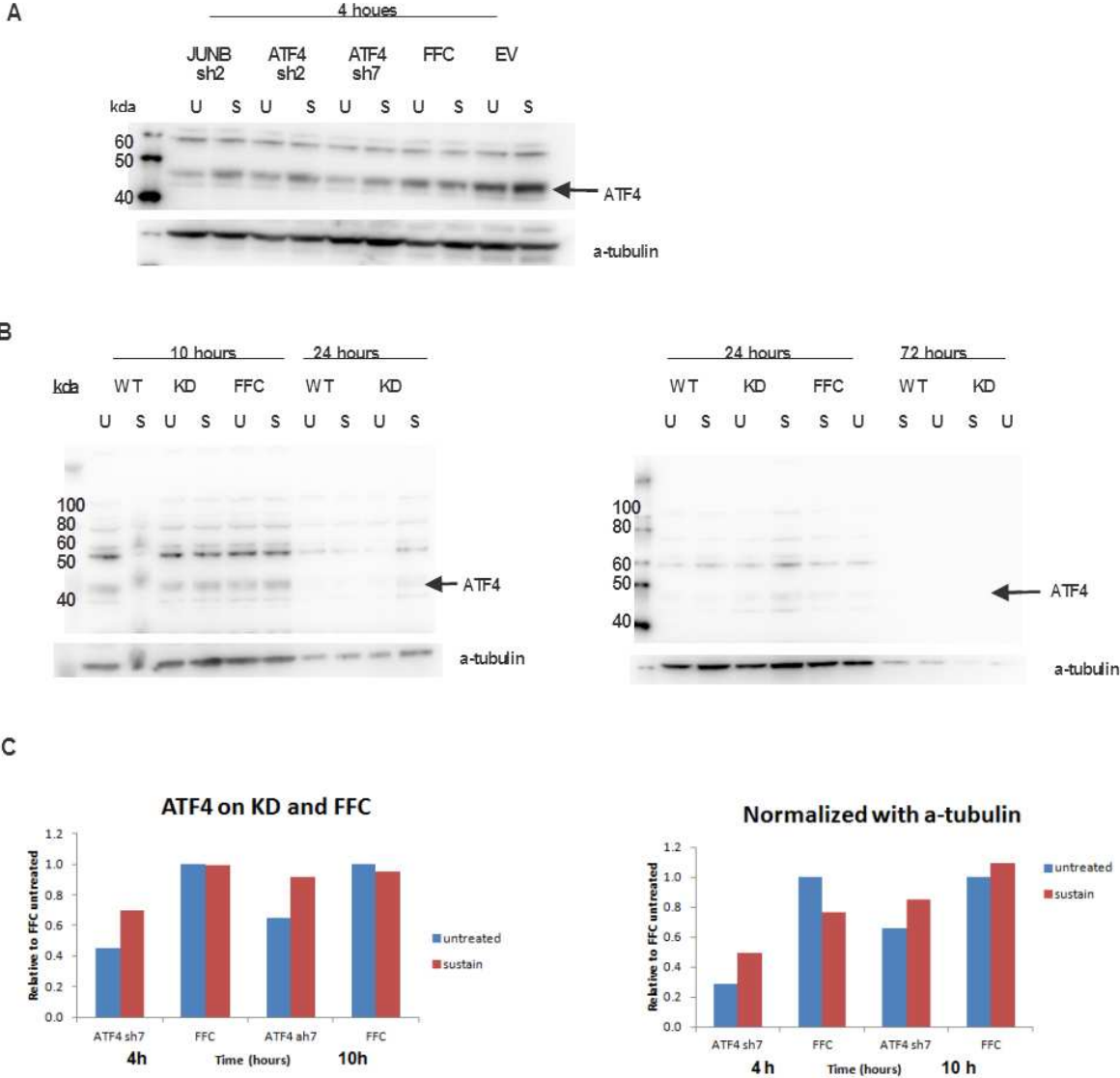


**Figure 23: Expression of gastrin receptor (CCKBR) in AR42J cells and in rat oxyntic mucosa upon elevated levels of gastrin. A) Left panel:** Temporal expression of CCKBR mRNA in AR42J cells treated with gastrin in a sustained (S) *versus* transient (T) mode (From Exp. 3 in Table1). **Right panel:** Temporal expression of CCKBR mRNA in sustained gastrin treated *versus* untreated cells (From Exp. 2 in Table 1). **B) Western blot analysis** of gastrin receptor protein in whole cell lysate at different time points in sustained (S) *versus* transiently (T) gastrin treated and untreated (U) AR42J cells. T0: time zero. Gastrin receptor's molecular weight is 75 kDa. The protocol is optimized by using 5% BSA and 5% Milk. The right most panel shows detection of the gastrin receptor in tissue lysate from hypergastrinemic (4 weeks) and control rats using different concentration of protein lysate and different blocking solutions.

### 3.4 Characterization of ATF4 knock down cells generated by retrovirus based RNAi

To investigate the impact of the ATF4-network in the gastrin biology, ATF4 knock-down cells were generated by retrovirus based RNAi (work performed by Linn Karina Selvik and Tonje Strømme Steigedal in 2012). In order to validate the knockdown effect we have performed western blot analysis at 4 hours together with Christina

Sæten Fjeldbo (Figure 24, panel A). To examine if the protein abundance of ATF4 changes at later time points, the ATF4 protein was detected at 10, 24 and 72 hours by Western blot (Figure 24, panel B).



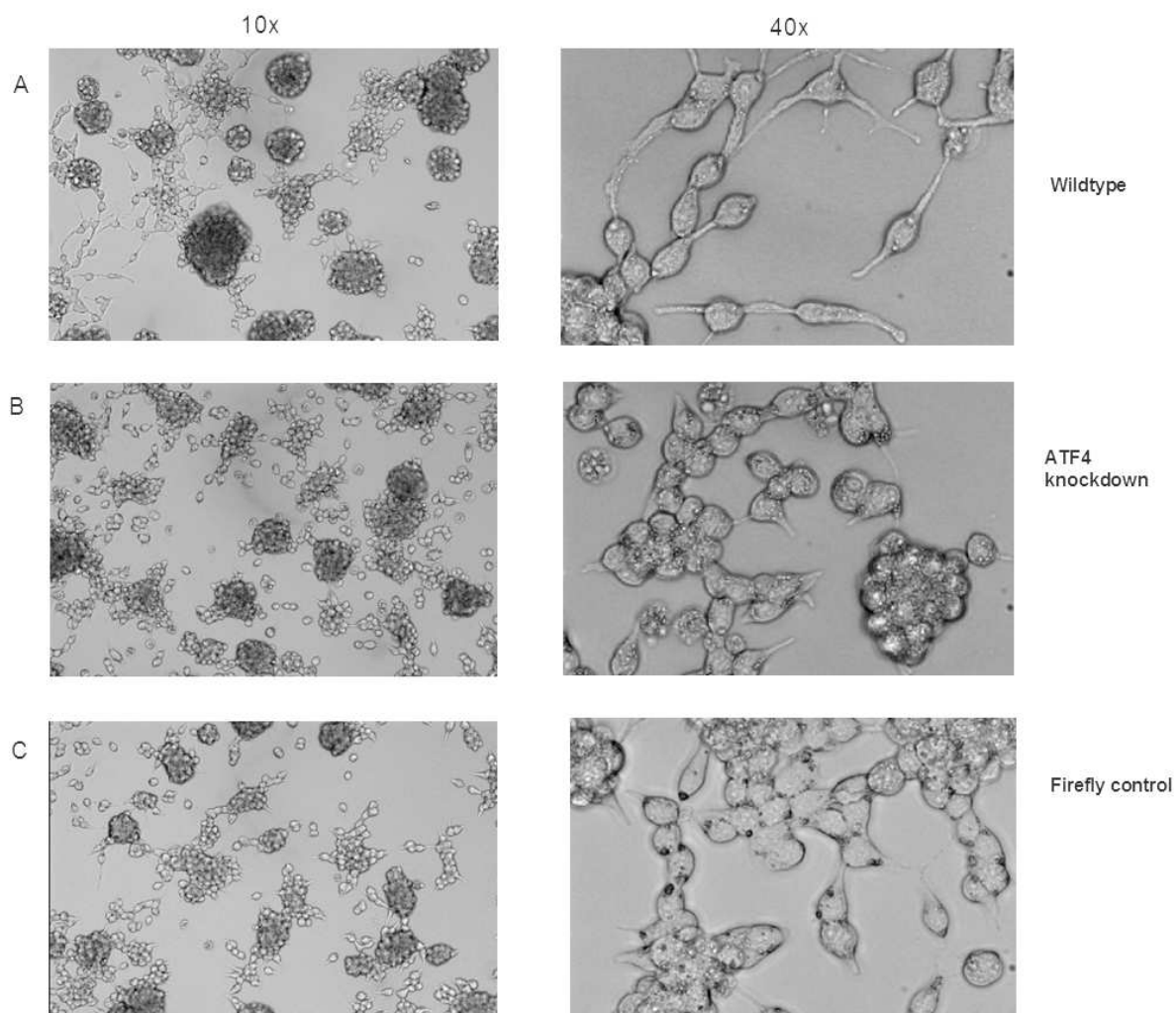
**Figure 24: Expression of ATF4 in wild type (WT), ATF4 knockdown (KD) and firefly control (FFC) cells with and without gastrin treatment. A)** Western blot image of ATF4 in knockdown and control (FF) cells together with JUNB sh2, ATF4 sh2 and EV (empty vector) at 4 hours of gastrin treatment and housekeeping protein ( $\alpha$ -tubulin) below. **B)** Western blot image of ATF4 in WT, KD and FFC cells at 10, 24 and 72 hours and image of housekeeping gene ( $\alpha$ -tubulin) below. **C) Left panel,** Quantified graph of relative intensity of ATF4 in ATF4 sh7 (KD) and FFC calculated in relation to the unstimulated Firefly control (FFC) at 4 and 10 hours. **Right panel,** graph shows relative intensities normalized with a-tubulin at 4 and 10 hours.

At 4 hours, the knock-down of ATF4 were calculated to 40-50% when normalized with  $\alpha$ -tubulin and 60% when normalized with  $\beta$ -actin. At 10 hours the knock-down ratio was 30% when normalized with  $\alpha$ -tubulin. The protein expression after 24 and 72 hours are too weak to calculate. Similar experiments were done at all-time points by using fluorescence in the western blot; and we found that protein expression was too weak to detect after 24 and 72 hours (data shown in the Supplementary Figure S2). Further optimization is needed for the fluorescence western blotting protocol. We concluded that the level of ATF4 protein was lower in the KD cells compared to wild type cells and FF control cells. This cell line can therefore be used as model system to investigate how reduced levels of ATF4 proteins may influence gastrin mediated cellular responses.

- **Cellular morphology of AR42J wt/control cells and ATF4 KD cells:**

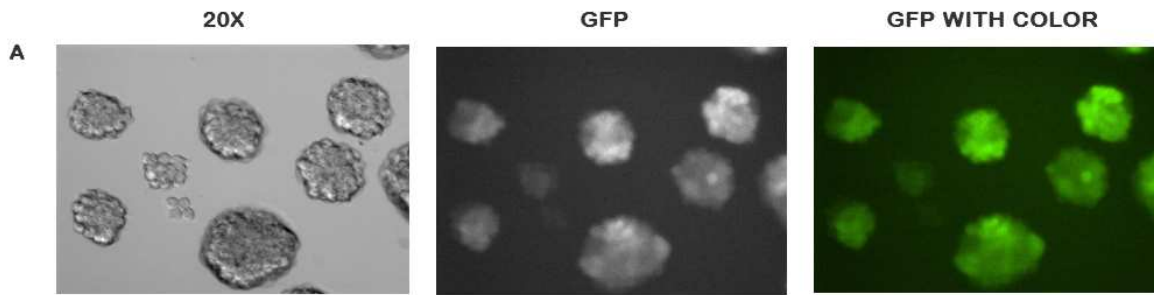
In order to examine whether reduced levels of ATF4 had an impact on cellular growth and morphology, we seeded AR42J wt cells and ATF4 KD cells into 75 cm<sup>2</sup> cell culture flasks and incubate them in the same condition. Both wt and ATF4 KD cells were observed to grow in cluster, but we observed that the ATF4 KD cells (Figure 25, panel B) appeared to pile up somewhat more than wt cells (panel A) and Firefly control (FFC) cells (panel C). However, overall we could not observe any drastically differences in cell growth and morphology between transfected and untransfected cells.





