Assessment of the basic molecular mechanisms underlying L-glutamine's cytoprotective effects after intestinal injury

Dissertation zur Erlangung des

naturwissenschaftlichen Doktorgrades

der Julius-Maximilians-Universität Würzburg

vorgelegt von

Stefanie Niederlechner

Prien am Chiemsee

Würzburg 2013

Eingereicht am: .....

Mitglieder der Promotionskommission:

Vorsitzender: .....

Gutachter: …….....

Gutachter: ……..

Tag des Promotionskolloquiums: .....

Doktorurkunde ausgehändigt am: .....

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die vorliegende Dissertation selbstständig und ohne Hilfe eines kommerziellen Promotionsberaters angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Denver,.....11.2.2013..... S. Niedzielski .....

Datum Unterschrift

Telluride, Colorado, USA

Before I came here I was confused about this subject.

Having listened to your lecture,

I am still confused.

But on a higher level.

~Enrico Fermi ~

Meinen Eltern

Table of contents

A INTRODUCTION 1

1 Critical illness 1

2 Gastrointestinal tract - Role in critical illness 2

3 Extracellular matrix – Role in critical illness 3

3.1 Fibronectin 4

3.2 Netrin-1 5

4 Sensors and transducers of cell volume changes 6

4.1 Integrins 6

4.1.1 Background and significance 6

4.1.2 Integrin signaling and apoptosis 7

4.2 EGFRs 9

4.2.1 Background and significance 9

4.2.2 EGFR signaling and apoptosis 9

4.2.3 EGFR - Integrin 'duet' 10

5 GLN 11

5.1 GLN utilization in the gut 11

5.2 GLN starvation and its role in critically ill patients 11

5.3 GLN's role in parenteral and enteral nutrition 12

5.4 GLN's cell protective mechanism 13

5.5 GLN transport and its osmoswelling effect 14

6 Hypotheses and aim of the study 14

B MATERIAL AND METHODS 17

1 Cell culture 17

1.1 Cell lines 17

1.2 Subculture of adherent cells 18

1.3 Cryopreservation and resuscitation 18

2 Experimental design 19

2.1 GLN starvation 19

2.2 Treatment with pharmaceutical compounds 20

2.2.1 Stimulation with inhibitors 20
2.2.2 Transfection with pre-designed Stealth siRNA 21
2.3 Treatment with GLN 23
2.4 Model of injury 23

3 MTS cell viability assay 24
3.1 Cell treatment 24
3.2 MTS assay 24

4 Protein extraction 25
4.1 Total protein extraction 26
4.2 Nuclear and cytoplasmic protein extraction 26

5 Determination of protein concentration via BCA 27
6 Western blot 28
6.1 SDS-polyacrylamid-gelelectrophoresis (SDS-PAGE) 29
6.2 Transfer 30
6.3 Protein detection 30
6.4 Restripping membranes 33

7 Hsp70 ELISA 33
8 Confocal fluorescence microscopy 35
8.1 Treatment 37
8.2 Fixing, staining, and mounting of slides 37
8.3 Fluorescence microscopy and post-processing 37
8.4 Analysis of cell area size and F-actin 38

9 LC-MS/MS 39
9.1 Treatment 39
9.2 Preparation of standards and samples 40
9.3 Method development 41
9.4 HPLC-chromatographic procedure 42
9.5 MS/MS 42
9.6 Quantification 43

10 Statistical analysis, illustrations, and references 43

C RESULTS 44

1 Involvement of FN-Integrin interaction in GLN's protective mechanism 44

1.1 GLN-mediated protection via expression of FN after HS 44
1.2 Involvement of FN-Integrin interaction in GLN-mediated protection 46
1.3 GRGDSP inhibits GLN-mediated increases in cell area size, disrupts F-actin organization, but has no impact on intracellular GLN-concentrations 48
1.4 GLN is protective by activating ERK1/2 via FN-Integrin pathway 51
1.5 GLN-mediated HSF-1 activation and expression is dependent on FN-Integrin/ERK1/2 signaling 53
1.6 GRGDSP attenuates increases in GLN-mediated HSP expression 55
1.7 GLN-mediated dephosphorylation of p38MAPK via FN-Integrin pathway ... 57
1.8 PI3-K/Akt is involved in GLN's protection independent of FN-Integrin signaling 59
1.9 PI3-K/Akt regulates FN expression 63
1.10 Involvement of Src-kinases in GLN-mediated protection 64

2 Involvement of Ntn-1 expression in GLN's protective mechanism 66
2.1 GLN prevents decreases in HS-mediated Ntn-1 expression 66
2.2 Ntn-1 expression is essential in GLN's protective mechanism 67

3 Involvement of EGFR expression and signaling in GLN's protective mechanism after HS 68
3.1 GLN is protective by preventing decreases in EGFR expression 68
3.2 Involvement of EGFR signaling in GLN-mediated cellular protection 71
3.3 GLN is protective by activating ERK1/2 via EGFR signaling 73
3.4 GLN is protective by dephosphorylating p38MAPK via EGFR signaling 74
3.5 EGFR signaling has no effect on GLN-mediated Akt activation 75

D DISCUSSION 76

1 Involvement of FN expression and FN-Integrin signaling via ERK1/2 in GLN's protective mechanism after intestinal injury 76
2 PI3-K, FN-Integrin-p38MAPK, and Src signaling are essential in GLN-mediated protection 81
3 Involvement of EGFR expression and signaling in GLN's protection 85
4 Ntn-1 expression is important in GLN's protection in the intestine 89

E SUMMARY - ZUSAMMENFASSUNG 91
F REFERENCES 95

G ACKNOWLEDGMENT 103
H ABBREVIATIONS 104

A Introduction

1 Critical illness

Any condition requiring support of failing vital organ function with supportive aids (such as mechanical ventilation, hemodialysis or cardiac assist devices) or pharmacological agents (such as vasopressors or inotropes) is defined as critical illness (CI) [41]. CI, often manifested by sepsis and inflammation, can be caused by many different types of diseases, such as cancer, surgical/traumatic injury, or diseases of an infectious origin, e.g. encephalitis and poliomyelitis. CI is one of the leading causes of morbidity and mortality in the USA and around the world. Mortality of critically ill patients (CIP) is high. The risk of death for adult patients with an intensive care unit (ICU) stay of more than 5 days (d) is around 20 % and around 30 % for those with an ICU stay of more than 21 d with a higher risk for males, the etiology of which remains obscure [155]. According to data from the United States Center for Disease Control and Prevention, the total number of death rates from sepsis has increased more than 90 % in the last 20 years and increases continuously [105, 166]. An estimated 750,000 patients develop sepsis annually and one third die because of this condition each year [8, 105, 124, 161]. In 1993, the percentage of severe sepsis death was 25.6 % and increased to 43.8 % in 2003 ($P< .001$). Within these years, the rate of severe sepsis hospitalization almost doubled from 66.8 to 132 per 100,000 patients ($P< .001$) and the mortality rate increased from 30.3 to 49.7 per 100,000 patients ($P< .001$) [43, 166]. Angus et al. estimate that the associated costs of care are around 16.7 billion dollars [8]. Sepsis and inflammation commonly lead to multiple organ failure syndromes, which causes death in many CIP. Despite appropriate parenteral and/or enteral nutrition, these CIP suffer from hypercatabolism, characterized by increased catabolism of certain tissues, such as muscle, bone, and fat, to mobilize stored nutrients. Protein energy malnutrition results if such high energy and nitrogen loss cannot be counterbalanced by enhanced dietary intake [19]. After this early phase of severe illness, patients no longer efficiently use fatty acids as metabolic

substrates [146]. They store fat from nutrition sources in adipose tissue and as infiltrates in the liver and the pancreas [28, 154]. This energetic failure is the consequence of multiple factors: increased levels of cytokines and catabolic hormones, elaboration of nitrogen monoxide, generation of reactive oxygen species, mitochondrial and endothelial dysfunction, and deficiencies in key nutrients, e.g. glutamine (GLN) and selenium. This is conducive to decreased vital functions, weakness, and increased morbidity and mortality [28, 146, 162]. It leads to persistent dependency on intensive medical care and patients become increasingly susceptible to multiple organ failure syndromes and infections. Novel approaches to improve the outcome of sepsis and inflammation and to reduce the risk of multiple organ failure syndromes are urgently needed. For this reason, nutritional and other anticatabolic therapies have recently become an interesting field of investigation.

2 Gastrointestinal tract - Role in critical illness

The gut consists of three entities, the epithelium, the mucosal immune system, and the commensally flora [32]. The single-layer epithelium has a major role in the digestion and absorption of nutrients and at the same time constitutes the organism's most important barrier. Under normal conditions only a small, restricted amount of intact antigens are allowed to permeate the mucosa where they interact with the mucosal immune system to downregulate inflammation [39, 97]. Together with the gut-associated lymphoid tissue and the neuroendocrine network, the intestinal epithelial barrier controls the equilibrium. Infectious and inflammatory stimuli disrupt this equilibrium, which lead to barrier dysfunction [16, 138]. Thus, the gastrointestinal tract, especially the small intestine, plays a crucial role in the stress response during CI and must be maximally supported. It is acknowledged that gastrointestinal tissues require direct contact with nutrients to support their own rapid cellular turnover rate and carry out a multitude of metabolic and immunologic functions to adapt to stress [97]. However, if the gastrointestinal tract is not successful in adapting to severe physical stress (e.g. trauma, burns, major surgery, inflammatory bowel disease (IBD),

pancreatitis, ischemia, intestinal perforation), gastrointestinal dysfunction will occur [140]. These stresses lead to disruption of the mucosal barrier, which results in increased mucosal permeability for microorganisms and toxins [52, 62]. These ecologic equilibrium changes in mucosal permeability, changes in intestinal immune system and endogenous bacteria lead to stress ulcers, intestinal ischemia, bacterial overgrowth, aspiration pneumonia, sepsis, bacterial translocation, and the systemic inflammatory response syndrome, which activate inflammatory pathways leading to multiple organ failure syndromes in CIP [32, 97, 148].

3 Extracellular matrix – Role in critical illness

The intestinal epithelium, which is in constant and rapid renewal, lies on a thin and continuous sheet of specialized extracellular matrix (ECM), which consists of the interstitial matrix and the basement membrane. The ECM separates parenchymal cells from the interstitial connective tissue [14]. Resident epithelial cells secrete polysaccharides and proteins, like collagen, elastin, fibronectin (FN), and laminin into the ECM via exocytosis [91]. Their duty is to fill the interstitial space and to act as a compression buffer against the stress placed on the ECM [4]. Hence, the primary function of the basement membrane is to anchor down the epithelium to its loose connective tissue underneath. This is achieved by cell-matrix adhesions through cell adhesion molecules, such as integrins and growth factor receptors. The basement membrane defines the necessary microenvironment for multiple functions such as adhesion, proliferation, migration, and cell survival [1, 14, 76, 92, 128], mediated by various receptors, including integrins and epidermal growth factor receptors (EGFRs). Therefore, intestinal epithelial cells represent an attractive focus for the study of the mechanisms of these functions. Fig. 1 illustrates an overview of the structure of the ECM.

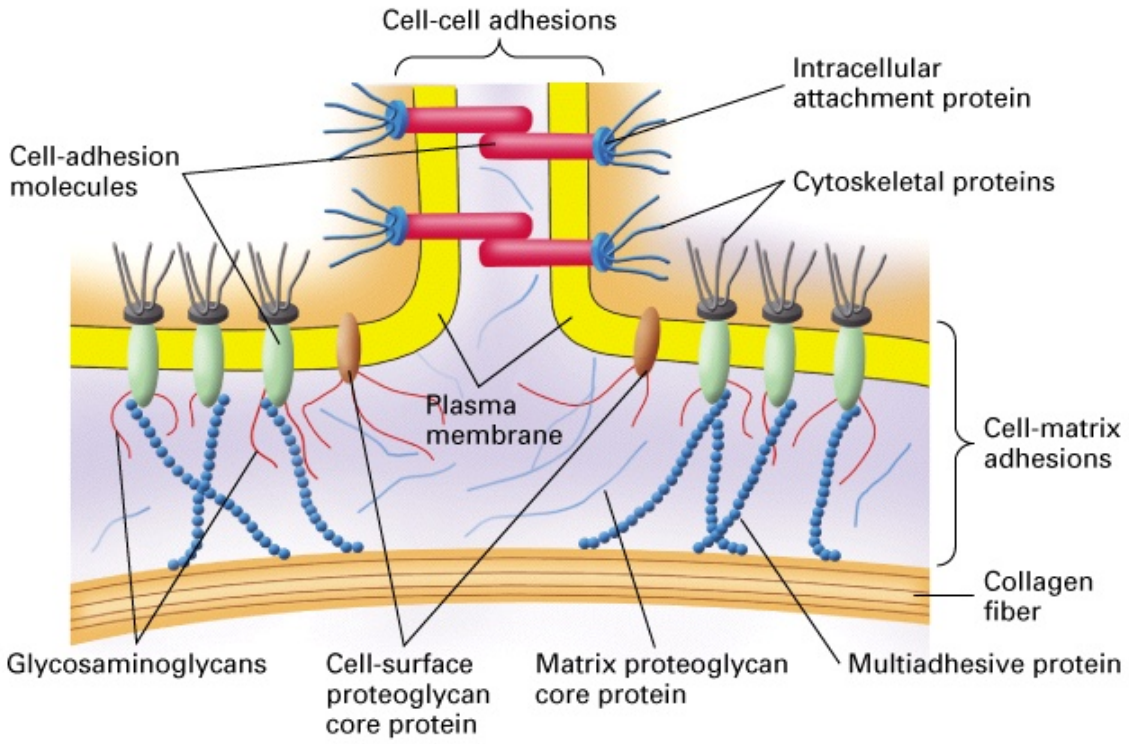


Fig. 1. Schematic of the extracellular matrix with its polysaccharides and proteins, interacting with cell receptors [96].

3.1 Fibronectin

FN, a high molecular weight adhesive glycoprotein in the basement membrane and connective tissue matrix of the intestine, is known to be vital to cell adhesion, migration, growth, and wound repair. These effects are dependent on binding of FN to the integrin receptor [61]. FN expression is important for the maintenance of normal epithelial integrity as well as for the regulation of epithelial response to intestinal injury [85]. FN contains an Arg-Gly-Asp (RGD) attachment site, which can bind to integrin receptors, forming an important recognition system for cell adhesion and survival signaling pathways [131, 149].

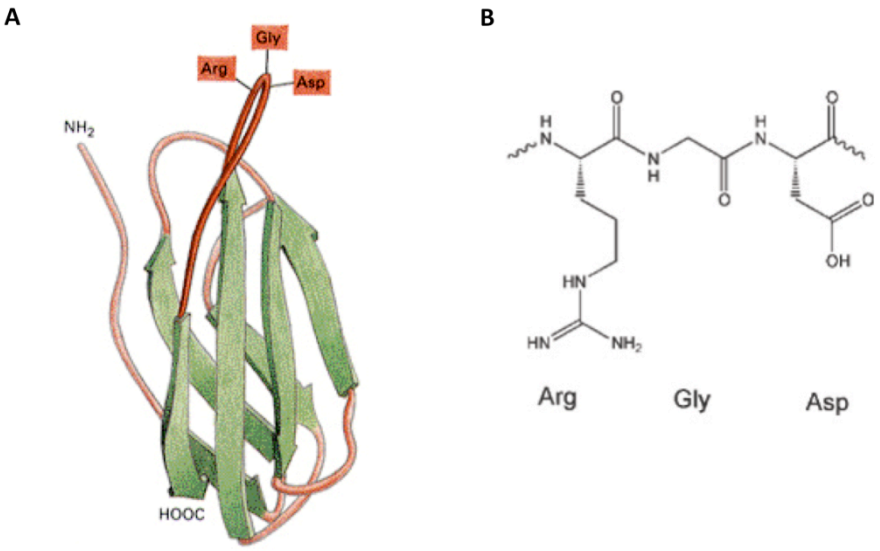


Fig. 2. Fibronectin structure with its RGD sequence. A) FN structure with the RGD sequence (http://jpkc.scu.edu.cn/ywwy/zbsw(E)/edetail4.htm). B) Chemical structure of the RGD sequence [181].

3.2 Netrin-1

The ECM harbors multiple diffusible proteins, including netrins, providing informational cues for many cellular functions including cell adhesion, proliferation, migration, differentiation, and cell survival [31]. Netrins affect these functions through classical netrin receptors such as the transmembrane receptors of the DCC and UNC5H families and integrins [31, 175]. Named after the Sanskrit word *netr,* which means 'one who guides', netrins provide migrational cues in the developing central nervous system. The secreted netrins are bifunctional, acting as attractants for some cell types and repellents for others during the development of brain.

Netrin-1 (Ntn-1) is a diffusible laminin-related, neuronal guidance protein and belongs to one of the five members of netrins (secreted Netrin 1, 3, 4 and membrane-bound G1 and G2) [31]. Ntn-1 was originally identified as diffusible protein released by neural floor plate cells in the developing spinal cord regulating axonal outgrowth. Recently, it has been shown that Ntn-1 also plays an important protective role in non-neural cells and peripheral tissues, such as intestinal epithelial, kidney, and lung cells [31].

4 Sensors and transducers of cell volume changes

In the early 1990's, it was determined that cell volume changes play a role in the regulation of metabolism, gene expression, and in stress response [67, 134]. The influence of cell volume on cell function requires osmosensors, which activate signaling pathways. In fungi, yeast, and plant cells, osmoreceptors, belonging to the family of histidine kinases, have been identified. However, these histidine kinases are rarely expressed in mammalian cells [172]. At present, little is known about the mechanism of osmosensing in mammalian cells. A number of integral proteins including EGFRs and integrins have been assigned roles as upstream sensors of cell volume changes [68].

4.1 Integrins

4.1.1 Background and significance

Specific cell surface adhesion molecules regulate adhesion of cells to the ECM. Integrins along with the immunoglobulin superfamily, selectins, cadherins, and mucins comprise the five major groups of cell adhesion molecules [29]. Integrins are a highly conserved family of heterodimeric adhesion molecules that connect the ECM, such as FN, to intracellular signaling proteins and the cytoskeleton [68]. Integrins are cell surface transmembrane heterodimeric glycoproteins, consisting of two non-covalently bound subunits, an α subunit (120-180 kD) and a β subunit (90-110 kD) [72]. Integrins in the small intestine mainly belong to the β1 and β4 classes and are localized along the crypt-villus axis [14]. There is a wide diversity of integrin receptors on the cell surface: 18 known α subunits and 8 known β subunit form 24 different integrin receptors. Integrins are the structural foundation of focal adhesion complexes, which link the cytoskeleton and signaling molecules to the ECM [87] (Fig. 3).

These protein complexes can reorganize the cytoskeleton and can modulate multiple signaling pathways [95]. Integrins were found to activate Src and other signaling pathways that transactivate EGFRs [68]. Integrin heterodimers contain binding sites for divalent magnesium and calcium that facilitate their binding of ligands [73, 150, 158]. Integrins

differentially bind with their extracellular domain to ECM proteins. The binding site consists of a portion of an α subunit and a portion of a β subunit. One important cellular binding site linking integrins to FN is called RGD, as mentioned above.

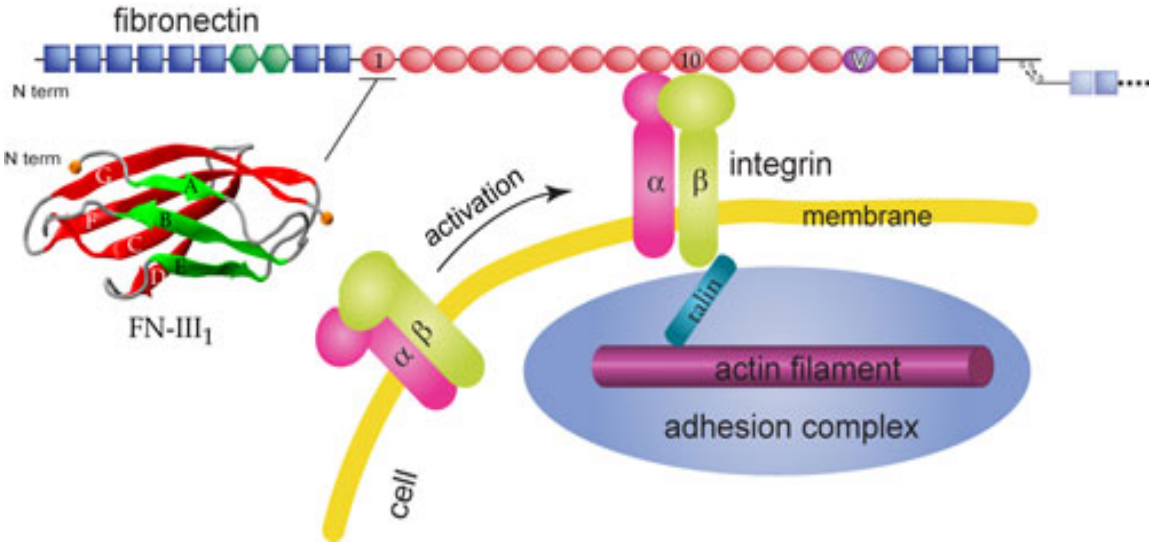


Fig. 3. Fibronectin-Integrin interaction and formation of the focal adhesion complex. FN binds to integrins via the recognition site RGD. FN and integrin assembly is activated and they form adhesion complexes, connecting the cytoskeleton to the ECM. (http://www.ks.uiuc.edu/Research/fibronectin/)

4.1.2 Integrin signaling and apoptosis

Signals are transduced bidirectionally through inside-out and outside-in mechanisms modulating proliferation, survival or apoptosis, shape, polarity, motility, gene expression, and differentiation. These functions are important in the regulation of multiple processes, such as inflammation, tissue development, tumor cell growth, angiogenesis, and programmed cell death. Reciprocal crosstalk between integrins and other receptors, e.g. EGFRs, can induce multiple, intracellular signaling pathways via matrix proteins [87], which are intertwined [102]. Furthermore, different integrins can activate different signaling pathways, and depending on the cell type, state, and environment, signaling through the same integrin may be different [14]. Ligand-binding affinity is regulated by the inside-out mechanism and can be activated by a change in the integrin conformation, regulated by the cytoplasmic tails of integrins [112].