**Figure 25: Morphological overview of the AR42J wild type cells, ATF4 knockdown and Firefly control cells.** **A:** growth of the wild type of cells (x10 and x40). **B:** Growth of ATF4 knockdown cells. **C:** Growth of Firefly control cell. Magnifications: x10 (left panels) and x40 (right panels). The cells were grown in 75 cm<sup>2</sup> cell culture flasks.

The retrovirus transfer vectors used for transfection of shRNAs designed to target ATF4 and the Firefly control included also DNA encoding GFP (green fluorescent protein) as marker for transfection. Thus, cells that are successfully transfected will express GFP and can be detected in the fluorescent microscope, as shown in Figure 26. The expressions of GFP appeared strong in some clusters of cells and weaker or diffuse in some single cells. However, as discussed in 3.6 and chapter 4, the expression of GFP is overall so strong that it will be problematic to use a green secondary antibody for ICC in these cells.



**Figure 26: Morphology of ATF4 sh7 knockdown cells.** The left panel shows the ATF4 sh7 cells under x20 magnification light microscopy, middle panel: detection of green fluorescent protein (GFP) in ATF4 sh7 cells and right panel shows ATF4 sh7 knockdown cells with green color indicating that GFP is expressed.

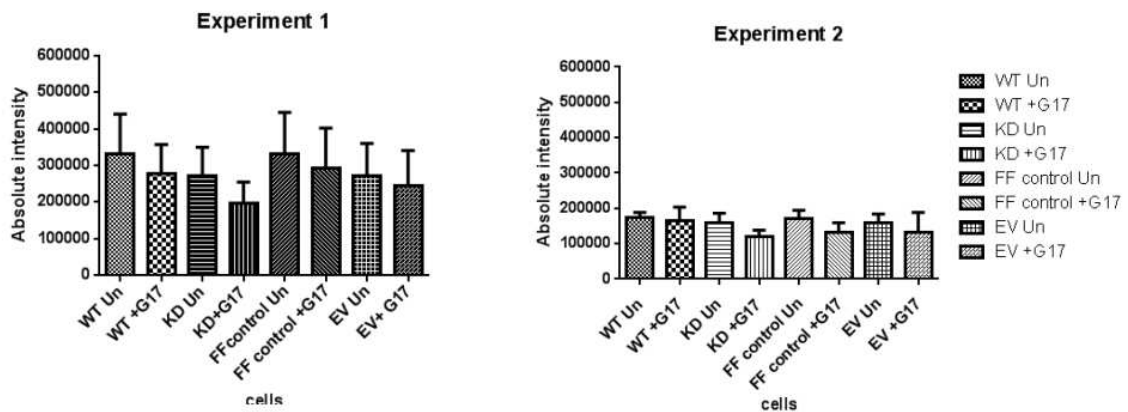
### 3.5 Impact of ATF4 in gastrin mediated survival response:

- **The gastrin induced anti-apoptotic effect is not abolished by reduced ATF4 protein level**

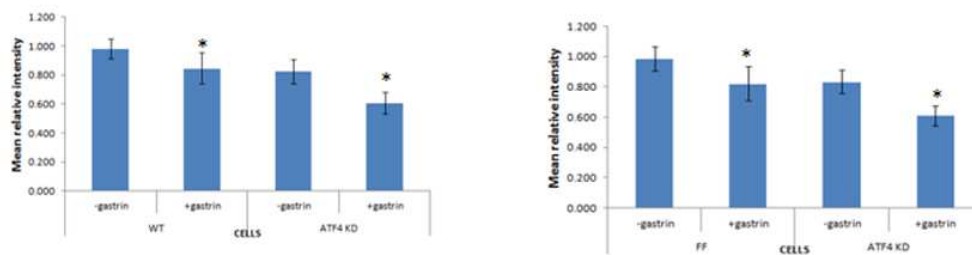
Gastrin has been shown to have an anti-apoptotic effect in AR42J cells which is dependent on sustained gastrin treatment [41, 60]. In order to observe if knocking down ATF4 reduces gastrin-mediated transcriptional activation of the genes in the ER stress and survival pathways, we performed caspase assay by growing the cells (WT, ATF4 KD, Firefly control and Empty vector cells) for 24 hours and then serum starve them for 72 hours with and without gastrin treatment. After 72 hours the caspase activity was measured by adding caspase-Glo 3/7 assay solution. In accordance with previous findings [41, 60], the caspase activity was lower in gastrin treated cells than in untreated cells (Figure 27, panel A).

Although we observed some technical and biological variations, the anti-apoptotic effect of gastrin was also observed in ATF4 KD cells as well as in the control cell lines FFC and EV (Figure 27A). When combining the results from the two biological replicates, we observed that the basal levels of caspase activities were slightly lower in the ATF4 knockdown cells than in the WT cells (Figure 27 B). Significant differences were observed in WT *versus* ATF4 knockdown cells and FF *versus* ATF4 knockdown cells, both with and without gastrin treatment. In conclusion, gastrin has anti-apoptotic effect both in wild type and ATF4 knockdown cells and slightly less caspase activity is detected in untreated ATF4 knockdown cells. This might be interesting to explore further.

A



B



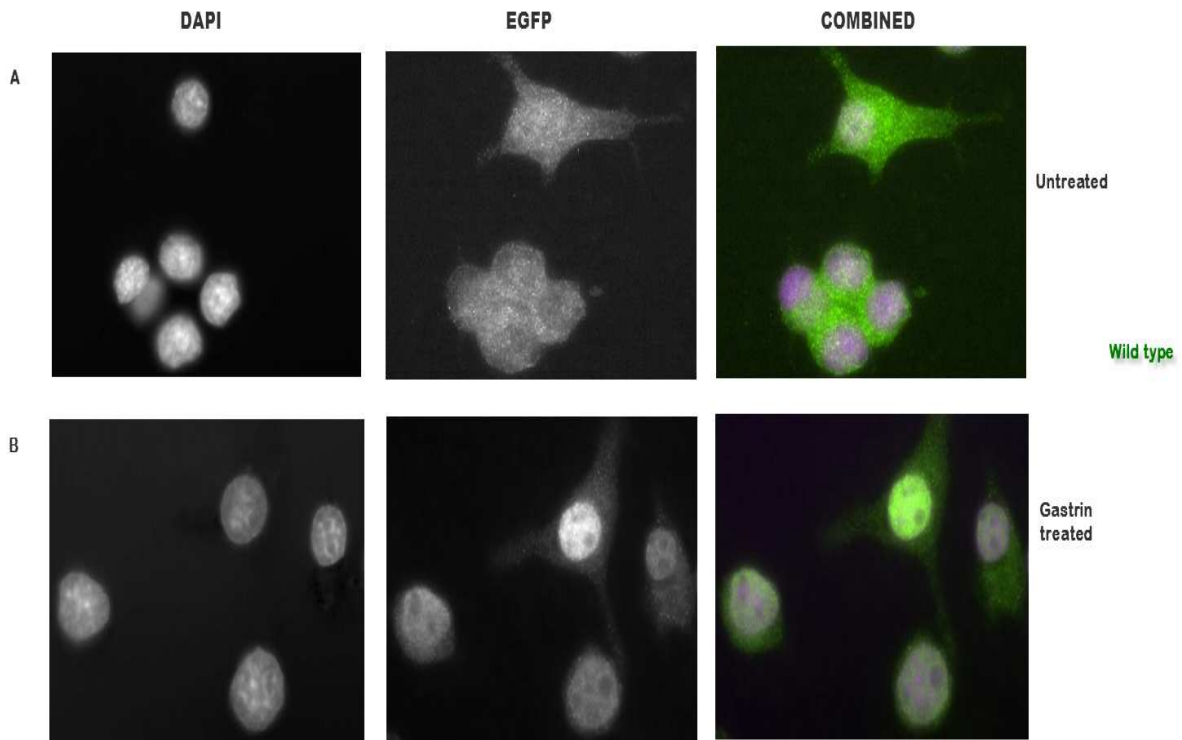
**Figure 27: Survival responses in wild type (WT), ATF4 knockdown (KD), firefly control (FFC) and empty vector (EV) cells upon gastrin treatment.** Apoptosis was induced in untreated and sustain gastrin treated cells by 72 hours of serum starvation and measured using Caspase Glo 3/7 assay. **A)** The statistical analysis (mean +/- SD) of the absolute intensity of two independent experiments (6 technical replicates in each independent experiment) is plotted in the graph. Error bars represents 95% CI. **B)** Relative intensity of the mean of the two independent experiments is statistically analyzed (mean +/- SD). Left panel: wild type AR42J cells *versus* ATF4 knockdown cells are shown in the graph. Right panel: Firefly control (FFC) cells *versus* ATF4 knockdown cells.\* P value is significant (P<0.05).

### 3.6 Cellular localization of proteins in the ATF4 network:

To perceive the localization and the expression of proteins in the ATF4 network in both wild type AR42J and ATF4 knockdown cells and their effect upon gastrin stimulation, we performed ICC and immunofluorescence staining using green fluorescence Alexa 488 as secondary antibody. Unfortunately, since the ATF4 KD cells express GFP proteins, the ICC protocol used in the present thesis is not optimal for transfected cells and we could therefore not conclude whether the proteins in the ATF4 network are differently expressed in wt *versus* ATF4 KD cells. The results from wild type AR42J cells are described below. The results from ATF4 KD cells are shown in the Supplementary Figures S3-S7.

### 3.6.1 ATF4:

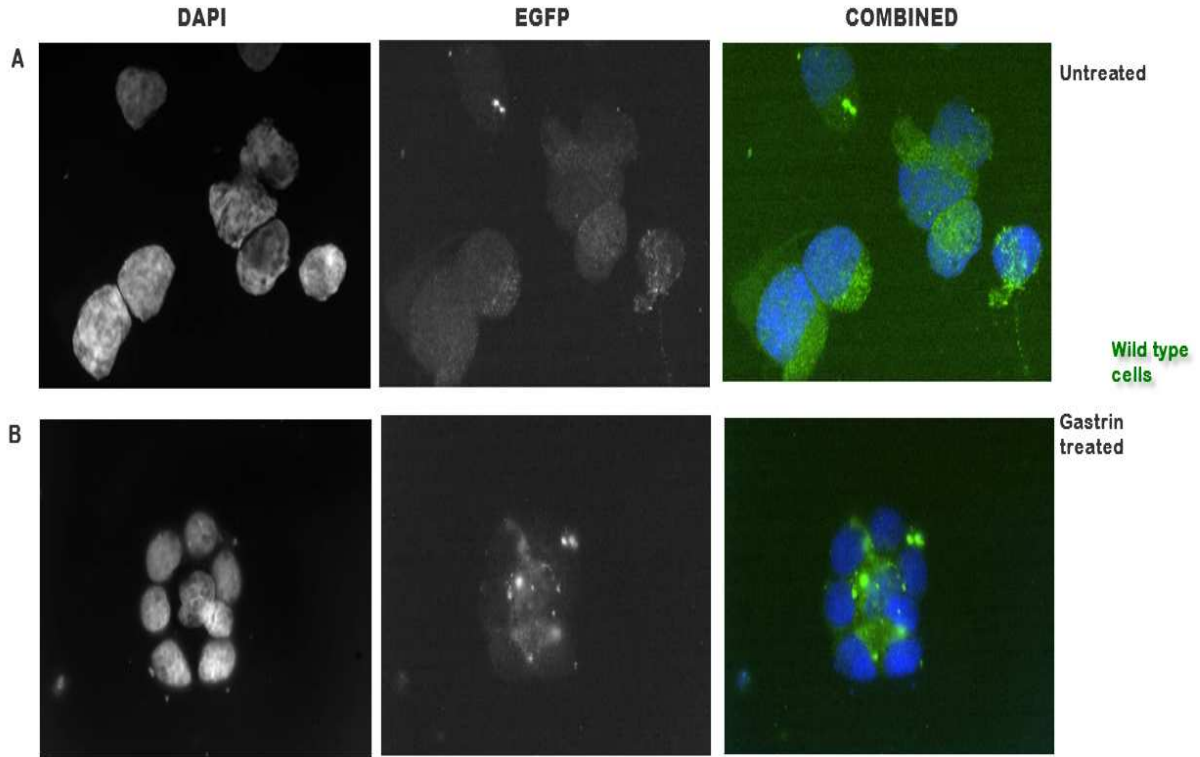
We first examined the expression of ATF4 protein using primary antibody from Santa Cruz. ATF4 protein was detected in WT cells and seemed to be expressed both in the nucleus and cytoplasm (Figure 28). In some cells treated with gastrin there appeared to be stronger staining in the nucleus than in untreated cells.