Integrin affinity can also be increased through integrin clustering [102]. Integrins can activate a number of intracellular signaling pathways following ECM adhesive interactions. They mediate signaling from the extracellular space into the cell via adaptor molecules, such as Src [87]. These signal transduction pathways are of high complexity. For further details see reviews of Gilcrease 2006 [54] and Miranti and Brugge 2002 [102],

Mitogen-activated protein kinase (MAPK) pathways, such as extracellular signal-related kinase 1 and 2 (ERK1/2, also named p44/p42MAPK) and p38MAPK, as well as the phosphatidylinositol 3-kinase (PI3-K) pathway are important downstream survival signaling cascades from the membrane to the nucleus [21, 77, 90]. Activated MAPKs regulate the activities of transcription factors and/or kinases downstream via phosphorylation, control of gene expression, and other key cellular functions [11, 47, 48, 99]. It is well established that the ERK1/2 signaling pathway is stimulated by growth factors and adhesion signals from integrins [176] for cell proliferation, differentiation, and cell survival [84, 135]. In contrast, p38MAPK is considered to be a stress-related kinase and its activation often leads to apoptosis. However, p38MAPK activation is highly divergent and its pro- or anti-apoptotic function appears to be dependent on the cell type and cellular content [88, 99]. PI3-K is an ubiquitous lipid kinase comprised of a large and complex family with multiple subunits and isoforms. Together these subunits catalyze upstream effectors, which, in turn, phosphorylate Akt kinases [7, 25, 26]. The PI3-K/Akt pathway is an intracellular signaling pathway important in apoptosis. PI3-K phosphorylation activates Akt, reducing apoptosis, and allowing proliferation [90]. Recent studies have demonstrated that Akt1 is responsible for maintaining cell size and survival by increasing nutrient uptake [45].

4.2 EGFRs

4.2.1 Background and significance

The EGF family of receptor tyrosine kinases include four members: EGFR (also known as ErbB1), ErbB2, ErbB3, and ErbB4 [27]. Several reports have pointed to a role for EGFR tyrosine kinases in osmosensing in both the swelling- and shrinkage-activated responses [68]. Evidence indicates that activation of the EGF receptor family through multiple downstream pathways leads to increased cell survival [17, 34], decreased inflammation [59], and improved epithelial barrier function [33].

4.2.2 EGFR signaling and apoptosis

EGFR is a pleiotropic signal transducer that is dependent on tyrosine phosphorylation of its cytoplasmic domain. Phosphorylation provides specific docking sites for intracellular signal transducers and adaptors that can then recruit other intracellular signaling proteins [75]. EGFR interacts with multiple intracellular signaling pathways in a manner that promotes cell survival [38]. Moreover, EGFR has been shown to be important in overall intestinal homeostasis regulating multiple cellular functions such as proliferation, ion transport, cell survival, and cell growth [116, 119, 139, 160, 173, 174]. This is probably best demonstrated in studies using transgenic mice with defective EGFR (waved-2 mice), which display an increased susceptibility to colitis induced by the administration of dextran sulfate DSS [46]. These results suggest a key role for EGFR in protecting against the development of chronic intestinal disease. Furthermore, it has been shown that there is reduced EGFR signaling in IBD patients [5, 44], indicating that impairment of EGFR might contribute to disease etiology. Other reports have supported the role of EGFR in the recovery of the small intestine [94, 106]. Furthermore, it was shown that EGFR gets internalized into the nucleus; nuclear EGFR is crucial for DNA repair because of its potential role as transcription factor [42, 93].

4.2.3 EGFR - Integrin 'duet'

ECM interactions mediated by integrins are closely linked to growth factor signaling and to complex modulation of the cellular cytoskeleton. This complex interplay controls the specific gene expression program at any given time. A number of growth factor receptors are known to enhance integrin signaling and vice-versa [3, 54]. EGFR and integrins work as a 'duet' with similar downstream signaling pathways [3] (Fig. 4). Evidence for the formation of integrin and growth factor receptor macromolecular complexes has been suggested by co-clustering and immunofluorescence experiments [18]. Moreover, studies exist suggesting that ECM proteins, such as FN, are involved in EGFR activation, mediated by integrins [36].

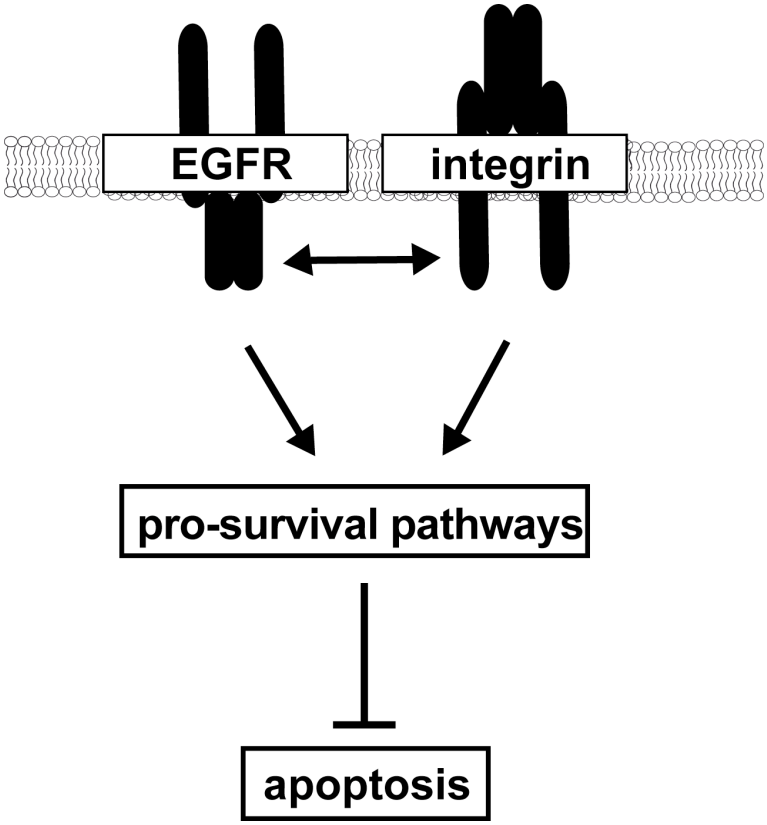


Fig. 4. The EGFR - Integrin 'duet' modified with Adobe Illustrator from http://www.grin.com/object/external_document.259304/e52f1b5818a1375f12979f70e905f467_LARGE.png

5 GLN

GLN is the most abundant amino acid in plasma and many tissues [86, 170]. Sir Hans Krebs, the pioneer of biochemical research, once stated that “most amino acids have multiple functions, but glutamine appears to be the most versatile” [113]. GLN is a polar amino acid that is uncharged at a neutral pH and can exist in two stereoisomers, the L- and the D-glutamine. In protein synthesis the L-glutamine stereoisomer is used.

5.1 GLN utilization in the gut

GLN is a crucial substrate for the small intestine and has a central role in numerous metabolic processes in the gut because of its preferential substrate status in this organ [159, 170]. It serves not only as a precursor for proteins, polyamines, glutathione, and nucleotide synthesis, and as a nitrogen carrier, but also as the primary metabolic fuel for enterocytes [37, 113, 127, 145, 170]. Windmueller and Spaeth showed GLN´s contribution to the provision of energy in the intestine in numerous experiments [163-165]. Complete oxidation of GLN results in 27 ATP equivalents, one of the highest rates of all nonessential amino acids. GLN´s oxidation provides approximately one third of the ATP production in cultured enterocytes [113].

5.2 GLN starvation and its role in critically ill patients

In CIP, the GLN plasma level is significantly decreased and remains decreased for more than 21 days [115]. Oudemans-van Straaten et al. associated the GLN plasma depletion with increased mortality in CIP [114]. These low blood GLN levels are related with a poor prognosis [129, 144, 145, 179]. Catabolic states, caused by stress, are characterized by an increased GLN use by GLN-consuming cells and a decrease in nutrient uptake, resulting in a higher GLN demand. This GLN demand is counterbalanced by increased GLN efflux from lung and muscle tissue. In CI, the provision of GLN is not sufficient, which leads to a

decreased GLN plasma level and GLN-utilizing cells suffer from GLN starvation. Reduced availability of GLN affects energy metabolism, protein synthesis, cell protective mechanisms, and cell viability. GLN starvation leads to reduced ATP and intracellular glutathione levels, cell shrinkage, which affects the osmosensitive pathways, and reduced protein synthesis rate. Several of these catabolic pathways lead to an increased sensitivity of the cell to apoptosis activators, which increases mortality in CIP [113].

5.3 GLN's role in parenteral and enteral nutrition

CIP are rarely able to take in a normal diet orally. Therefore, they have to be fed either by a feeding tube, which is called enteral feeding, or intravenously, called parenteral feeding. Meta-analyses demonstrate that GLN-supplemented enteral and parenteral feeding can significantly reduce mortality in seriously ill patients [110, 121]. During severe illness and malnutrition, GLN-supplemented parenteral and enteral nutrition become essential for the gut because GLN reduces intestinal permeability, decreases bacterial translocation, enhances immune function, protects gut mucosa against injury, accelerates healing of the small intestine, and improves nitrogen balance in animal models of intestinal atrophy, injury, and adaption [53, 82, 142, 178, 179]. For example, Klimberg and Souba demonstrated in radiation-injured animal models that GLN was able to improve the intestine morphology and survival [82, 83, 143]. Additionally, Wischmeyer and his colleagues showed that GLN can protect the ileal mucosa in humans after inflammation [167]. *In vivo* studies also revealed that the effect of GLN, given enterally and parenterally, could maintain mucosal proliferation, protein synthesis, expression of ornithine decarboxylase, and polyamine synthesis [156]. Furthermore, clinical trials in humans have shown decreases in infectious complications and shorter hospital stays in severely ill patients after GLN supplementation [56, 69, 89, 110, 169, 180]. Thus, GLN, considered a non-essential amino acid, now appears to be conditionally essential during CI or serious injury, and can be supplemented via enteral GLN-enriched solutions in diverse patient populations [20].

5.4 GLN's cell protective mechanism

Our laboratory has previously shown that GLN enhances transactivation of transcription factor HSF-1 following cellular injury, which activates key protective stress response pathways, such as enhanced heat shock protein (HSP) expression [168-170]. Further, GLN can decrease caspase-3 and poly (ADP-ribose) polymerase (PARP) cleavage after heat shock (HS), leading to a significant reduction of cellular apoptosis [132].

During inflammation, cells have to deal with a number of cytotoxic mediators, such as reactive oxygen species, endotoxins, and cytokines. Therefore, cells express HSPs [113]. HSPs are a family of highly conserved proteins and are involved in the most basic mechanisms of cellular protection. They were first discovered in 1962 in the salivary glands of *Drosophila* larva by Ritossa in chromosomal puffs when increasing the temperature 5°C-7°C above normal [126]. They were named HSPs because they were thought to be induced only by heat, but many agents and stresses, such as anoxia, sodium arsenite, ethanol, amino analogs, and drugs, to name a few, induce similar response [111, 152]. HSPs belong to multigene families that range in molecular size from 10 to 105 kDa and are found in all major cellular compartments. They are identified according to their molecular weight and can be grouped into two classes, constitutive and inducible. The constitutive HSPs are often named chaperones, because they are involved in the process of protein synthesis in the endoplasmatic reticulum and in protein delivery to particular cellular compartments [74, 130]. Inducible HSPs are synthesized predominantly under stress conditions [15]. Their expression, which is regulated transcriptionally, is increased when cells are exposed to elevated temperatures or other stress [40] and is induced primarily by heat shock factors (HSFs) [171]. The exact mechanism by which HS or other stressors activate HSFs has not yet been determined. Stress proteins protect cells in part by binding to denatured proteins and preventing further denaturation. Alternatively they bind denatured protein and promote their degradation [103, 158, 170]. HSP inducing agents have not yet been exploited clinically because, to date, they are toxic and, thus, have not been clinically relevant. More recent literature shows that GLN can enhance HSP expression without having a toxic effect. Our

laboratory and others have demonstrated in a variety of *in vitro* and *in vivo* studies that GLN is protective and can be a potent enhancer of HSPs, especially two of the major protective HSPs, 25 and 70 [30, 49, 101, 109, 117, 120, 170]. It has also been shown that GLN's protective effect is mediated by metabolic and non-metabolic (GLN's analog 6-diazo-5-oxo-L-norleucine) mechanisms.

5.5 GLN transport and its osmoswelling effect

In intestinal epithelial cells, GLN is primarily transported by Na^+-dependent systems. Transport of GLN into cells via a Na^+-dependent mechanism causes osmotic cell swelling. It has been hypothesized that GLN-mediated cell swelling may lead to changes in gene expression and inhibition of proteolysis [58, 63-65, 100]. Huang and his group demonstrated that cell swelling causes HSF-1 binding to its HSP gene promoter [70]. However, it is not evident if GLN-mediated cell swelling causes expression of these and other proteins.

6 Hypotheses and aim of the study

The previous chapters imply that a relationship between GLN`s osmoswelling, ECM expression, integrin and EGFR osmosignaling, survival signaling pathways, and HSP expression might exist in the intestine after hyperthermia. However, at present, no direct connection between GLN´s effect on cell swelling and protein expression has been proven. Furthermore, it is unclear if GLN´s effect on cell swelling alone leads to HSP expression or if other survival pathways are involved in mammalian cells.

Our laboratory has previously shown that GLN enhances transactivation of transcription factor HSF-1 after HS, which activates key protective stress response pathways, such as enhanced Hsp70 expression [168-170]. Further, GLN can decrease caspase-3 and PARP cleavage after HS, leading to a significant reduction of cellular apoptosis [132]. As previously described, the integrin cell volume-sensing pathway has also been shown to be a key

protective pathway following stress [63, 66]. Given this existing data, I hypothesized that the mobilization of GLN into the circulation following stress may serve as a “cytoprotective signal” leading to GLN-mediated cell swelling and modulation of FN-Integrin-mediated signaling. Thus, inhibition of FN-Integrin signaling may reduce cell size and attenuate GLN-mediated protection by decreasing intracellular GLN concentration. Activation of FN-Integrin signaling may modulate GLN’s activation of key stress response proteins, such as Hsp70, via ERK1/2. Therefore, this study specifically investigated the role of FN-Integrin signaling via ERK1/2, HSF-1, and HSPs in GLN’s molecular mechanisms of cellular protection.

Moreover, GLN appears to mediate protection against CI-related gut injury via multiple pathways. These also include FN-Integrin, PI3-K, and p38MAPK pathways. I hypothesized that there may be a relationship between these pathways mediating GLN’s protection in intestinal epithelial-6 (IEC-6) cells after HS. I assessed whether p38MAPK and PI3-K signaling are involved in GLN’s cytoprotective mechanisms and which role they play in GLN-mediated protection in conjunction with FN-Integrin osmosignaling after HS.

FN has been shown to play a critical role in tissue repair and survival in intestinal injury [61]. Thus, I considered that FN expression is essential in GLN’s protective mechanism after hyperthermia in intestinal epithelial-6 (IEC-6) cells. Since Ntn-1 has been shown to be another very important protective ECM protein in the intestine and known to signal via integrins [2, 31, 175], I looked at Ntn-1 expression to see whether only FN is involved in GLN-mediated cellular protection, or other ECM proteins as well.

Since EGFR expression and signaling is known to induce cellular protection after intestinal inflammation and since literature showed that integrins crosstalk and intertwine with EGFRs, I was interested whether GLN-mediated EGFR signaling is protective via the same signaling pathways as FN-Integrin signaling. In this context ERK1/2, PI3-K/Akt, and p38MAPK signaling may be regulated via both, FN-Integrin and EGFR signaling. Moreover, I investigated whether EGFR expression played an essential role in GLN’s protective mechanism or just EGFR tyrosine kinase signaling.

By using different specific interaction and tyrosine kinase inhibitors, it was the aim of the study to show which survival signaling pathways were involved in GLN-mediated cellular protection and to see whether they were regulated via GLN-mediated FN-Integrin and/or EGFR signaling. Specific siRNAs were used to investigate the role of FN, EGFR, and Ntn-1 expression in GLN's protective mechanism. Moreover, cell area size was quantified and filamentous actin (F-actin) morphology was analyzed to determine if cell swelling and microtubules have direct relevance to GLN-mediated cellular protection. Finally, liquid chromatography–mass spectrometry (LC-MS/MS) experiments were performed to see if intracellular GLN concentration changed after inhibition of FN-Integrin signaling. IEC-6 cells were chosen because of their vital role in the gut and because of their ability to produce high amounts of ECM proteins [122].

B Material and Methods

1 Cell culture

Used materials:	Company; Registered office
IEC-6 cells	ATCC, Manassas, VA, USA
DMEM	Cellgro Mediatech Inc., Herndon, VA, USA
Insulin	Sigma-Aldrich, St. Louis, MO, USA
FBS	Cellgro Mediatech Inc., Herndon, VA, USA
Penicillin	Cellgro Mediatech Inc., Herndon, VA, USA
Streptomycin	Cellgro Mediatech Inc., Herndon, VA, USA
L-Glutamine	Sigma-Aldrich, St. Louis, MO, USA
Trypsin-EDTA	Life Technologies, Grand Island, NY, USA
PBS	Cellgro Mediatech Inc., Herndon, VA, USA
DMSO	Sigma-Aldrich, St. Louis, MO, USA
Tubes 50 mL/15 mL	Fisher Scientific, Pittsburgh, USA
Tissue culture flasks 70 mL	Fisher Scientific, Pittsburgh, USA
96 well plates	Fisher Scientific, Pittsburgh, USA
10 cm dishes	Fisher Scientific, Pittsburgh, USA
4 well chamber slides	Fisher Scientific, Pittsburgh, USA
Cryovails	Fisher Scientific, Pittsburgh, USA
Hemacytometer	Hausser Scientific, Horsham, PA, USA

1.1 Cell lines

Full media

DMEM	440 mL
Insulin	500 µL; final concentration 1 %
FBS	50 mL; final concentration 10 %
Penicillin (10.000 U/mL) / streptomycin	5 mL
200 mM GLN	146 mg

IEC-6 cells were used in this study and purchased from ATCC at passage number 15. They are non-transformed crypt-like cells isolated from the whole small intestine [123]. Cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10 % fetal bovine serum (FBS), 2 mM L-GLN, 10 mL/L of antibiotic solution containing penicillin G (10,000 U/mL) and streptomycin (10,000 µg/mL), and 0.01 mg/mL insulin. Cultured cells were maintained in a humidified 37°C incubator (Thermo Scientific, Waltham, MA, USA) with 5 % CO_2 until passage number 23.

1.2 Subculture of adherent cells

IEC-6 cells were split under a cell culture laminar air flow hood (Labconco, Kansas City, MI, USA) twice a week in order to prevent the culture from becoming overpopulated. Cells were washed with 7 mL PBS and brought into suspension using 7 mL of trypsin (0.25 % trypsin-EDTA) to release the cells from the flask. After 2-5 min, when 80-90 % of the cells were detached, cells were resuspended in 15 mL of serum-containing full DMEM to neutralize the trypsin. A cell count with a hemacytometer was performed and the required number of cells was transferred to either a new labeled T-75 flask (2,000 cells), 96 well plates (approximately 7,000 cells per well for survival assays), 10 cm dishes (around 200,000 cells per dish for Western blots), or 4 well chamber slides (around 40,000 cells per chamber for fluorescence microscopy).

1.3 Cryopreservation and resuscitation

Freezing media:

Full medium	80 %
FBS	10 %
DMSO	10 %

To bring frozen cells, stored in cryovails in liquid nitrogen (-196°C), back into culture, they needed to be thawed first. It is essential to thaw the cultures quickly because DMSO is toxic. To minimize the toxic effect, it was crucial to dilute the culture in full growth medium. The frozen ampoule of cells was thawed in a water bath (Precision, Winchester, Virginia, USA) at 37ºC and was immediately transferred into a T-75 flask containing 15 mL of pre-warmed full growth medium. After 24 h, when the cells were attached to the bottom of the flask, the culture medium was changed to remove the cells from the toxic DMSO. For cryopreservation of cells, they were detached from the flask as described above, and centrifuged for 5 min at 1200 rpm in a 50 mL tube. The cell pellet was resuspended in 4 mL of freezing media and was stored in 4 separate cryovails (1 mL each) at -196°C in liquid nitrogen.

2 Experimental design

Used materials:	Company; Registered office
DMEM	Cellgro Mediatech Inc., Herndon, VA, USA
Insulin	Sigma-Aldrich, St. Louis, MO, USA
FBS	Cellgro Mediatech Inc., Herndon, VA, USA
L-Glutamine	Sigma-Aldrich, St. Louis, MO, USA
GRGDSP	Bachem, Torrance, CA, USA
GRGESP	Bachem, Torrance, CA, USA
AG1478	Sigma-Aldrich, St. Louis, MO, USA
LY294002	Sigma-Aldrich, St. Louis, MO, USA
PD98059	Calbiochem, Philadelphia, PA, USA
UO126	Cell signaling, Danvers, MA, USA
SB203580	Sigma-Aldrich, St. Louis, MO, USA
PP2	Enzo life science, Biomol, Plymouth, PA, USA
EGFR Stealth RNAi™ siRNA	Invitrogen Inc., Carlsbad, CA, USA
FN Stealth RNAi™ siRNA	Invitrogen Inc., Carlsbad, CA, USA
Ntn-1 Stealth RNAi™ siRNA	Invitrogen Inc., Carlsbad, CA, USA
Stealth RNAi™ Negative Control Duplexes Low GC	Invitrogen Inc., Carlsbad, CA, USA
Stealth RNAi™ Negative Control Duplexes Medium GC	Invitrogen Inc., Carlsbad, CA, USA
Stealth RNAi™ Negative Control Duplexes High GC	Invitrogen Inc., Carlsbad, CA, USA
1X Annealing/Dilution buffer	Invitrogen Inc., Carlsbad, CA, USA
SilentFect (1 mg/mL)	Bio-Rad, Hercules, CA
DMSO	Sigma-Aldrich, St. Louis, MO, USA
10 cm dishes	Fisher Scientific, Pittsburgh, USA
6 well dishes	Fisher Scientific, Pittsburgh, USA
4 well chambers	Fisher Scientific, Pittsburgh, USA
96 well plates	Fisher Scientific, Pittsburgh, USA

2.1 GLN starvation

Pretreatment media

DMEM	500 mL
FBS	10 %
Insulin	0,1 %

24 h before the experiment, when the cells were approximately 80 % confluent, the standard culture full media was replaced by GLN-free, serum containing DMEM with only 10 % FBS and 0.01 mg/mL insulin for 24 h to standardize GLN content at the start of each experiment and to mimic the severe GLN depletion occurring in CI.

2.2 Treatment with pharmaceutical compounds

2.2.1 Stimulation with inhibitors

Preparation of stock solutions:

GRGDSP

GRGDSP inhibits the interaction between FN and integrins. 25 mg of FN-Integrin interaction inhibitor GRGDSP (MW: 587.59 g/mol) was diluted in 2.5 mL sterile, ultra-pure dH_2O to receive a 10 mg/mL stock solution (= 6.8 mM). Aliquots were stored at -20°C. A final concentration of 50 µM was used for the following IEC-6 cell experiments.

GRGESP

GRGESP is the inactive control peptide of GRGDSP. It is an inactive FN fragment analog. 25 mg of GRGESP (MW: 601.62 g/mol) was reconstituted in 2.5 mL of dH_2O to receive a 10 mg/mL stock solution (= 6.6 mM). Aliquots were stored at -20°C. Final concentration for IEC-6 cell treatment was 50 µM.