**Figure 28: Cellular localization of ATF4 protein in wild type cells with and without gastrin treatment.** Pictures with DAPI stained nuclei to the left, EGFP fluorescence in the middle, and combined DAPI and EGFP fluorescence to the right. **A-B:** Expression of ATF4 (Sc-20) in untreated wild type **(A)** and gastrin treated **(B)** cells respectively, shown at magnification  $\times 60$ .

### 3.6.2 GRP78:

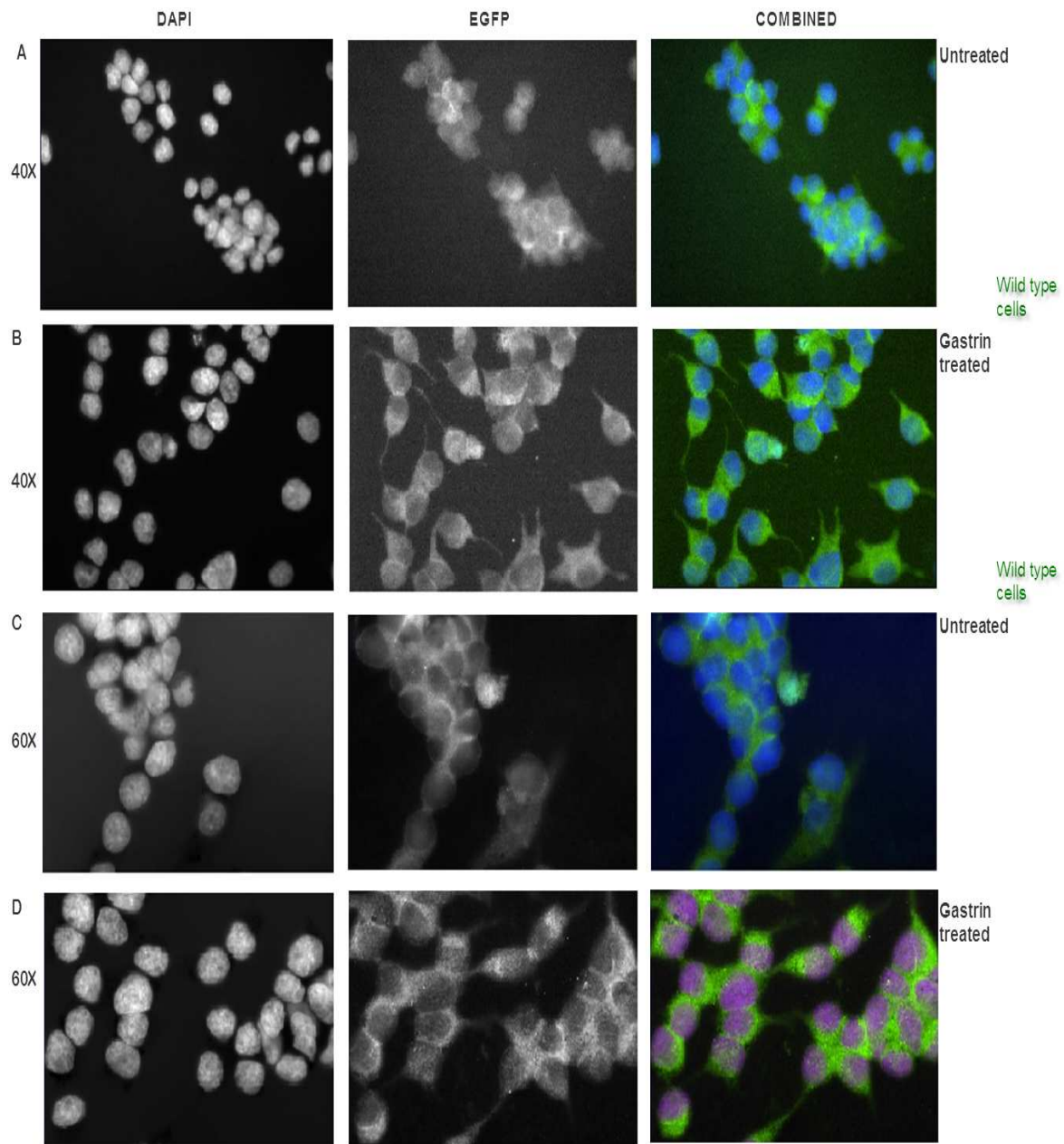
We then examined if we could detect the major ER stress regulator, GRP78 [17], in AR42J cells by ICC. As illustrated in Figure 29 panel A & B, there was a detectable expression of GRP78 on wild type of cells in both untreated and gastrin treated cells. The protein was observed in the cytoplasm close to the nucleus, which indicates that the protein is expressed in ER. There appeared to be enhanced expression of GRP78 around the nucleus in gastrin treated cells compared to untreated cells, but this needs to be explored further.



**Figure 29: Cellular localization of GRP78 (Bip) protein in wild type cells with and without gastrin treatment.** Pictures with DAPI stained nuclei to the left, EGFP fluorescence in the middle, and combined DAPI and EGFP fluorescence to the right. Expression of GRP78 in **A)** untreated wild type cells, **B)** gastrin treated wild type cells, shown at magnification x60.

### 3.6.3 pPERK:

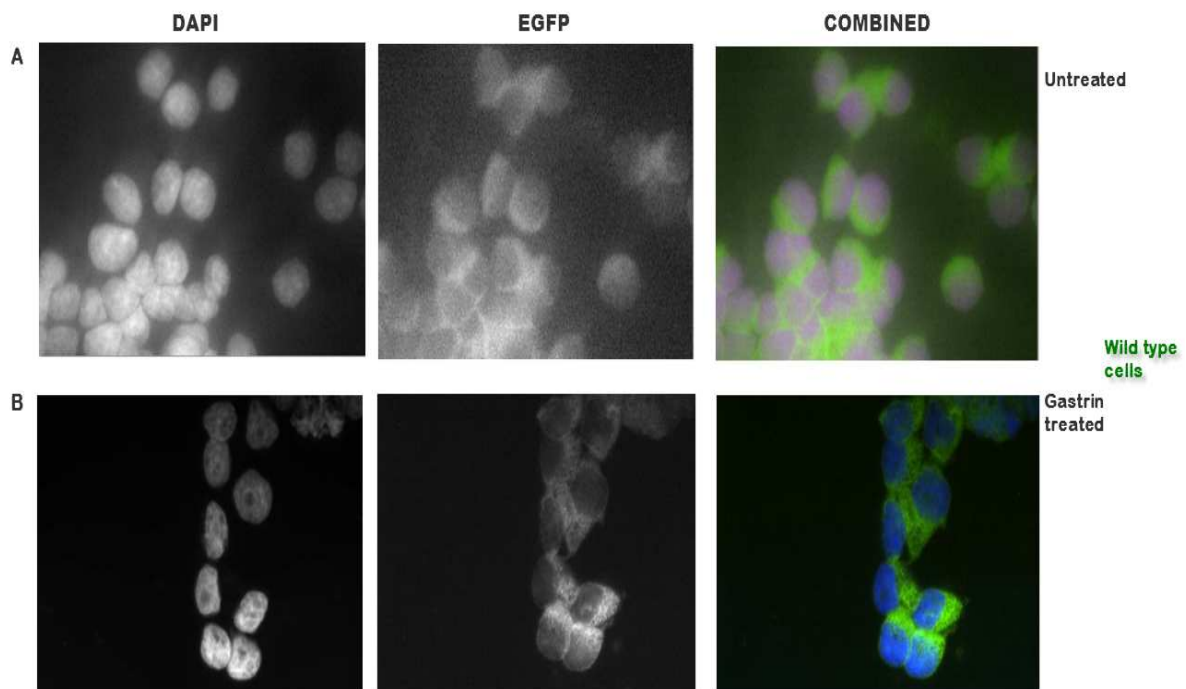
As illustrated in Figure 4, when unfolded proteins accumulate in the endoplasmic reticulum (ER), they are bound by the ER chaperone GRP78, and e.g., PERK will become free and phosphorylated and activate downstream signal transduction [37]. We therefore examined if we could detect phosphorylated PERK in gastrin treated and untreated cells by ICC. Also phosphorylated PERK (pPERK) was expressed in the cytoplasm, close to the nucleus, indicating that the phosphorylated kinase is localized in ER (Figure 30). We could not detect any clear difference in pPERK expression levels in gastrin treated *versus* untreated cells, but we observed that gastrin treated cells shows cytoplasmic protrusions (Figure 30, panels B, D ), and seems to have some stronger green spots close to the nucleus.



**Figure 30: Cellular localization and expression of pPERK in wild type cells with and without gastrin treatment.** Pictures with DAPI stained nuclei to the left, EGFP fluorescence in the middle, and combined DAPI and EGFP fluorescence to the right. **A-B:** Expression of pPERK (x40) in untreated wild type (**A**) and gastrin treated wild type cells (**B**); **C-D:** Expression of pPERK (x60) in untreated (**C**) and gastrin treated (**D**) wild-type cells respectively.

### 3.6.4 Herpud1:

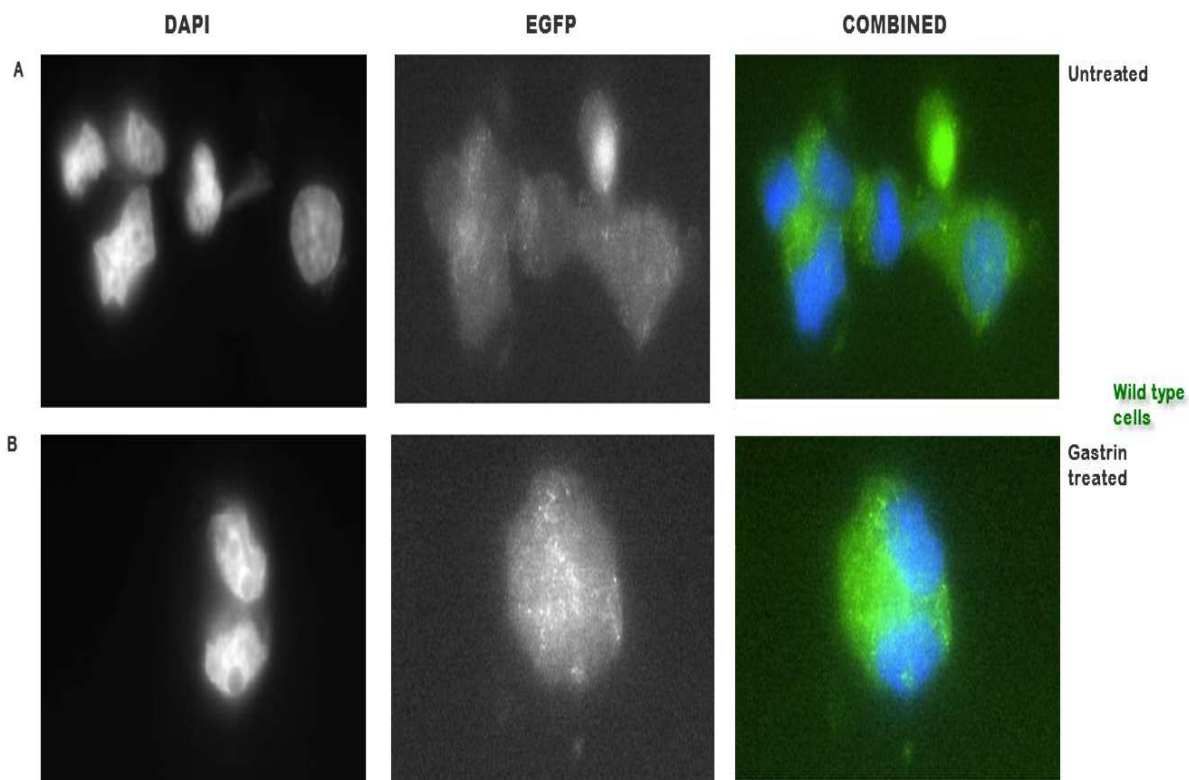
In AR42J cells both Herpud1 mRNA and protein are highly expressed (Figure 22); and therefore it was of interest to study the cellular expression and localization of Herpud1 in WT and ATF4 KD cells (supplimentary Figure S6). From the pictures in the Figure 31, panel A and B, Herpud1 seems to be mostly expressed in cytoplasm in WT cells with slightly higher expression have been observed upon gastrin stimulation.