PP2

PP2 is a potent and selective inhibitor of the Src family tyrosine kinases. 1 mg of PP2 (MW: 301.8 g/mol) was diluted in 1 mL sterile DMSO to receive a 1 mg/mL stock solution (= 3.3 mM). Aliquots were stored at -20°C. A final concentration of 75 µM was used for the following IEC-6 cell experiments.

AG1478

AG1478 is a tyrophostin, which inhibits EGFR tyrosine kinase activity. 5 mg of AG1478 (MW: 315.75 g/mol) were reconstituted in 1 mL DMSO to a 5 mg/mL stock solution (= 15.8 mM). Aliquots were stored at -20°C. A final concentration of 5, 10, and 20 µM was used for the following IEC-6 cell experiments.

LY294002

LY294002 is a specific cell permeable phosphatidylinositol 3-kinase inhibitor. 5 mg of LY294002 (MW: 343.8 g/mol) were diluted in 1 mL DMSO to a 5 mg/mL stock solution (= 14.5 mM). Aliquots were stored at -20°C. A final concentration of 25 µM was used for the following IEC-6 cell experiments.

PD98059

PD98059 is a selective, reversible, and cell-permeable inhibitor of MAP kinase kinase (MEK)

that acts by inhibiting the activation of MAP kinase and subsequent phosphorylation of MAP kinase substrates. An InSolution™ PD98059 inhibitor was purchased from Calbiochem. It consists of a 5 mg/mL solution of PD98059 in anhydrous DMSO. Aliquots were stored at -20°C. A final concentration of 50 µM was used for the following IEC-6 cell experiments.

UO126

UO126 is a highly selective inhibitor of MEK1 and MEK2. 5 mg of UO126 (MW: 380.5 g/mol) were diluted in 1 mL DMSO to a 5 mg/mL stock solution (= 13.1 mM). Aliquots were stored at -20°C. A final concentration of 30 µM was used for the following IEC-6 cell experiments.

SB203580

SB 203580 is an inhibitor of SAPK2 (p38MAPK) and acts as an anti-inflammatory drug. 1 mg of SB203580 (MW: 377.4 g/mol) was reconstituted in 1 mL DMSO to receive a 1 mg/mL stock solution (= 2.6 mM). Aliquots were stored at -20°C. A final concentration of 10 µM was used for the following IEC-6 cell experiments.

Treatment

After 24 h GLN starvation, IEC-6 cells were either treated with DMEM and 0.01 % insulin or with the final inhibitor concentrations for 1 h before GLN treatment.

2.2.2 Transfection with pre-designed Stealth siRNA

Pre-designed double-strand Stealth RNAi™ siRNA uses RNAi chemistry that provides higher specificity and increased stability in serum and cell culture than standard siRNA. This chemistry produces clearer results while eliminating unwanted off-target effects providing effective knockdown, higher specificity, greater stability, and less cellular toxicity.

Transfection media

DMEM with 2 mM GLN	Cellgro Mediatech Inc., Herndon, VA, USA
10 % FBS	Cellgro Mediatech Inc., Herndon, VA, USA

Preparation of Stealth RNAi™ siRNA:

Fn Stealth RNAi™ siRNA (rat)	Set of 3 (20 nmole each)
Ntn-1 Stealth RNAi™ siRNA (rat)	Set of 3 (20 nmole each)
EGFR Stealth RNAi™ siRNA (rat)	Set of 3 (20 nmole each)
Stealth RNAi™ Negative Control Duplexes for FN siRNA	2x Medium GC and 1x High GC (20 µM each)
Stealth RNAi™ Negative Control Duplexes for Ntn-1 siRNA	2x Medium GC and 1x High GC (20 µM each)
Stealth RNAi™ Negative Control Duplexes for EGFR siRNA	3x Low GC (20 µM each)

1X Annealing/Dilution buffer (pH 8.0)

Tris-HCl	10 mM
NaCl	20 mM
EDTA	1 mM

Stealth RNAi™ dry pellets were stored at -20°C until use. Before reuse they were thawed and centrifuged briefly to allow the contents to settle at the bottom. 20 nmole of FN, Ntn-1, and EGFR Stealth siRNA (set of 3) were each resuspended in 1 mL of RNase-free water to make a 20 µM solution. Final concentrations of 20 nM FN siRNA and Ntn-1 siRNA and 40 nM EGFR siRNA were used in IEC-6 cell experiments. Stealth siRNA negative controls were supplied in a ready-to-use format (20 µM in 1X RNA Annealing/Dilution buffer).

Transfection

Cells were seeded in 10 cm dishes and allowed to grow for 24 h until 50 % of confluence in full media. Media was changed to transfection media, and cells were transfected for 48 h using SilentFect (30 µL SilentFect per 12 mL media) with no siRNA, FN, Ntn-1 (20 nM), EGFR siRNA (40 nM), or their corresponding control non-coding (NC) oligos (20 nM; 40 nM) with a comparable GC content to siRNAs. SilentFect and siRNAs were pipetted together 30 min prior to the experiment and incubated at 37°C before use. After 48 h transfection, cells were GLN-starved 24 h before HS, with the transfection reagents still present before GLN treatment.

2.3 Treatment with GLN

GLN stock solutions

200 mM GLN: 146 mg in 5 mL DMEM

100 mM GLN: 146 mg in 10 mL DMEM

20 mM GLN: 146 mg in 50 mL DMEM

After pharmaceutical compound treatment, IEC-6 cells were treated with DMEM control media or different concentrations of GLN (2 mM, 10 mM, 20 mM) for 15 min before injury was performed. Stock solutions of 0 mM, 20 mM, 100 mM, 200 mM of GLN were prepared. In 10 cm dishes with 7 mL of pharmaceutical compound media, 770 µL of according GLN stock solution concentrations were added to achieve the desired GLN concentrations of 2 mM, 10 mM, and 20 mM. In 96 well plates, 11 µL of GLN stock solutions were added to 100 µL and in 4 well chambers 33 µL in 3 mL.

2.4 Model of injury

A well-established model of HS injury was used for all IEC-6 cell experiments to mimic injury and inflammation [60]. For cell viability, 96 well plates were submerged to a lethal HS in a 44°C Precision water bath Model 260 (Winchester, VI) for 50 min and allowed to recover at 37°C for 24 h [60]. For protein expression, microscopy, ELISA, and LC-MS/MS experiments, cells were subjected to a non-lethal HS at 43°C for 45 min [60]. Cells were allowed to recover for either 0 min for measurement of phosphorylated and their total proteins, FN and EGFR expression, and transfection experiments, 15 min for microscopy and LC-MS/MS experiments, or 3 h for assessment of FN, EGFR, Ntn-1, HSP, total HSF-1, caspase-3, bax, bcl-2, and PARP expression.

3 MTS cell viability assay

Used materials:	Company; Registered office
MTS/PMS reagent	Promega, Madison, WI, USA
Phenol-red free DMEM	Cellgro Mediatech Inc., Herndon, VA, USA
Insulin	Sigma-Aldrich, St. Louis, MO, USA
96 well plates	Fisher Scientific, Pittsburgh, USA

MTS solution

MTS/PMS reagent	5 mL
Phenol-red free DMEM	20 mL
Insulin	5 µL

3.1 Cell treatment

To measure cell viability of IEC-6 cells, cells were cultured in 96 well plates and treated as mentioned in the experimental design section of this thesis with different concentrations of inhibitors, siRNAs, and GLN concentrations to determine an optimal inhibitory concentration, that was non-toxic in control cells, and also to optimize the inhibitory effect on GLN-mediated protection in IEC-6 cells. After HS IEC-6 cells were allowed to recover without removal or addition of media at 37°C for 24 h. However, all HS groups were also studied in non-HS contol groups to account for differences in cell growth.

3.2 MTS assay

After 24 h of recovery, a soluble tetrazolium salt assay was used as per manufacturer's instructions. One part phenazine methosulfate (PMS) was added immediately to 20 parts 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) before the solution was diluted 1:5 in phenol red-free DMEM and insulin. After the prepared MTS/PMS solution was added to cells, MTS was bioreduced by cells into a colored, soluble formazan product. After 3 h the absorbance values were read at 490 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader (Thermo Electron Corporation, San Jose, CA, USA). References included readings at 650 nm and no-cell blank wells.

Higher absorbance values reflect more cell proliferation and more cell viability. After normalizing every well to their individual non-HS controls, to account for possible differences in cell growth, the normalized values for each treatment group from multiple experiments were averaged.

The MTS assay is a standard colorimetric assay for measuring the activity of enzymes that reduce MTS and PMS to formazan, giving a brown-purple color. MTS produces, in the presence of PMS, a water-soluble formazan product that has an absorbance maximum at 490-500 nm in phosphate-buffered saline. These reductions only occur when reductase enzymes are active and subsequently conversion is often used as a measure of viable cells. When the amount of brown-purple formazan, produced by cells treated with an agent, is compared with the amount of formazan produced by untreated control cells, the effectiveness of the agent in causing death or changing metabolism of cells can be inferred from the production of a dose-response curve.

4 Protein extraction

Used materials:	**Company; Registered office**
M-PER Mammalian Protein Extraction Reagent	Thermo Scientific, Waltham, MA, USA
Complete, Mini, EDTA-free Protease Inhibitor Cocktail Tablets (EASYpacks)	Roche, Mannheim, Germany
PhosSTOP Phosphatase Inhibitor Cocktail Tablets	Roche, Mannheim, Germany
NE-PER Nuclear and Cytoplasmic Extraction Reagents	Pierce, Rockford, IL
Cell Scraper	Fisher Scientific, Pittsburgh, USA

Complete, Mini, EDTA-free Protease Inhibitor Cocktail Tablets

Each tablet is sufficient for a volume of 10 mL extraction solution

PhosSTOP Phosphatase Inhibitor Cocktail Tablets

1 tablet contains phosphatase inhibitor sufficient for 10 mL cell extract or buffer

4.1 Total protein extraction

M-PER Mammalian Protein Extraction Reagent

Proprietary detergent in 25 mM bicine buffer, pH 7.6

Thermo Scientific M-PER Mammalian Protein Extraction Reagent provides highly efficient total soluble protein extraction from cultured mammalian cells.

After the experimental treatment with inhibitors, siRNAs, NC, GLN, and HS, medium was removed from 10 cm dishes of IEC-6 cell monolayers, and cells were immediately washed in 6 mL of PBS. For total protein extraction cells were scraped in 180 µL Mammalian Protein Extraction Reagent (M-PER) with inhibitor protease and phosphatase cocktail, transferred into an Eppendorf tube, and incubated for 15 min on a shaker followed by a 15 min centrifugation step at 14,000 x g. The supernatant, containing total protein was transferred to a new labeled, chilled Eppendorf tube and stored at -80°C.

4.2 Nuclear and cytoplasmic protein extraction

NE-PER Nuclear and Cytoplasmic Extraction Reagents

Cytoplasmic Extraction Reagent I (CER I)	10 mL
Cytoplasmic Extraction Reagent II (Cer II)	550 µL
Nuclear Extraction Reagent (NER)	5 mL

The Thermo Scientific NE-PER Nuclear and Cytoplasmic Extraction Reagents enabled stepwise separation and preparation of cytoplasmic and nuclear extracts from cultured cells or tissue in less than 2 h. Protease inhibitor cocktail was added to CER I and NER from concentrated stocks to maintain extract integrity and function. After the cells were treated and heat-stressed, they were washed in 6 mL of PBS. They were scraped off using 100 µL ice-cold CER I and transferred into pre-chilled Eppendorf tubes. The tubes were vigorously vortexed on the highest setting for 15 s to fully suspend the cell pellet. The tubes were incubated on ice for 10 min. Ice-cold CER II was added to the tubes, vortexed for 5 s on the highest setting and centrifuged for 5 min at maximum speed (16,000 x g). Immediately, the

supernatants were transferred to clean pre-chilled tubes (cytoplasmic extract). The tubes were stored at -80ºC until use. Addition of CER I and Cer II to the cell pellet caused cell membrane disruption and release of cytoplasmic contents. After recovering the intact nuclei from the cytoplasmic extract by centrifugation, the nuclei were lysed with NER to yield the nuclear extract. The insoluble pellet fraction, which contained nuclei, was resuspended in ice-cold NER, vortexed for 15 s on the highest setting and placed on ice. Every 10 min the samples were vortexed for 15 s for a total of 40 min. Then, the tubes were centrifuged at maximum speed (16,000 x g) for 10 min. The supernatant fractions were transferred to clean pre-chilled tubes, placed on ice and stored at -80ºC until use. Extracts obtained with this kit generally have less than 10 % contamination between nuclear and cytoplasmic fractions.

5 Determination of protein concentration via BCA

Used materials:	**Company; Registered office**
BCA protein assay kit	Thermo Scientific, Waltham, MA, USA
Albumin BSA standard	Thermo Scientific, Waltham, MA, USA

BCA working reagent:

BCA reagent A (sodium carbonate, sodium bicarbonate, bicinchoninic acid, and sodium tartrate in 0.1 M sodium hydroxide)	20 mL
BCA reagent B (4 % cupric sulfate)	400 µL

Protein concentrations (0 µg/mL to 2 mg/mL) were assessed by utilizing a two-component Thermo Scientific Pierce bicinchoninic acid protein assay (BCA) according to the manufacturer's instructions. A 9 step dilution series of albumin standards (BSA) was prepared (0 µg/mL, 25 µg/mL, 125 µg/mL, 250 µg/mL, 500 µg/mL, 750 µg/mL, 1000 µg/mL, 1500 µg/mL, 2000 µg/mL) and duplicate volumes (25 µL) of each standard and protein sample were incubated with 200 µL of BCA working reagent at 37°C for 25 min. Protein concentrations were determined by measuring the absorbance at 562 nm on an ELISA plate reader (Thermo Electron Corporation, San Jose, CA, USA). The BCA assay relies on two

reactions. First, the peptide bonds in proteins reduce Cu^{2+} ions from the cupric sulfate to Cu^{+}, which is a temperature dependent reaction. The amount of Cu^{2+} reduced is proportional to the amount of protein present in the solution. The next step is that two molecules of bicinchoninic acid chelate with each Cu^{+} ion form a purple-colored product that strongly absorbs light at a wavelength of 562 nm. The amount of protein can be quantified by measuring the absorption spectra (562 nm) and by comparing them with BSA standards with known concentrations.

6 Western blot

Used materials:	**Company; Registered office**
20X NuPage MES SDS running buffer	Invitrogen Inc., Carlsbad, CA, USA
Tris/Cl	Sigma-Aldrich, St. Louis, MO, USA
SDS	Sigma-Aldrich, St. Louis, MO, USA
Glycerol	Sigma-Aldrich, St. Louis, MO, USA
2-mercapto-ethanol	Sigma-Aldrich, St. Louis, MO, USA
Bromphenol blue	Sigma-Aldrich, St. Louis, MO, USA
Full Range Rainbow molecular weight marker	GE Healthcare, Waukesha, WI, USA
NuPage 4 %-12 % Bis-Tris Gel	Invitrogen Inc., Carlsbad, CA, USA
Mini-gel system	Invitrogen Inc., Carlsbad, CA, USA
Methanol	Fisher Scientific, Pittsburgh, USA
Tris/ Glycine Buffer 10X	Bio-Rad Laboratories, Inc.; Hercules, CA, USA
Mini Trans-Blot Electrophoretic Transfer Cell	Bio-Rad Laboratories, Inc.; Hercules, CA, USA
PVDF membrane	Millipore, Billerica, MA, USA
Semi-dry transfer system	Invitrogen Inc., Carlsbad, CA, USA
NaCl	Sigma-Aldrich, St. Louis, MO, USA
KCL	Sigma-Aldrich, St. Louis, MO, USA
Na_2HPO_4	Fisher Scientific, Pittsburgh, USA
KH_2PO_4	Fisher Scientific, Pittsburgh, USA
Tween 20	Fisher Scientific, Pittsburgh, USA
Non-fat dry milk	LabScientific, Livingston, NJ, USA
Bovine serum albumin (BSA)	Sigma-Aldrich, St. Louis, MO, USA
Restore Western Blot Stripping Buffer	Fisher Scientific, Pittsburgh, USA
UVP chemiluminescent darkroom system	UVP, Upland, Ca, USA
LabWorks 4.0 Image Acquisition and Analysis Software BioImaging Systems	UVP, Upland, Ca, USA

SuperSignal West Dura Extended Duration Substrate	Pierce, Rockford, IL, USA
SuperSignal West Femto Maximum Sensitivity Substrate	Pierce, Rockford, IL, USA
β-actin	Sigma-Aldrich, St. Louis, MO, USA
Hsp32	StressGen, Victoria, BC, Canada
Hsp70	StressGen, Victoria, BC, Canada
Caspase-3	Cell signaling, Danvers, MA, USA
PARP	Cell signaling, Danvers, MA, USA
Bax	Cell signaling, Danvers, MA, USA
Bcl-2	Cell signaling, Danvers, MA, USA
[T(P)^{202}/Y(P)^{204}]ERK1/2	Cell signaling, Danvers, MA, USA
Total ERK	Cell signaling, Danvers, MA, USA
[S(P)^{473}]Akt	Cell signaling, Danvers, MA, USA
Total Akt	Cell signaling, Danvers, MA, USA
[T(P)^{180}/Y(P)^{182}]p38MAPK	Cell signaling, Danvers, MA, USA
Total p38MAPK	Cell signaling, Danvers, MA, USA
[Y(P)^{418}]Src	Cell signaling, Danvers, MA, USA
[Y(P)^{418}]Src	Invitrogen Inc., Carlsbad, CA, USA
Total Src	Cell signaling, Danvers, MA, USA
[S(P)^{303}]HSF-1	Abcam, Cambridge, MA, USA
Total HSF-1	Cell signaling, Danvers, MA, USA
FN	Sigma-Aldrich, St. Louis, MO, USA
EGFR	Cell signaling, Danvers, MA, USA
Ntn-1	Santa Cruz Biotechnology, Santa Cruz, CA, USA
Phospho-HER2/ERB2 (Tyr1248)/EGFR (Tyr1173)	Cell signaling, Danvers, MA, USA
Phospho-EGF Receptor (Tyr845)	Cell signaling, Danvers, MA, USA
Phospho-EGFR Receptor (Tyr1086)	Cell signaling, Danvers, MA, USA
Peroxidase-conjugated goat anti-mouse IgG	Fisher Scientific, Pittsburgh, USA
Peroxidase-conjugated donkey anti-rabbit IgG	Santa Cruz Biotechnology, Santa Cruz, CA, USA
Peroxidase-conjugated donkey anti-goat IgG	Santa Cruz Biotechnology, Santa Cruz, CA, USA

6.1 SDS-polyacrylamid-gelelectrophoresis (SDS-PAGE)

4X treatment buffer (for 10 mL), pH 6.8

Tris/Cl	250 mM
SDS	8 %
Glycerol	27,5 %
2-mercapto-ethanol	20 %
Bromphenol blue	0.1 %

Running buffer (for 1 L)

20X NuPage MES SDS running buffer	50 mL
dH_20	950 mL

Following determination of the protein concentration via BCA, 20 µg of protein from each sample was added to a 4X treatment buffer (TB), heat-denatured at 100°C for 5 min, and loaded into a NuPage 4 %-12 % Bis-Tris Gel with 10 µL of Full Range Rainbow molecular weight marker. Proteins were electrophoretically separated with a mini-gel system, filled with running buffer. The gels were run for 1 h at 200 V until sample buffer band reached the bottom of the gel.

6.2 Transfer

Transfer buffer (1X); pH 8.3

Tris	25 mM
Glycine	192 mM
Methanol	20 %

In order to make the proteins accessible to antibody detection, they were transferred from within the gel onto a methanol-activated polyvinylidene difluoride (PVDF) membrane at 300 mA for 3 h, using the semi-dry transfer system or the Mini Trans-Blot Electrophoretic Transfer Cell from Bio-RAD, filled with transfer buffer.

6.3 Protein detection

PBS-Tween (for 10 L)

NaCl	80 g
KCL	2 g
Na_2HPO_4	11.5 g
KH_2PO_4	2 g
dH_2O	10 L
Tween 20	10 mL

Blocking and antibody solution 1

Non-fat dry milk	5 %
PBS-Tween	0.1 %

Blocking and antibody solution 2 (for phosphorylated protein)

Bovine serum albumin (BSA)	5 %
PBS-Tween	0.1 %

Antibody list

Epitop	Species	Characteristics	Dilution
Primary antibodies			
β-actin	mouse	monoclonal	1:10,000
Hsp32	rabbit	polyclonal	1:5,000
Hsp70	rabbit	polyclonal	1:5,000
Caspase-3	rabbit	polyclonal	1:5,000
PARP	rabbit	polyclonal	1:5,000
Bax	rabbit	polyclonal	1:1,000
Bcl-2	rabbit	polyclonal	1:1,000
[T(P)^{202}/Y(P)^{204}]ERK1/2	rabbit	polyclonal	1:1,000
Total ERK	rabbit	polyclonal	1:1,000
[S(P)^{473}]Akt	rabbit	polyclonal	1:1,000
Total Akt	rabbit	polyclonal	1:1,000
[T(P)^{180}/Y(P)^{182}]p38MAPK	rabbit	polyclonal	1:1,000
Total p38MAPK	rabbit	polyclonal	1:1,000
[Y(P)^{418}]Src	rabbit	polyclonal	1:1,000
Total Src	rabbit	polyclonal	1:1,000
[S(P)^{303}]HSF-1	rabbit	polyclonal	1:1,000
Total HSF-1	rabbit	polyclonal	1:1,000
FN	rabbit	polyclonal	1:10,000
EGFR	rabbit	polyclonal	1:5,000
Ntn-1	goat	polyclonal	1:500
Phospho-HER2/ERB2 (Tyr1248)/EGFR (Tyr1173)	rabbit	polyclonal	1:1,000
Phospho-EGF Receptor (Tyr845)	rabbit	polyclonal	1:1,000
Phospho-EGFR Receptor (Tyr1086)	rabbit	polyclonal	1:1,000
Secondary antibodies			
Mouse IgG	goat	peroxidase-conjugated	1:3,000
Rabbit IgG	donkey	peroxidase-conjugated	1:3,000
Goat IgG	donkey	peroxidase-conjugated	1:3,000

SuperSignal West Dura Extended Duration Substrate/ SuperSignal West Femto Maximum

Sensitivity Substrate

Luminol/Enhancer (reagent 1)	100 mL
Stable Peroxide Buffer (reagent 2)	100 mL

Following transfer, membranes were blocked at room temperature (RT) on a shaker in 5 % non-fat dry milk in 0.1 % PBS-Tween, or in 5 % bovine serum albumin (BSA) in 0.1 % PBS-tween for phosphorylated protein antibodies for 2 h. Primary antibodies (see list above) were added to accordant antibody solutions and incubated overnight at 4°C on a shaker. After washing the PVDF membrane in PBS-tween 3 x for 10 min, membranes were incubated with either peroxidase-conjugated goat anti-mouse, donkey anti-rabbit, or donkey anti-goat IgGs at a 1:3,000 dilution for 2 h, depending on the primary antibodies species. Finally, another round of 3 x 10 min washing steps in 0.1 % PBS-tween were performed and membranes were added to a 1:1 chemiluminescence mixture of Pierce SuperSignal West Dura Extended Duration Substrate for all total protein levels or a 1:1 chemiluminescence mixture of Pierce SuperSignal West Femto Maximum Sensitivity Substrate for 2 min for the detection of phosphorylated proteins. Pierce SuperSignal West substrates are ultra-sensitive enhanced chemiluminescent substrates for detecting horseradish peroxidase on immunoblots. These substrates' extremely intense signal output enables detection of low femtogram amounts of antigen. Blots were removed from the 1:1 Pierce solution and placed into a plastic membrane protector. An absorbent tissue was used to remove excess liquid and to carefully press out any bubbles from between the blot and the surface of the membrane protector. Protein bands were exposed utilizing a UVP chemiluminescent darkroom system. The bands were analyzed with LabWorks 4.0 Image Acquisition and Analysis Software BioImaging Systems. Densitometry was normalized against β–actin.