**Figure 31: Cellular localization of Herpud1 in WT cells upon gastrin stimulation.** Pictures with DAPI stained nuclei to the left, EGFP fluorescence in the middle, and the combined DAPI and EGFP fluorescence to the right. Expression of Herpud1 protein in **A)** untreated WT cells, **B)** gastrin treated WT cells, shown at magnification  $\times 60$ .

### 3.6.5 CHOP:

CHOP is the another target gene in ATF4 network [61]. In western blot the expression of CHOP was very weak (Figure 21) and we can't see any gastrin effect on AR42J cells. ICC analysis indicated that the CHOP protein is expressed around the nucleus in both gastrin treated and untreated cells. So, ICC analysis confirmed that AR42J cells express CHOP.

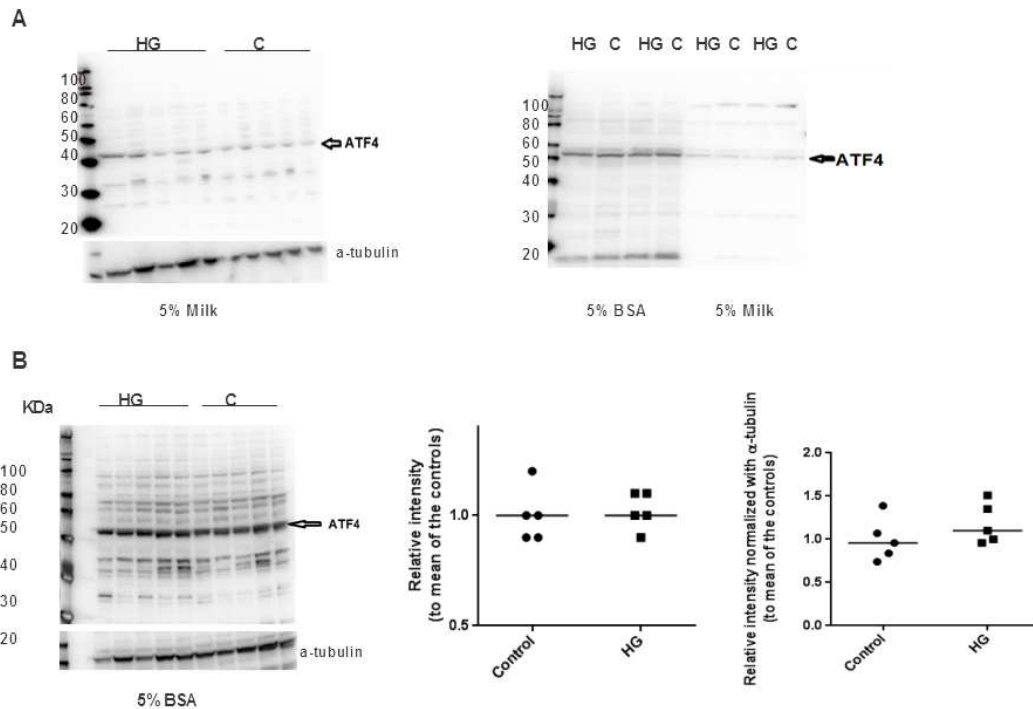


**Figure 32: Cellular localization of CHOP in wild type cells under gastrin stimulation.** Pictures with DAPI stained nuclei to the left, EGFP fluorescence in the middle, and combined DAPI and EGFP fluorescence to the right. Expression of CHOP in **A)** untreated wild type cells, **B)** gastrin treated wild type cells, shown at magnification  $\times 60$ .

### 3.7 The ATF4 protein levels in oxyntic mucosa of hypergastrinemic rats

In order to study the levels of ATF4 protein expression *in vivo* and the effect of hypergastrinemia, western blot analysis on tissue lysates of oxyntic mucosa from control rats and hypergastrinemic (HG) rats were performed. The two different ATF4 antibodies tested showed protein bands at 48 kDa when using the antibody from Protein Tech (Figure 33A, left panel) but above 50 kDa when using the antibody from Santa Cruz (Figure 33A, right panel). In any case, no clear differences between the hypergastrinemic and control tissues were found (Figure 33B).

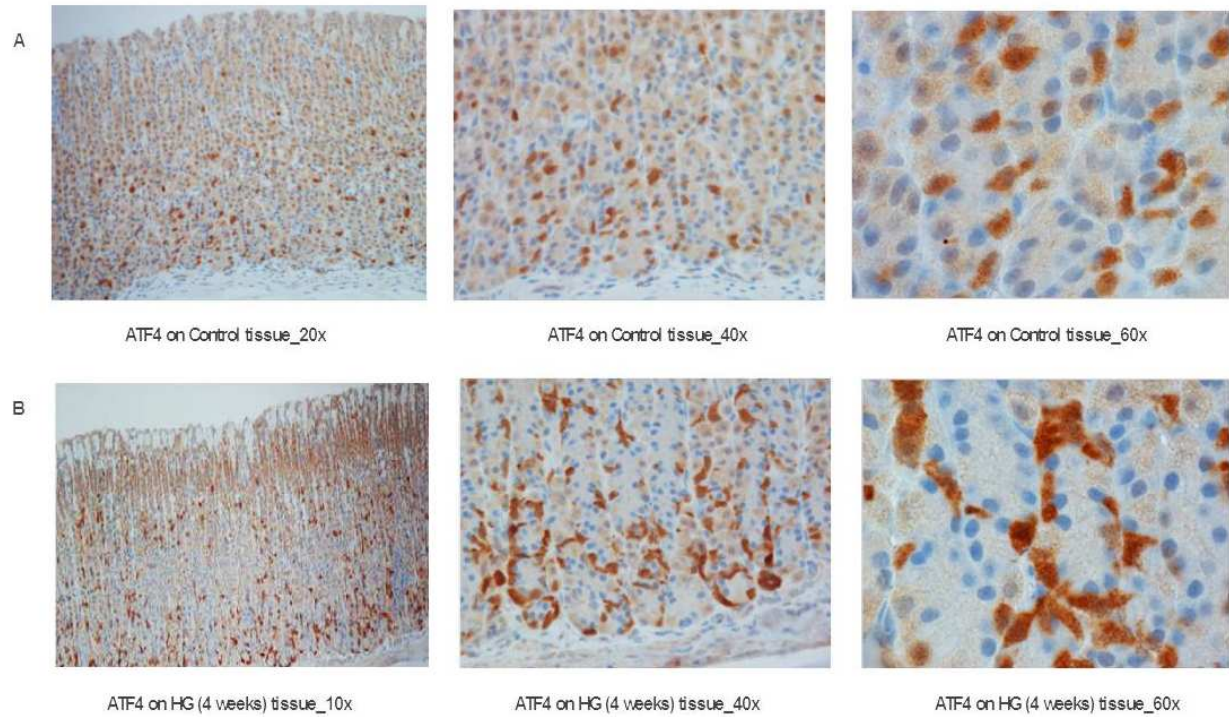




**Figure 33: Expression of ATF4 in oxyntic mucosa of control and hypergastrinemic (HG) rats by western blot.** **A) Left panel:** Western blot image of ATF4 (Protein tech.) at 48 kDa and the housekeeping protein ( $\alpha$ -tubulin) for normalization below. **Right panel:** Western blot image of ATF4 (Santa Cruz) above 50 kDa. The protocols were optimized using both BSA and milk for blocking and antibody diluent. **B) Left panel:** Western blot image of ATF4 (Protein tech.) at 48 kDa using BSA as blocking and antibody diluent, and the housekeeping protein ( $\alpha$ -tubulin) for normalization below. **Right panels:** relative intensity of ATF4 in hypergastrinemia compared to the mean intensity of the controls without and with normalization with the housekeeping protein ( $\alpha$ -tubulin).

We further wanted to examine the cellular localization of ATF4 in control *versus* hypergastrinemic oxyntic mucosa by doing immunohistochemistry. ATF4 protein was expressed strongly in scattered single cells predominantly in the basal half of the control mucosa, increasing in number and distribution in hypergastrinemic mucosa and also forming linear hyperplasia in some areas (Figure 34). The appearance, number and localization of these cells are indicative of neuroendocrine cells. The changes induced by hypergastrinemia, further indicate that these cells are ECL cells, which are the dominant neuroendocrine cell type in rat oxyntic mucosa and known to react with hyperplasia due to hypergastrinemia [1]. In addition, there was a weaker ATF4 expression in other epithelial cells, which did not seem to change by hypergastrinemia. In the neuroendocrine cells, ATF4 was detected in both cytoplasm and nucleus when using the Protein tech antibody. The antibody from Santa Cruz showed a similar expression of ATF4, but the intracellular localization in the

neuroendocrine cells with this antibody was detected only in cytoplasm (data not shown).



**Figure 34: Expression and localization of ATF4 in oxyntic mucosa of control and hypergastrinemic (HG) rats by immunohistochemistry.** Localization and expression of ATF4 (Protein Tech) in **A**) normal rat oxyntic mucosa and **B**) hypergastrinemic rat oxyntic mucosa, shown at magnifications  $\times 100$ ,  $\times 400$  and  $\times 1000$ .

## 4. DISCUSSION

Insight into molecular mechanisms of gastrin mediated regulation of gene expression can contribute to a better understanding of the importance of gastrin in normal as well as in tumor biology. In the present study, temporal protein expression of ATF4 in AR42J cells and their cellular localization in wild type AR42J cells (and ATF4 knockdown cells) regulated by gastrin were detected. The survival response upon gastrin treatment in both wild-type AR42J cells and ATF4 KD AR42J cells were examined. Finally, the expression of ATF4 proteins in normal and hypergastrinemic rats were studied. Establishment and optimization of western blot analysis has been a large part of this master's thesis; as briefly described below.