6.4 Restripping membranes

Restore Western Blot Stripping Buffer

Proprietary clear, colorless, acidic solution 500 mL

Performing gel electrophoresis and duplicate immunoblot assays to test new primary antibodies or antibody concentrations was time-consuming and expensive. Restore Western Blot Stripping Buffer eliminated this waste when detecting immunoblots with chemiluminescent Western blotting substrates. The reagent allowed clean and efficient removal of primary and secondary antibodies from immunoblots without removing or damaging the immobilized antigen. First, the chemiluminescent substrate was removed by washing the blots in PBS-tween 2 x for 5 min. After incubation in 4 mL of Restore Western Blot Stripping Buffer for 15 min at RT, these were removed from the stripping buffer and washed in PBS-tween for 10 min. Finally, the blots were blocked in blocking solution and probed for different proteins as described above.

7 Hsp70 ELISA

Used materials:	**Company; Registered office**
Anti-Hsp70 Immunoassay plate	Enzo Life Science, Farmingdale, NY, USA
5X Extraction Reagent	Enzo Life Science, Farmingdale, NY, USA
Recombinant Hsp70	Enzo Life Science, Farmingdale, NY, USA
Sample Diluent 2	Enzo Life Science, Farmingdale, NY, USA
20X Wash Buffer	Enzo Life Science, Farmingdale, NY, USA
Hsp70 Antibody	Enzo Life Science, Farmingdale, NY, USA
Hsp70 Conjugate	Enzo Life Science, Farmingdale, NY, USA
Tetramethylbenzidine Substrate	Enzo Life Science, Farmingdale, NY, USA
Stop Solution 2	Enzo Life Science, Farmingdale, NY, USA
LY294002	Sigma-Aldrich, St. Louis, MO, USA
L-Glutamine	Sigma-Aldrich, St. Louis, MO, USA

Another related technique, which includes using antibodies to detect proteins in cells, is the enzyme-linked immunosorbent assay (ELISA). To detect and quantify intracellular inducible Hsp70 levels, an Hsp70 ELISA kit from Enzo Life Science, a quantitative sandwich immunoassay, was used. A mouse monoclonal antibody specific for Hsp70 was pre-coated

on the wells of the provided Hsp70 Immunoassay plate. Inducible Hsp70 was captured by the immobilized antibody and was detected using an Hsp70 specific rabbit polyclonal antibody. The rabbit polyclonal antibody was subsequently bound by a horseradish peroxidase conjugated anti-rabbit IgG secondary antibody. The assay was developed with Tetramethylbenzidine Substrate and a blue color developed in proportion to the amount of captured Hsp70. The color development was stopped with acid Stop solution, which converted the endpoint color to yellow. The intensity of the color was measured in a microplate reader at 450 nm.

1X Wash buffer

20X Wash Buffer	100 mL
dH_20	1900 mL

IEC-6 cells were treated with chemical inhibitor LY294002 (25 μM) for 1 h, then 15 min with either 0 or 10 mM GLN, heat stressed, and allowed to recover for 3 h. Cells were collected, lysed, and assayed for total protein with BCA (as specified above). Ten μg of protein was used per well, and the ELISA was performed via the manufacturer's instructions. Thereby, Anti-Hsp70 Immunoassay plate, 20X Wash Buffer, Sample Diluent 2, Hsp70 Antibody, Hsp70 Conjugate, Tetramethylbenzidine Substrate, and Stop Solution 2 were brought to RT. Next, an Hsp70 standard curve with 7 points, ranging from 0.78 – 50 ng/mL, was prepared with Hsp70 Standard and Sample Diluent 2. Additionally, a standard blank was prepared, which only consisted of Sample Diluent and represent 0 ng/mL. Then, 100 μL standard and samples were added into wells of the Anti-Hsp70 Immunoassay plate in duplicate and subsequently covered at RT for 2 h. After incubation for 2h, the wells were washed 4 x with 1X wash buffer. After this and every following wash, the liquid was aspirated from all wells by inverting the plate and carefully patting them dry on a clean paper towel. One hundred μL Hsp70 antibody was added to each well except the standard blank. Hereafter, the plate was covered and incubated at RT for 1 h. The plate was washed 4 x with 1X Wash buffer. Next 100 μL Hsp70 Conjugate was pipetted into each well except the standard blank, incubated

for 1 h and washed 4 x. Finally, 100 µL Tetramethylbenzidine Substrate was added to each well (color change was visible after 1 min) and incubated for 30 min before 100 µL Stop Solution was pipetted. The absorbance of the plate was measured in a microplate reader (Thermo Electron Corporation, San Jose, CA, USA) at 450 nm. Hsp70 concentrations contained in the samples were quantified by interpolating absorbance readings from a standard curve generated with the calibrated Hsp70 protein standard. The value of the standard blank was subtracted from all sample concentrations.

8 Confocal fluorescence microscopy

Used materials:	**Company; Registered office**
HNa_2O_4P	Sigma-Aldrich, St. Louis, MO, USA
NaH_2PO_4	Sigma-Aldrich, St. Louis, MO, USA
NaCl	Sigma-Aldrich, St. Louis, MO, USA
PFA	Sigma-Aldrich, St. Louis, MO, USA
Acetone	Fisher Scientific, Pittsburgh, USA
Methanol	Fisher Scientific, Pittsburgh, USA
NDS	Jackson Immuno Research, West Grove, PA, USA
BSA	Sigma-Aldrich, St. Louis, MO, USA
PBS	Cellgro Mediatech Inc., Herndon, VA, USA
NaN_3	Sigma-Aldrich, St. Louis, MO, USA
O-phenylenediamine	Sigma-Aldrich, St. Louis, MO, USA
Glycerol	Sigma-Aldrich, St. Louis, MO, USA
Alexa Fluor 488 phalloidin	Invitrogen Inc., Carlsbad, CA, USA
Bis-benzimide	Sigma-Aldrich, St. Louis, MO, USA
4 well chamber slides	Fisher Scientific, Pittsburgh, USA
Glass Slides	Fisher Scientific, Pittsburgh, USA
Confocal microscope	Carl Zeiss Inc., Thornhood, New York, USA
Intelligent Imaging Innovations SlideBook software	Intelligent Imaging Innovations Inc., Denver, CO, USA
GraphPad Prism 5 for Mac OS X Version 5.0b	GraphPad Software Inc.; San Diego, CA, USA
Cooke CCD SensiCam	Cooke, Eugene, OR, USA
GRGDSP	Bachem, Torrance, CA, USA
GRGESP	Bachem, Torrance, CA, USA
L-Glutamine	Sigma-Aldrich, St. Louis, MO, USA

1X PBS solution (for 10 L)

HNa_2O_4P	58.8 g
NaH_2PO_4	9.54 g
NaCl	90 g
dH_2O	10 L

Fixing solution

PFA	4 % in PBS

Permeabilization solution

Acetone	70 %
Methanol	30 %

Blocking solution

NDS	10 %
BSA	1 % in PBS

Antibody solution

BSA	1 % in PBS

Antiquenching media

NaN_3	20 mg
O-phenylenediamine	10 mg
Glycerol	9 mL
PBS	1 mL

Antibodies for fluorescence microscopy

Staining antibody	**Dilution**
Bis-benzimide (0.1 mg/mL)	1:100
Alexa Fluor 488 phalloidin (6.6 µM)	1:100

8.1 Treatment

To determine GLN´s involvement in cell swelling and actin polymerization, 4 well chamber slides, containing IEC-6 cells, were GLN-starved, treated with GRGDSP (50 µM), GRGESP (50 µM) or control DMEM for 1 h. Then GLN (0, 2, or 10 mM) was added to the specific treatment groups for 15 min. A total of 18 different groups were analyzed: 0 mM GLN (CT), 2 mM GLN, and 10 mM GLN alone and in combination with GRGDSP or GRGESP in either control or HS conditions.

8.2 Fixing, staining, and mounting of slides

After 15 min recovery time in the incubator, the media was gently suctioned off and the cells were fixed with 4 % paraformaldehyde (PFA) in PBS at RT for 15 min. After fixation, cells were washed with PBS three times, permeabilized in ice-cold acetone (70 %) and methanol (30 %) for 10 min, and allowed to air dry. Cells were incubated with blocking solution at RT for 1 h. Blocking solution was removed and slides were stained with Alexa Flour 488 phalloidin for F-actin and bis-benzimide for nuclei in the dark to prevent fluorochrome fading at RT for 1 h. After three 10 min PBS washing steps, the slides were mounted in antiquenching media and coverslips were sealed with clear nail polish. Slides were stored at -20°C before they were examined for cell area size and F-actin morphology.

8.3 Fluorescence microscopy and post-processing

All images were acquired with a Zeiss Axiovert with appropriate filter set, using a 40X oil immersion objective. Images were acquired with a Cooke CCD SensiCam camera and were evaluated using Intelligent Imaging Innovations SlideBook software. Images were post-processed with Adobe Photoshop CS3 extended software (version 10.0). Image acquisition and analysis of F-actin by fluorescence microscopy was performed avoiding artifacts induced by changes in exposure time, camera gain, and lamp intensity. Importantly, all settings were

adjusted to avoid overexposure, while still providing a strong signal. This was achieved by optimizing the exposure time, based on a field with average stain intensity on all three channels. Furthermore, images were only compared to images taken during the same session. For F-actin analysis, cells were always stained with Alexa Fluor 488 phalloidin and visualized in the green channel. The blue channel showed the nuclei. Typically, ten to twenty cells, depending on the cell size, fit on one image. Five to six images were taken for each group to achieve the required 50 cells per group for each experiment. The experiment was repeated three times with 2 mM GLN and four times with 10 mM GLN.

8.4 Analysis of cell area size and F-actin

Images were analyzed with Slidebook 3.0 software using the following procedure: The draw tool was selected and each individual cell was marked. For cell area size changes, total cell area statistics were exported from Slidebook into GraphPad Prism 5. Cell area size was averaged and standard deviation and standard error were calculated for each group. For qualitative analysis, F-actin changes in structural stability and depolymerization were evaluated. Fifty cells per group were evaluated for all three (for 2 mM GLN) or four (for 10 mM GLN) experiments.