In the present work, the pancreatic adenocarcinoma cell line AR42J was used as the main model system. AR42J cells are derived from azaserine induced hyperplastic nodules from a male rat pancreas [62]. This cell line maintains similar characteristics as normal pancreatic acinar cells, such as calcium ( $Ca^{2+}$ ) signaling, the synthesis and secretion of digestive enzymes, protein expression, growth and proliferation. This is the only cell line now available with all these characteristics [54]. In AR42J cells, there are three types of secretions: 1) the basal secretion of digestive enzymes, also called 'constitutive' secretion, is released directly after synthesis and does not depend on secretagogues. The secretion is much higher compared to normal rat pancreatic acini; 2) the regulated secretory pathway of digestive enzymes, that have slow-exit kinetics emerges from secretory granules and reflects the sensitivity to secretagogues and have significant two fold increase in amylase from AR42J cells, when treated with dexamethasone; and 3) the secretion of amino acid and neurotransmitters [62]. If the AR42J cells are treated with dexamethasone (Dx), they differentiate to acinar cells. Treatment of activin (Act) and bethacellulin (BTC), a member of the epidermal growth factor family (EGF), induce differentiation into insulin producing cells [63]. AR42J cells also differentiate into  $\beta$  cells upon exposure to hepatocyte growth factor (HGF) [63].

Besides the exocrine properties, this cell line also displays some neuroendocrine (NE) properties. Like the expression of typical NE vesicle protein synaptophysin (Syn), the synaptic vesicle protein type 2 (SV2), voltage-activated ionic channels, as well as the neurotransmitters GABA, glutamate and glycine. They also express some transcription factors that typically found in NE cells [55]. In this way, these cells

provide an excellent in vitro model system to study both exocrine and endocrine properties [63] [62]. In my study, I have conducted my experiments in AR42J cell line as gastrin receptors are expressed endogenously in AR42J cells and also used in the experiments before as a model system to study the effects of gastrin in proliferation [14], differentiation [55] and apoptosis [16, 49, 64, 65].

Transiently increased gastrin levels have important physiological functions in the gastrointestinal and sustained high gastrin levels (hypergastrinemia) are related to the pathological process [5, 47, 48]. Therefore, it is of interest to examine how duration of gastrin treatment affects gene expression and molecular responses. We conducted present study to explore how adenocarcinoma cell respond to transient *versus* sustained gastrin signalling and to identify characteristic differences between protein levels and downstream biological responses. ATF4 protein was found to be up-regulated after 1.5 hours of gastrin treatment in sustained gastrin treated cells and peak up-regulation were found at 2,4,6 and 8 hours and remain increased upto 12 hours of gastrin treatment compared with transiently gastrin treated and untreated cells (Figure 17). At 14 and 24 hours, decreasing trends are seen in sustained gastrin treated cells (in the supplementary Figure S1).

Several of the network proteins in PERK-eIF2 $\alpha$ -ATF4 pathways were demonstrated by western blot analysis. Our results suggested that the proteins GRP78, PGCN2, pPERK, pEIF2 $\alpha$ , Herpud1 and CHOP were expressed in AR42J cells. But increased expression and phosphorylation upon gastrin stimulation was only seen for eIF2 $\alpha$  in the AR42J cell line. In order to examine if gastrin has an effect in its own receptor (CCKBR) we detected the gastrin receptor both in AR42J cells and hypergastrinemic rat tissue (Figure 23), but fail to observe any clear gastrin effects.

As western blot is a semi-quantitative method that needs protein specific optimization, we optimized western blot analysis for ATF4 network proteins and target genes as well as gastrin receptor and their effect after gastrin stimulation. The running times for gel electrophoresis were optimized for each protein, as shown in Table 3; and also the blotting time to transfer the proteins into the membrane was optimized (shown in the Table 3). For high molecular weight proteins like PERK (140 kDa), pPERK (170 kDa) and PGCN2 (181 kDa) we have difficulties in the running gel electrophoresis. We experienced that running the gel electrophoresis as well as blotting for 2 hours on ice we obtained a good assay. Optimizations of western blot

analysis were also done using different blocking agents; either 5% milk in TBST or 5% BSA (bovine serum albumin) in TBST. We demonstrated that by using milk we can obtain less unspecific bands and used this as an approach to find the correct band for ATF4, CHOP, Herpud1 and CCK2R proteins. But for the detection of phosphorylated proteins, we cannot use milk as milk solution contain casein which is itself a phospho-protein and biotin that will interfere with the assay results. We experienced a lot of difficulties when we used housekeeping genes as a loading control in western blot. We optimized the protocols by using different housekeeping gene in different concentrations both in manual method and using SNAP id. Protein detection system. We found out that if we use less antibody concentrations in case of Housekeeping genes, we obtained better result as shown in Table 6.

When designing the experiments for identification of possible ATF4 interacting proteins, one of the points that had to be taken under consideration was: Where are the proteins expected to interact, in the nucleus or in the cytosol? We performed immunocytochemical (ICC) analysis using fluorescence. In the ICC analysis we have stimulated the cells with gastrin for 6 hours for the early expressed genes like GRP78, pPERK, ATF4, pEIF2 $\alpha$ , and 20 hours of gastrin treatment for late response genes like Herpud1 and CHOP. Unstimulated cells were analyzed as controls. The expression of ATF4 was found in both nucleus and cytoplasm in both gastrin treated and untreated cells and expression. We detected expression of the proteins of ATF4 network and their locations. They are as follows; GRP78 and pPERK in the cytoplasm close to the nucleus; which indicates location in ER, Herpud1 in the cytoplasm and CHOP around the nucleus. The proteins were in the locations where they were expected to be and they didn't seem to have any gastrin effects.

Our aim was also to compare different protein expression upon gastrin stimulation in wild type AR42J cells and ATF4 knockdown cells. However, unfortunately we forgot that the ATF4 KD cells express GFP and used a green fluorescent labeled secondary antibody. Thus these analyses have to be repeated with another secondary antibody.

Depending upon the duration and degree of ER stress, the UPR can provide either survival response by activating adaptive and anti-apoptotic pathways or death signals by inducing cell death programs [17]. As mentioned in 1.3, ATF4 is a stress responsive gene [28, 30] and an important mediator of the UPR [28]. In diverse micro environmental conditions ATF4 expression promotes cell survival and adaptive

responses by transcriptional upregulation of genes involved in redox homeostasis, autophagy and angiogenesis [33]. ATF4 can act in the balance between survival and apoptosis. When ATF4 gives a inhibitory signals to the prosurvival transcriptional activators like cAMP response element binding proteins activates the cell death program [29]. In many cellular stresses like arsenite, hypoxia and amino acid deprivation, ATF4 upregulates CHOP which is a transcriptional factor related to proapoptotic effects depending upon expression of CHOP [31]. Downstream mediator Herpud1 is related to survival response [33] [43]. Our results showed that sustained gastrin treatment was required for increased expression of important regulators of UPR, the transcription factor ATF4, eIF2 $\alpha$  and probably the downstream mediator Herpud1.

As gastrin has an anti-apoptotic effects [16, 49, 64, 65], it was of interest to look at the relation to apoptosis in wild type AR42J cells and ATF4 knockdown cells in sustained gastrin treated *versus* untreated mode. To examine the duration of gastrin signaling on cell survival, we induced apoptosis in AR42J cells, ATF4 knockdown, Firefly control and Empty vector cells by serum starvation for 72 hours and measured caspase activity in the cells with sustained gastrin treatment and untreated cells. We found slightly decreased caspase activity in ATF4 knockdown cells compared to wild type AR42J cells, Firefly control and Empty vector cells (Figure 27). Also observed significant difference in WT *versus* ATF4 knockdown cells and FF *versus* ATF4 knockdown cells in both with and without gastrin treatment. This result indicates anti-apoptotic action of gastrin both in wild type and ATF4 knockdown cells and slightly less caspase activity in untreated ATF4 knockdown cells.

To validate the ATF4 expression in an *in vivo* model, we examined whether the level of ATF4 expression was increased in oxyntic mucosa of hypergastrinemic rats compared with control rats. We performed western blot analysis and optimized the protocol. But it was difficult to draw any conclusion whether there is any difference between hypergastrinemic and control rats. To further investigate the possible biological function of ATF4 in the oxyntic mucosa, immunohistochemistry was performed to examine the localization of ATF4 protein. In mucosa of control rats, strong ATF4 expression was found in scattered single cells in the basal half of the gastric glands. Increase in number and distribution and linear hyperplasia were seen in hypergastrinemic mucosa, which suggest that these cells are neuroendocrine cells,

and particularly ECL cells. Although it seemed like there was an increased amount of ATF4 proteins in hypergastrinemic mucosa by looking at the IHC results, we could not find a significant increase in proteins by western blot even if the gel image could give that impression. This could be explained by the general hypertrophy of the mucosa due to gastrin stimulation and only a small relative increase of the ATF4 protein. The intracellular localization of ATF4 was detected in the neuroendocrine cells in both cytoplasm and nucleus when using the Protein Tech antibody (Figure 34) but only in the cytoplasm when using the antibody from Santa Cruz (not shown, done by other members of the research group). These differences in cellular localization by IHC, and also in detected bands on western blots, could be interesting to explore further.

In conclusion, the protein level of ATF4 was shown up-regulated by gastrin. In addition, ATF4 was found in the cytoplasm and nucleus in both stimulated and unstimulated AR42J cells and rat tissue, indicating presence of ATF4 protein. Looking at the serum-starved cells, we found that the level of caspase activity was lower in gastrin stimulated cells compared to unstimulated cells in both WT and ATF4 KD cells. Interestingly, slightly less caspase activity was detected in untreated ATF4 knockdown cells; suggesting that ATF4 plays a role in the gastrin-induced circumvention of serum starvation induced apoptosis. This might be interesting to explore further. Our results also documented that sustained gastrin treatment has a role in phosphorylation of eIF2 $\alpha$  and up-regulated upon various duration of gastrin treatment. Even though the upstream and downstream genes of ATF4 seem to have no impact upon gastrin stimulation, we found out that phosphorylation of eIF2 $\alpha$  plays an important role in PERK-ATF4 pathway. The role of the eIF2 $\alpha$  in this pathway may be explored further.

**Future work:**

- The protocol for the western blot using fluorescence should be optimized further.
- Further optimization of western blot protocol to detect the downstream target genes of ATF4.
- Optimization of Immunocytochemical analysis of the ATF4 related proteins.
- The role of ATF4 in gastrin induced anti-apoptotic effect can be explored further.
- ATF4 network proteins can be detected in *vivo*, especially the expression in ECL cells and the possible involvement in ECL-hyperplasia.



## 5. REFERENCES

1. Jain, R.N. and L.C. Samuelson, *Differentiation of the Gastric Mucosa II. Role of gastrin in gastric epithelial cell proliferation and maturation*. American Journal of Physiology - Gastrointestinal and Liver Physiology, 2006. **291**(5): p. G762-G765.
2. Modlin, I., et al., *The pivotal role of John S. Edkins in the discovery of gastrin*. World Journal of Surgery, 1997. **21**(2): p. 226-234.
3. Dockray, G.J., et al., *THE GASTRINS: Their Production and Biological Activities*. Annual Review of Physiology, 2001. **63**(1): p. 119-139.
4. Schubert, M.L. and D.A. Peura, *Control of Gastric Acid Secretion in Health and Disease*. Gastroenterology, 2008. **134**(7): p. 1842-1860.
5. Watson, S.A., et al., *Gastrin - active participant or bystander in gastric carcinogenesis?* Nature Reviews Cancer, 2006. **6**(12): p. 936-946.
6. Koh, T.J. and D. Chen, *Gastrin as a growth factor in the gastrointestinal tract*. Regulatory Peptides, 2000. **93**(1-3): p. 37-44.
7. Dockray, G., R. Dimaline, and A. Varro, *Gastrin: old hormone, new functions*. Pflügers Archiv, 2005. **449**(4): p. 344-355.
8. Schubert, M.L., *Gastric secretion*. Current Opinion in Gastroenterology. **26**(6): p. 598-603 10.1097/MOG.0b013e32833f2010.
9. Waldum, H.L., et al., *Gastrin-histamine sequence in the regulation of gastric acid secretion*. Gut, 1991. **32**(6): p. 698-701.
10. Fourmy, D., V.r. Gigoux, and J.C. Reubi, *Gastrin in Gastrointestinal Diseases*. Gastroenterology. **141**(3): p. 814-818.e3.
11. Dimaline, R. and A. Varro, *Attack and defence in the gastric epithelium - a delicate balance*. Experimental Physiology, 2007. **92**(4): p. 591-601.
12. Dufresne, M.n., C. Seva, and D. Fourmy, *Cholecystokinin and Gastrin Receptors*. Physiological Reviews, 2006. **86**(3): p. 805-847.
13. Ferrand, A. and T.C. Wang, *Gastrin and cancer: A review*. Cancer Letters, 2006. **238**(1): p. 15-29.

14. Stepan VM, D.C., del Valle J, Matsushima M, Todisco A., *Cell type-specific requirement of the MAPK pathway for the growth factor action of gastrin*. 1999.
15. Ferrand, A., et al., *Involvement of JAK2 upstream of the PI 3-kinase in cell-cell adhesion regulation by gastrin*. *Experimental Cell Research*, 2004. **301**(2): p. 128-138.
16. Todisco, A., et al., *Molecular mechanisms for the antiapoptotic action of gastrin*. *American Journal of Physiology - Gastrointestinal and Liver Physiology*, 2001. **280**(2): p. G298-G307.
17. Gorman, A.M., et al., *Stress management at the ER: Regulators of ER stress-induced apoptosis*. *Pharmacology & Therapeutics*. **134**(3): p. 306-316.
18. Wang, G., Z.Q. Yang, and K. Zhang, *Endoplasmic reticulum stress response in cancer: molecular mechanism and therapeutic potential*. *Am J Transl Res*. **2**(1): p. 65-74.
19. Schröder, M. and R.J. Kaufman, *ER stress and the unfolded protein response*. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, 2005. **569**(1-2): p. 29-63.
20. Naidoo, N. and M. Brown, *The endoplasmic reticulum stress response in aging and age-related diseases*. *Frontiers in Physiology*. **3**.
21. Malhotra, J.D. and R.J. Kaufman, *The endoplasmic reticulum and the unfolded protein response*. *Semin Cell Dev Biol*, 2007. **18**(6): p. 716-31.
22. Chakrabarti, A., A.W. Chen, and J.D. Varner, *A review of the mammalian unfolded protein response*. *Biotechnology and Bioengineering*. **108**(12): p. 2777-2793.
23. Luo, S., et al., *Induction of Grp78/BiP by Translational Block: ACTIVATION OF THE Grp78 PROMOTER BY ATF4 THROUGH AN UPSTREAM ATF/CRE SITE INDEPENDENT OF THE ENDOPLASMIC RETICULUM STRESS ELEMENTS*. *Journal of Biological Chemistry*, 2003. **278**(39): p. 37375-37385.
24. Shen, X., K. Zhang, and R.J. Kaufman, *The unfolded protein response--a stress signaling pathway of the endoplasmic reticulum*. *J Chem Neuroanat*, 2004. **28**(1-2): p. 79-92.

25. Rutkowski, D.T. and R.J. Kaufman, *A trip to the ER: coping with stress*. Trends in cell biology, 2004. **14**(1): p. 20-28.
26. Bernales, S., F.R. Papa, and P. Walter, *Intracellular signaling by the unfolded protein response*, in *Annual Review of Cell and Developmental Biology*. 2006, Annual Reviews: Palo Alto. p. 487-508.
27. Li, X., K. Zhang, and Z. Li, *Unfolded protein response in cancer: the physician's perspective*. J Hematol Oncol. **4**: p. 8.
28. Ameri, K. and A.L. Harris, *Activating transcription factor 4*. Int J Biochem Cell Biol, 2008. **40**(1): p. 14-21.
29. Lange, P.S., et al., *ATF4 is an oxidative stress-inducible, prodeath transcription factor in neurons in vitro and in vivo*. The Journal of Cell Biology, 2008. **181**(4): p. i13.
30. Blais, J.D., et al., *Activating Transcription Factor 4 Is Translationally Regulated by Hypoxic Stress*. Molecular and Cellular Biology, 2004. **24**(17): p. 7469-7482.
31. Ye, J., *The role of the transcription factor ATF4 in tumor progression under nutrient deprivation and hypoxia*, in *ProQuest Dissertations and Theses*, University of Pennsylvania: United States -- Pennsylvania. p. 110.
32. Berlanga, J.J., J. Santoyo, and C. de Haro, *Characterization of a mammalian homolog of the GCN2 eukaryotic initiation factor 2 $\alpha$  kinase*. European Journal of Biochemistry, 1999. **265**(2): p. 754-762.
33. Singleton, D.C. and A.L. Harris, *Targeting the ATF4 pathway in cancer therapy*. Expert Opin Ther Targets. **16**(12): p. 1189-202.
34. de Haro, C., R. Mandez, and J. Santoyo, *The eIF-2 $\alpha$  kinases and the control of protein synthesis*. The FASEB Journal, 1996. **10**(12): p. 1378-87.
35. Fels, D.R. and C. Koumenis, *The PERK/eIF2 $\alpha$ /ATF4 module of the UPR in hypoxia resistance and tumor growth*. Cancer Biology & Therapy, 2006. **5**(7): p. 723-728.
36. Teske, B.F., et al., *The eIF2 kinase PERK and the integrated stress response facilitate activation of ATF6 during endoplasmic reticulum stress*. Molecular Biology of the Cell. **22**(22): p. 4390-4405.

37. Donnelly, N., et al., *The eIF2Î± kinases: their structures and functions*. Cellular and Molecular Life Sciences: p. 1-19.
38. Hamanaka, R.B., et al., *PERK and GCN2 contribute to eIF2alpha phosphorylation and cell cycle arrest after activation of the unfolded protein response pathway*. Mol Biol Cell, 2005. **16**(12): p. 5493-501.
39. Ye, J., et al., *The GCN2-ATF4 pathway is critical for tumour cell survival and proliferation in response to nutrient deprivation*. EMBO Journal. **29**(12): p. 2082-2096.
40. Wek, S.A., S. Zhu, and R.C. Wek, *The histidyl-tRNA synthetase-related sequence in the eIF-2Î± protein kinase GCN2 interacts with tRNA and is required for activation in response to starvation for different amino acids*. Molecular and Cellular Biology, 1995. **15**(8): p. 4497-4506.
41. Selvik, L.F., CS; Flatberg, A; Steigeda, TS; Misund, K; Anderssen, A; Doseth, B; Langaas, M; Tripathi, S; Beisvag, V; Læg Reid, A; Thommesen, L; Bruland, T, , *The duration of gastrin treatment affects global gene expression and molecular responses involved in ER stress and anti-apoptosis*. Submitted to BMC Genomics, 2012.
42. Dang Do, A.N., et al., *eIF2Î± kinases GCN2 and PERK modulate transcription and translation of distinct sets of mRNAs in mouse liver*. Physiological Genomics, 2009. **38**(3): p. 328-341.
43. Belal, C., et al., *The homocysteine-inducible endoplasmic reticulum (ER) stress protein Herp counteracts mutant Î±-synuclein-induced ER stress via the homeostatic regulation of ER-resident calcium release channel proteins*. Human Molecular Genetics. **21**(5): p. 963-977.
44. Schulze, A., et al., *The Ubiquitin-domain Protein HERP forms a Complex with Components of the Endoplasmic Reticulum Associated Degradation Pathway*. Journal of Molecular Biology, 2005. **354**(5): p. 1021-1027.
45. Kim, T.-Y., et al., *Herp enhances ER-associated protein degradation by recruiting ubiquilins*. Biochemical and Biophysical Research Communications, 2008. **369**(2): p. 741-746.

46. Ameri, K., et al., *Anoxic induction of ATF-4 through HIF-1-independent pathways of protein stabilization in human cancer cells*. *Blood*, 2004. **103**(5): p. 1876-1882.
47. Dockray, G.J., *Gastrin*. *Best Practice & Research Clinical Endocrinology & Metabolism*, 2004. **18**(4): p. 555-568.
48. Burkitt, M.D., A. Varro, and D.M. Pritchard, *Importance of gastrin in the pathogenesis and treatment of gastric tumors*. *World J Gastroenterol*, 2009. **15**(1): p. 1-16.
49. Fjeldbo, C.S.t., et al., *Gastrin upregulates the prosurvival factor secretory clusterin in adenocarcinoma cells and in oxyntic mucosa of hypergastrinemic rats*. *American Journal of Physiology - Gastrointestinal and Liver Physiology*. **302**(1): p. G21-G33.
50. Bruland, T., Flatberg A, Andersen E, Misund K, Fjeldbo CS, Beisvåg V, Sandvik AK, Thommesen L, Lægreid A, *Exploring signal-induced cellular regulatory subnetworks by use of partial least square regression (PLSR) multidimensional analysis of gene expression time series data*
51. Walter, P. and D. Ron, *The Unfolded Protein Response: From Stress Pathway to Homeostatic Regulation*. *Science*. **334**(6059): p. 1081-1086.
52. Mahadevan, N.R. and M. Zanetti, *Tumor Stress Inside Out: Cell-Extrinsic Effects of the Unfolded Protein Response in Tumor Cells Modulate the Immunological Landscape of the Tumor Microenvironment*. *The Journal of Immunology*. **187**(9): p. 4403-4409.
53. SavkoviÄž, V., et al., *Clusterin is protective in pancreatitis through anti-apoptotic and anti-inflammatory properties*. *Biochemical and Biophysical Research Communications*, 2007. **356**(2): p. 431-437.
54. Antonio Gonzalez, P.S.-C.a.G.M.S., *Culture of pancreatic AR42J cell for use as a model for acinar cell function*. *THE PANCREAPEDIA*, 2011: p. METHOD.
55. Hofslis, E., et al., *Expression of chromogranin A and somatostatin receptors in pancreatic AR42J cells*. *Mol Cell Endocrinol*, 2002. **194**(1-2): p. 165-73.
56. Todisco, A., et al., *Molecular mechanisms for the antiapoptotic action of gastrin*. *Am J Physiol Gastrointest Liver Physiol*, 2001. **280**(2): p. G298-307.

57. Stepan, V., et al., *Role of small GTP binding proteins in the growth-promoting and antiapoptotic actions of gastrin*. Am J Physiol Gastrointest Liver Physiol, 2004. **287**(3): p. G715-25.
58. Fjeldbo CS, B.I., Erlandsen SE, Holmseth J, Lægreid A, Sandvik AK, Thommesen L, Bruland T., *Gastrin upregulates the prosurvival factor secretory clusterin in adenocarcinoma cells and in oxyntic mucosa of hypergastrinemic rats*. 2012.
59. Ramamoorthy, S., V. Stepan, and A. Todisco, *Intracellular mechanisms mediating the anti-apoptotic action of gastrin*. Biochem Biophys Res Commun, 2004. **323**(1): p. 44-8.
60. Fjeldbo, C.S., et al., *Functional studies on RNA-transfected cell microarrays*. Methods Mol Biol. **629**: p. 175-91.
61. Dey, S., et al., *Both Transcriptional Regulation and Translational Control of ATF4 Are Central to the Integrated Stress Response*. Journal of Biological Chemistry. **285**(43): p. 33165-33174.
62. Christophe, J., *Pancreatic tumoral cell line AR42J: an ampicrine model*. Am J Physiol, 1994. **266**(6 Pt 1): p. G963-71.
63. Mashima, H., et al., *Genes expressed during the differentiation of AR42J cells into insulin-secreting*. Diabetes. **46**: p. 1378-1378.
64. Stepan, V., et al., *Role of small GTP binding proteins in the growth-promoting and antiapoptotic actions of gastrin*. American Journal of Physiology - Gastrointestinal and Liver Physiology, 2004. **287**(3): p. G715-G725.
65. Saravanan Ramamoorthy, V.S., Andrea Todisco, *Intracellular mechanisms mediating the anti-apoptotic action of gastrin*. 2004.

**Web links:**

66. <http://bestpractice.bmj.com/best-practice/monograph/871/basics/pathophysiology.html>
67. <http://nikamin.blogspot.no/2011/05/defense-mechanism-gastroduodenal.html>
68. <http://physrev.physiology.org/content/86/3/805/F4.expansion.html>
69. <http://home.hiroshima-u.ac.jp/imaizumi/index-e.html#top>
70. <http://www.genecards.org/cgi-bin/carddisp.pl?gene=ATF4>
71. <http://www.sciencedirect.com/science/article/pii/S0163725812000435>
72. <http://www.ptglab.com/support/technicalsupport/learningcenter/westernblot.aspx>
73. <http://www.tumblr.com/tagged/western%20blot>
74. [http://www.leinco.com/general\\_wb](http://www.leinco.com/general_wb)
75. <http://www.scribd.com/doc/57248323/SNAP-i-d-Protein-Detection-System>
76. <http://ibidi.com/xtproducts/en/ibidi-Labware/sticky-Slides/sticky-Slide-8-well>
77. <http://www.bing.com/images/search?q=microscope&FORM=HDRSC2#view=detail&id=45D9E20F9E20F24744F5034572DDEE2F7866CAFB&selectedIndex=21>
78. <http://www.bing.com/images/search?q=fluorescence+microscopy&qs=IM&form=QBIR&pg=fluorescence+microscopy&sc=1-23&sp=1&sk=#view=detail&id=CC86DE89DE5C81E70798AB041195EF7BAB907B96&selectedIndex=173>
79. <http://www.bing.com/images/search?q=fluorescence+microscopy&qs=IM&form=QBIR&pg=fluorescence+microscopy&sc=1-23&sp=1&sk=#view=detail&id=C3CB02777519A709F6B791880B08BDC3C5D2E415&selectedIndex=20>
80. <http://www.bing.com/images/search?q=caspase+assay&qs=n&form=QBIR&pg=caspase+assay&sc=0-8&sp=-1&sk=#view=detail&id=F9AAEC2ED781D23690BEE526EC22088F5AC0F8B1&selectedIndex=72>

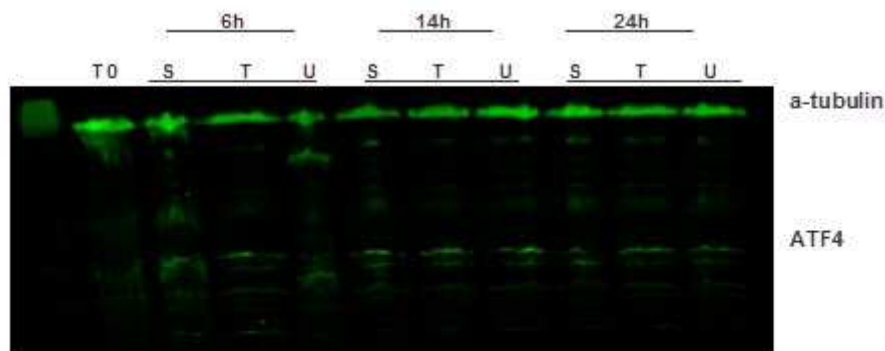




## 6. SUPPLEMENTARY FILE

### 6.1 Expression of ATF4 on AR42J cells in varying duration of gastrin treatment by using fluorescence western blot analysis:

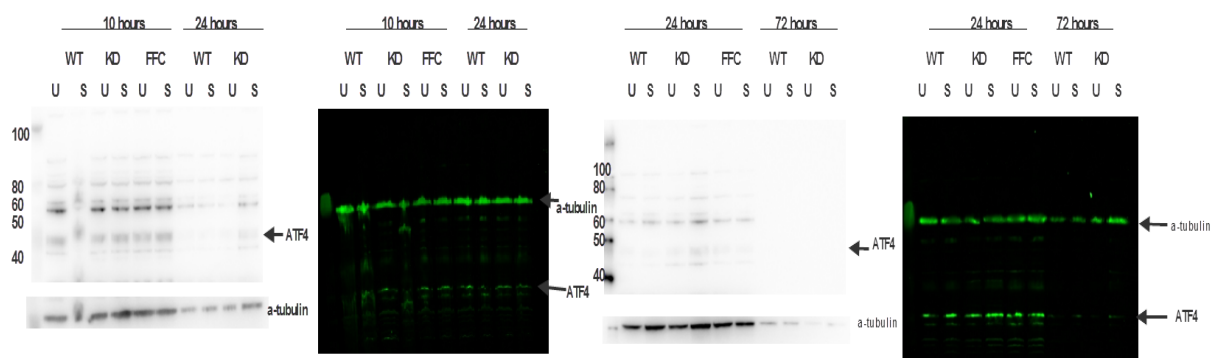
In western blot analysis we observed ATF4 protein in varying duration of gastrin treatment and found an up-regulation of ATF4 level in sustained gastrin treated cells. To support the result we further analyzed ATF4 protein by using fluorescence western blot technique (Figure S1). The ATF4 protein seems to be up-regulated by gastrin, but this experiment was done in only one biological replicate and need further optimization of the protocol.



**Figure S1: Expression of ATF4 on AR42J cells in varying duration of gastrin treatment by using fluorescence western blot analysis. A)** Upper panel: fluorescence western blot image at 6hrs, 14 hrs and 24 hours of gastrin treatment in sustained (S), transiently (T) gastrin treated cells and untreated (U) cells. Molecular weight of the ATF4 protein is 48 kDa (shown in the image) with housekeeping gene  $\alpha$ -tubulin at the top.

## 6.2 Expression of ATF4 on wild type AR42J cells, ATF4 (sh7) knockdown cells and Firefly control (FFC) cells to different duration of gastrin treatment:

We performed experiments by using both western blots in order to see if knocking down of ATF4 has expression of ATF4 protein compared to wild type and firefly control cells. We want to see if the results were similar in western blot and fluorescence western blot and seem to have same results. After 24 hours of gastrin treatment the bands become too weak to conclude anything.



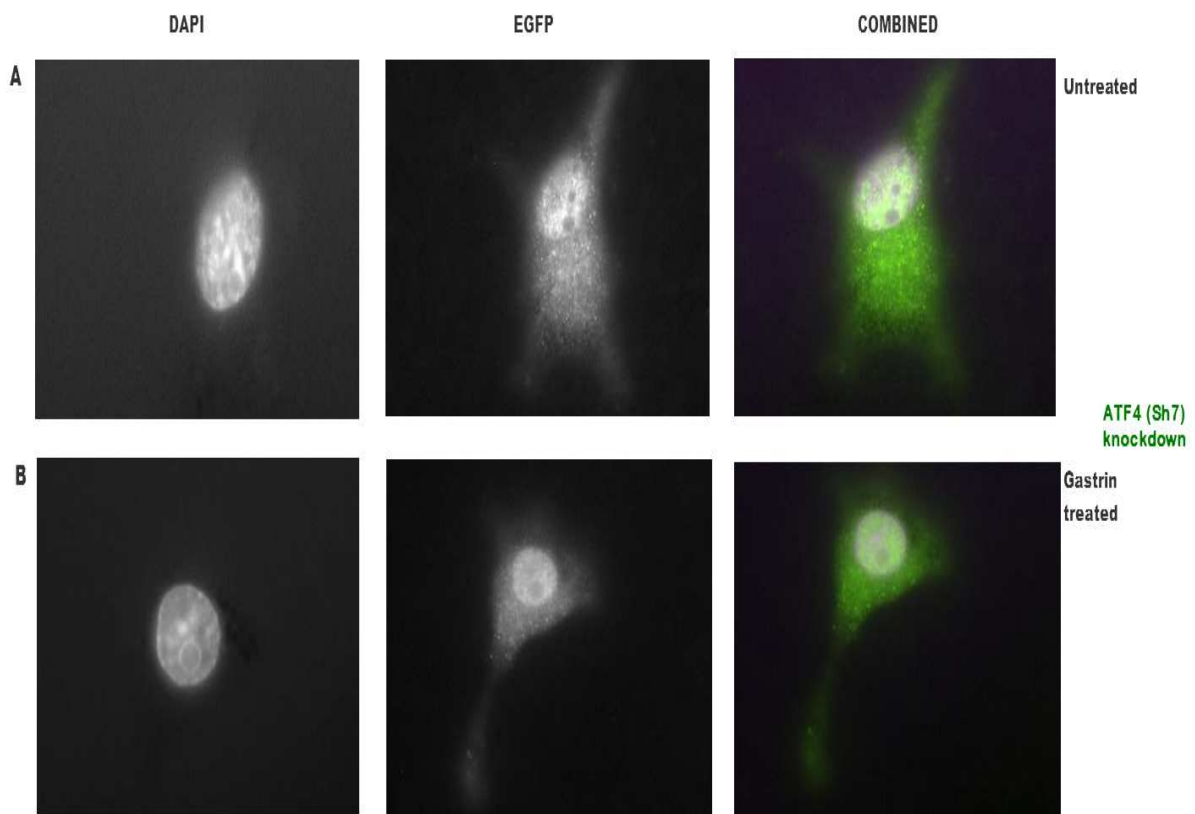
**Figure S2: Expression of ATF4 on wild type (WT) , ATF4 knockdown (KD) and firefly control (FFC) cells with and without gastrin treatment.** Western blot image of ATF4 on wild type, knockdown and firefly control cells at 10, 24 and 72 hours and fluorescence western blot image at the same time points normalization with a-tubulin.

### 6.3 Cellular localizations of proteins of ATF4 network on ATF4 (sh7)

#### Knockdown cells:

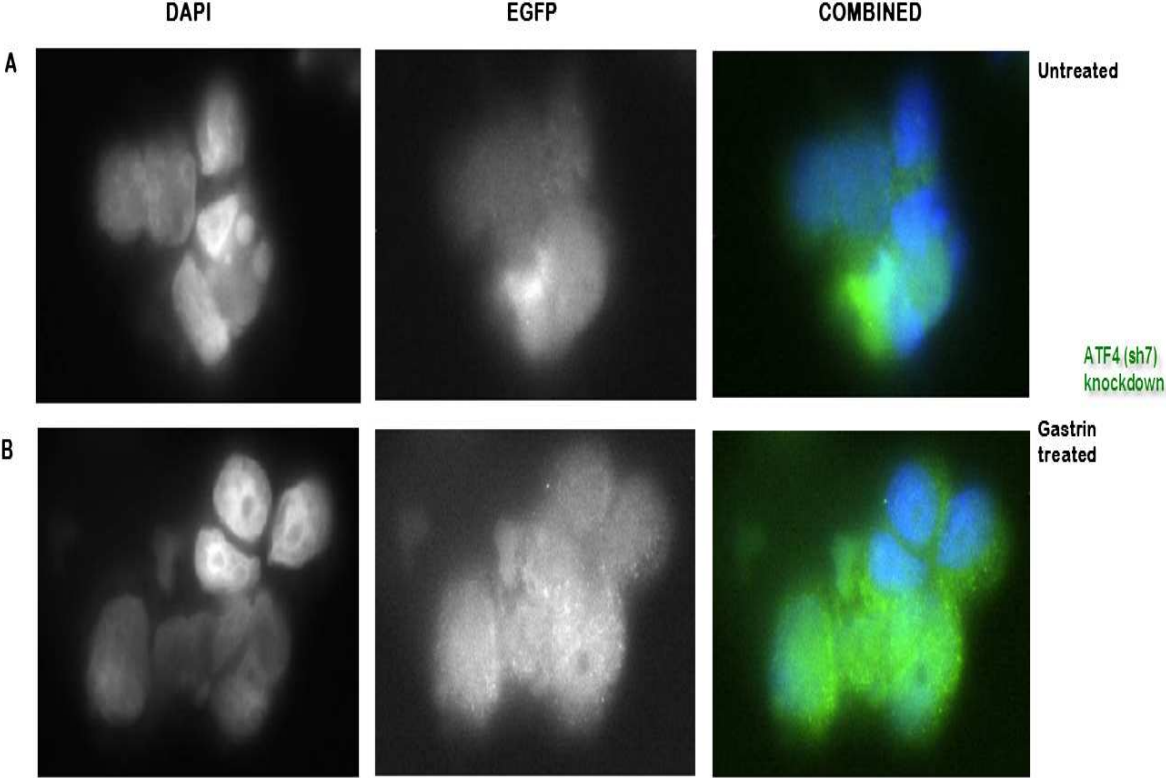
We detect ATF4 and its network proteins in wild type AR42J cells and found positive expression of ATF4 and its network proteins GRP78, pPERK, Herpud1 and CHOP. So, we repeated the same experiments in ATF4 (sh7) knockdown cells in order to see any difference in protein expression and their effect upon gastrin stimulation. Unfortunately, since the cells express GFP proteins it is difficult to analyze the expression of proteins when using a green secondary antibody. The analysis must therefore be repeated with another secondary antibody. The ICC pictures with Alexa 488 (green fluorescent) as secondary antibody are shown in Supplementary Figures S3-S7 below:

#### ATF4:



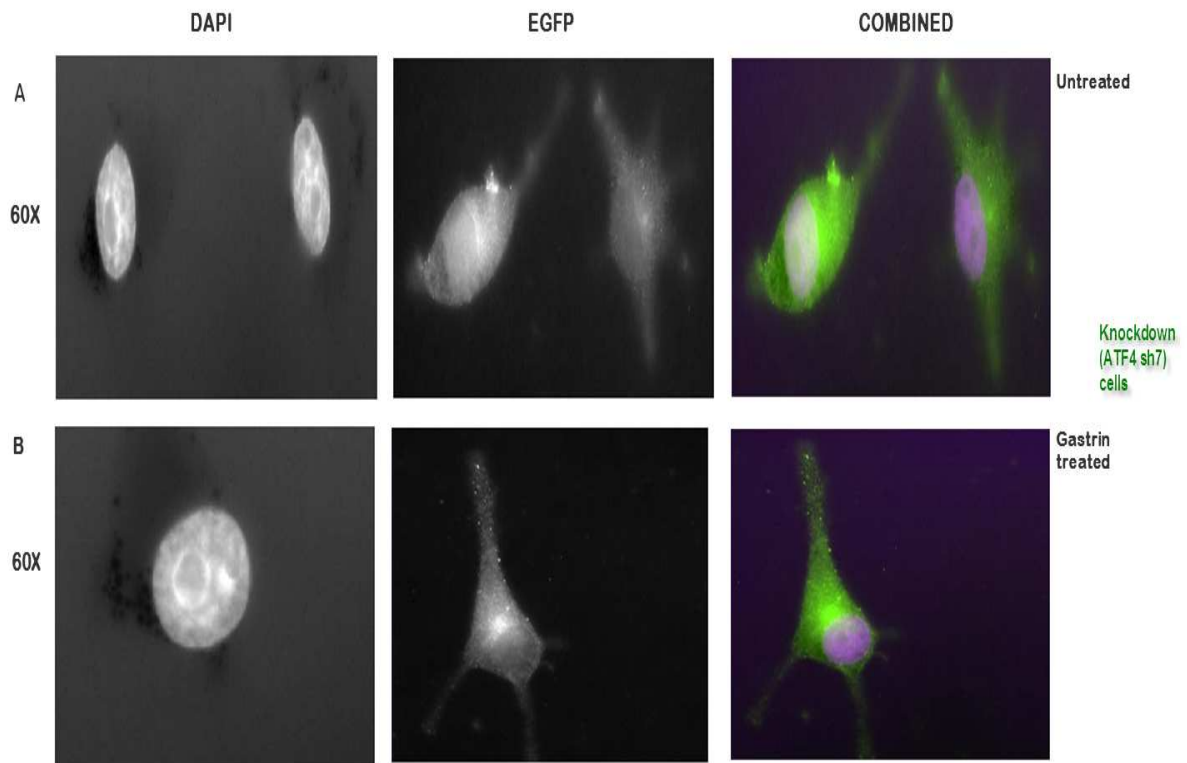
**Figure S3: Cellular localization of ATF4 protein in ATF4 knockdown cells with and without gastrin treatment.** Pictures with DAPI stained nuclei to the left, EGFP fluorescence in the middle and combined DAPI and EGFP fluorescence to the right. **A-B:** Expression of ATF4 (10835-1-AP, Protein tech.) in untreated ATF4 knockdown (**A**) and gastrin treated ATF4 knockdown (**B**) cells.

**GRP78:**



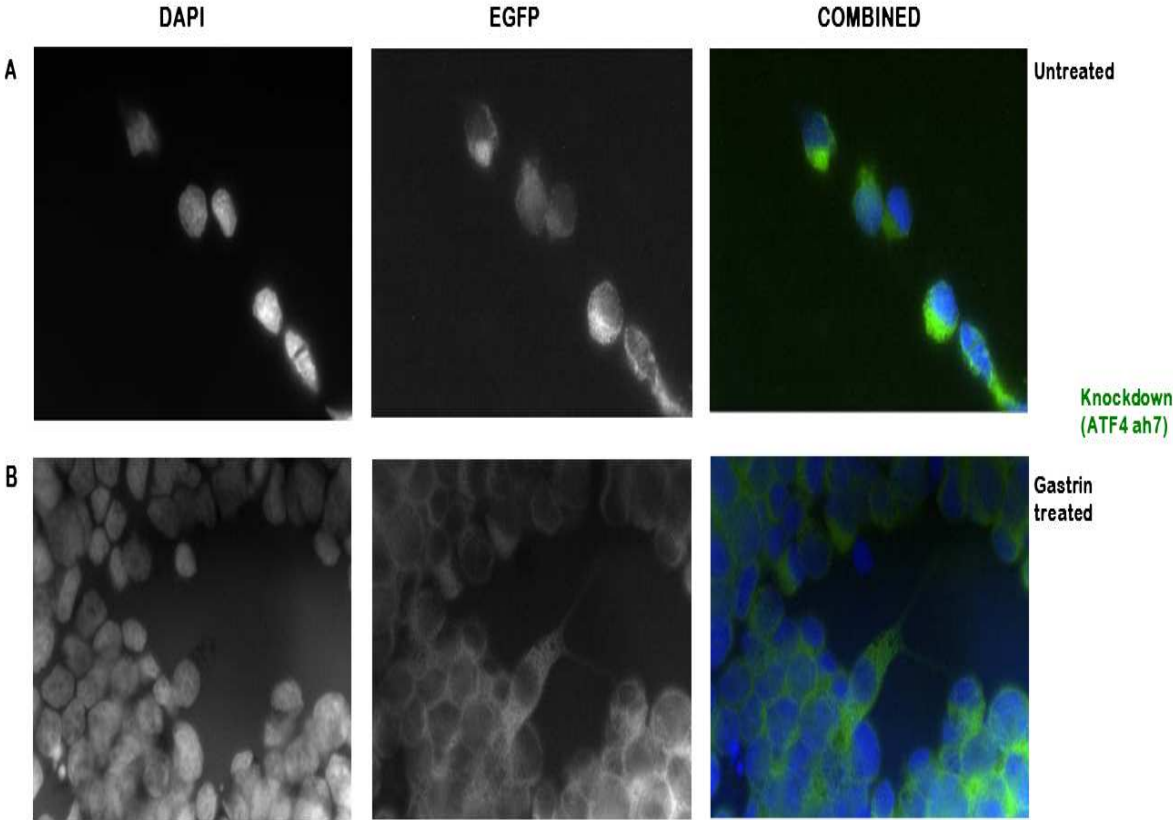
**Figure S4: Cellular localization of GRP78 (Bip) protein in ATF4 KD cells with and without gastrin treatment.** Pictures with DAPI stained nuclei to the left, EGFP fluorescence in the middle, and combined DAPI and EGFP fluorescence to the right. Expression of GRP78 in **A)** untreated ATF4 KD cells and **B)** gastrin treated ATF4 KD cells.

**pPERK:**



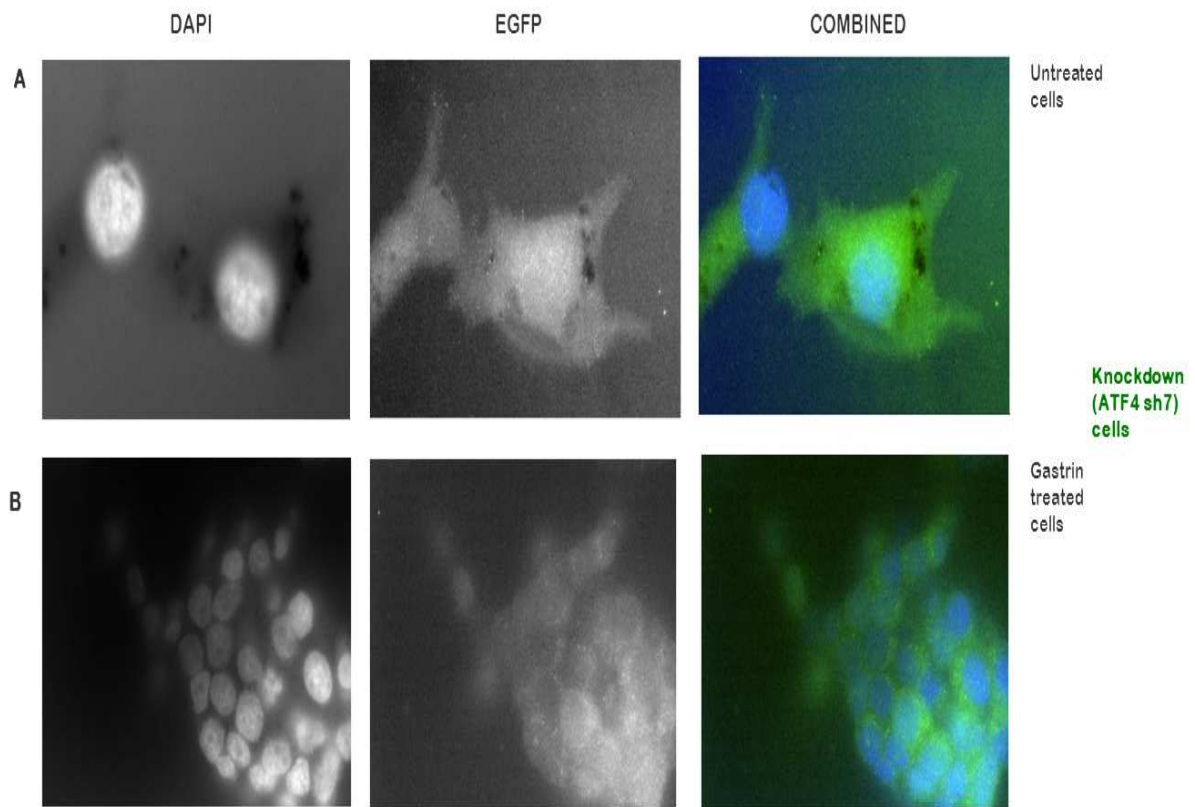
**Figure S5: Cellular localization and expression of pPERK in ATF4 knockdown cells with and without gastrin treatment.** Pictures with DAPI stained nuclei to the left, EGFP fluorescence in the middle, and combined DAPI and EGFP fluorescence to the right. **A-B:** Expression of pPERK (x60) in untreated (**A**) and gastrin treated (**B**) ATF4 knockdown cells respectively.

**Herpud1:**



**Figure S6: Cellular localization of Herpud1 in ATF4 knockdown cells upon gastrin stimulation.** Pictures with DAPI stained nuclei to the left, EGFP fluorescence in the middle, and the combined DAPI and EGFP fluorescence to the right. Expression of Herpud1 protein in **A)** untreated ATF4 KD cells and **B)** gastrin treated ATF4 KD cells.

## CHOP:



**Figure S7: Cellular localization of CHOP in ATF4 knockdown cells under gastrin stimulation.** Pictures with DAPI stained nuclei to the left, EGFP fluorescence in the middle, and combined DAPI and EGFP fluorescence to the right. Expression of CHOP in **A)** untreated ATF4 knockdown cells and **B)** gastrin treated knockdown cells.