9 LC-MS/MS

Used materials:	Company; Registered office
Acetonitrile-HPLC grade	Sigma-Aldrich, St. Louis, MO, USA
Methanol-HPLC grade	Sigma-Aldrich, St. Louis, MO, USA
Cell Scraper	Fisher Scientific, Pittsburgh, USA
PBS	Cellgro Mediatech Inc., Herndon, VA, USA
Stable-isotope labeled glutamine U-^{13}C5-glutamine	Cambridge Isotope, Inc., Andover, MA
L-Glutamine	Sigma-Aldrich, St. Louis, MO, USA
Glutamate	Sigma-Aldrich, St. Louis, MO, USA
Glucose	Sigma-Aldrich, St. Louis, MO, USA
Leap CTC Autosampler	Leap Technologies, Carrboro, NC, USA
Agilent 1100 HPLC	Agilent Technologies, Santa Clara, CA, USA
Synergi Polar 4u column	Phenomenex, Torrance, CA, USA
TFA	Sigma-Aldrich, St. Louis, MO, USA
Ammonium acetate	Sigma-Aldrich, St. Louis, MO, USA
API4000 triple–stage quadrupole MS system	Applied Biosystems Inc, Concord, ON, Canada
Analyst software 1.4.2	Applied Biosystems Inc, Concord, ON, Canada
GRGDSP	Bachem, Torrance, CA, USA
GRGESP	Bachem, Torrance, CA, USA
Nexcelom Cellometer^{TM} Auto T4	BD Bioscience, San Jose, CA, USA

An API4000 triple-stage quadrupole MS system coupled to an Agilent 1100 HPLC and a Leap CTC Autosampler was used for the LC-MS/MS analysis of intracellular GLN and Glutamate concentrations in IEC-6 cells. LC-MS/MS is a bioanalytical technology that combines the physical separation capabilities of liquid chromatography (or HPLC) with the sensitivity and specificity of mass spectrometry detection.

9.1 Treatment

After IEC-6 cells were GLN-starved, they were treated with and without 10 mM GLN for 15 min, with or without prior 1 h treatment with 50 µM GRGDSP or 50 µM GRGESP and subjected to HS.

9.2 Preparation of standards and samples

Extraction/ Protein precipitation solution

Acetonitrile-HPLC grade	10 %
Methanol-HPLC grade	90 %

Standard preparation

Compound stock solutions of 10 mM GLN, 10 mM glutamate, 10 mM glucose and 1 µM of the internal standard U-^{13}C5-glutamine were prepared in acetonitrile/water (10/90) solvent mixture. For the preparation of the calibration standards, GLN, glutamate, and glucose were diluted 1:10 (1 mM) and internal standard was added. A 15 step calibration curve was prepared with the following dilutions: 0 µM ; 0.01 µM; 0.05 µM; 0.1 µM; 0.25 µM; 0.5 µM; 1 µM; 2.5 µM; 5 µM; 10 µM; 25 µM; 50 µM; 100 µM; 250 µM; 500 µM.

Sample preparation:

For quantitation of amino acids in cell extracts, cell monolayers in 10 cm dishes were washed five times with ice-cold PBS after 15 min recovery time following HS, scraped into PBS (1000 µL), and centrifuged (14,000 rpm, 10 min) to remove cell debris. PBS was discarded, and 1 mL fresh PBS was added. One hundred µL was taken for a Nexcelom cell count; the rest was centrifuged again (14,000 rpm, 10 min) and PBS was discarded. Five hundred µL of acetonitrile/methanol mix was added to the cell pellets, snap frozen two times, and sonicated on ice for 5 min and stored at -80ºC until LC-MS/MS measurement. For LC-MS/MS measurement, the samples were diluted 1:10 (v/v) with the acetonitrile/water (10/90, v/v) solvent mixture containing the internal standard (1 µM). After centrifugation, 20 µL of the diluted samples were injected and analyzed by LC-MS/MS.

9.3 Method development

The majority of currently available analytical methods use lengthy derivatization processes prior to quantitation of amino acids in biological matrices. Recently published methods have described the use of perfluorinated acids as ion-pair agents on C18 columns without the requirement for special columns or pre-/post-column derivatization [10, 118]. In order to avoid the process of amino acid derivatization and prevent potential interferences between GLN and glutamate, several HPLC columns were tested during the method development phase with the help of Dr. Jelena Klawitter. The best chromatographic separation was achieved on a Synergi Polar 4u column (250 x 3 mm, 4 µm particle size) and representative ion chromatograms are shown in the following figure.

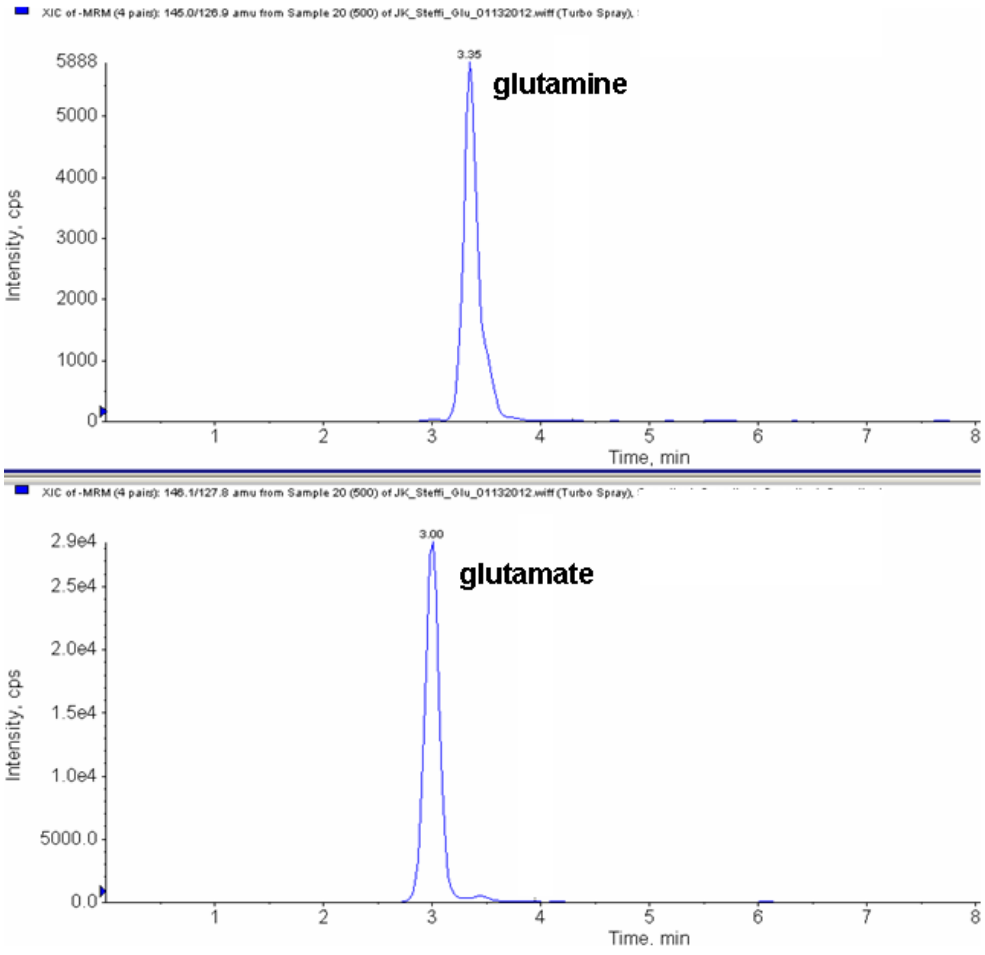


Fig. 5. Ion chromatograms for L-glutamine and glutamate

9.4 HPLC-chromatographic procedure

As shown in Fig. 5 the amino acids were baseline HPLC separated on a Synergi Polar 4u column (250 x 3 mm, 4 µm particle size). The LC mobile phases consisted of 5 mM ammonium acetate supplemented with 0.05 % trifluoric acid (TFA) in water (mobile A) and methanol (mobile B). The following LC gradient was run:

Table 1: LC-gradient

Time [min]	A: ammonium acetate with 0.05% TFA	B: methanol [%]
0	99	1
0.8	99	1
3	94	6
5	50	50
6	99	1

Abbreviations: TFA: trifluoric acid

9.5 MS/MS

MS data was acquired in negative electrospray ionization (ESI) mode with an ion voltage of -4.2 kV. Nebulizer gas was at 40 (arbitrary units, ABSciex Analyst software), and so was the heater gas (both nitrogen). The collision gas (CAD) and curtain gas (CUR) were at 12 and 15, respectively (both nitrogen, all arbitrary units). The source temperature was kept at 400°C.

Table 2: MS-parameters

CUR	15 units
GS1	40 units
GS2	40 units
IS	-4.2 kV
Temperature	400°C
CAD	12 units

Abbreviations: CUR: curtain gas; GS1: gas 1; GS2: gas 2; IS: ionization voltage; CAD: collision gas

The multiple reaction monitoring (MRM) transitions as well as the MS specifics were as follows:

Table 3: MRM-parameters

Compound	Transition	DP [V]	CE [V]	EP [V]	EXP [V]
glutamine	**145.1→126.9**	**-69**	**-15**	**-10**	**-15**
glutamate	**146.1→127.8**	**-55**	**-16**	**-10**	**-15**
glucose	**179.1→88.9**	**-75**	**-11**	**-10**	**-15**
U-13C^5-glutamine	**149.1→113.9**	**-58**	**-18**	**-10**	**-15**

Abbreviations: DP: declustering potential; CE: collision energy; EP: entrance potential; EXP: exit potential

9.6 Quantification

Data were acquired and processed for calibration and quantification of all analytes using the AB Sciex Analyst software 1.4.2. After sample analysis, the analytes were normalized based on cell number counts as measured by using a Nexcelom Cellometer™ Auto T4.

10 Statistical analysis, illustrations, and references

Used materials:	Product No.; Company; Registered office
GraphPad Prism 5 for Mac OS X Version 5.0b	GraphPad Software Inc.; San Diego, CA, USA
Microsoft Office 2008 Version 12.2.4	Microsoft Corporation; Redmond, WA, USA
EndNote X3	Thomson Reuters; Philadelphia, PA, USA
Adobe Illustrator CS4 Version 14.0.0	Adobe Systems GmbH; Munich, Germany
Adobe Photoshop CS3 extended software (version 10.0).	Adobe Systems GmbH; Munich, Germany

All data analysis, spreadsheet calculations, and graphs were generated with Microsoft Excel for Mac 2008 (version 12.2.4), and GraphPad Prism 5 for Mac OS X (version 5.0b). For the statistical analysis, one-way ANOVA, followed by Turkey's post-hoc test was used, whenever multiple groups were compared, and two-tailed student's *t*-test was used, when two groups were compared. If not mentioned otherwise, data are given as means±SEM (number of experiments) and differences were considered significant at $P < .05$. All panels were created with Adobe Photoshop CS3 extended software (version 10.0). Microsoft Word for Mac 2008 (version 12.2.4) was used for text processing and page layout, all figures were created using Adobe Illustrator CS4 (version 14.0.0), and references were included using EndNote X3.

C Results

1 Involvement of FN-Integrin interaction in GLN's protective mechanism

The majority of the following data about the involvement of FN-Integrin interaction in GLN's protective mechanism was published in *PLoS One* [108]. Certain sections have already been investigated in my master thesis and will be pointed out.

1.1 GLN-mediated protection via expression of FN after HS

FN expression is important for the maintenance of normal epithelial integrity as well as for the regulation of epithelial response to intestinal injury [85]. Therefore, we investigated what effect HS or HS in combination with GLN treatment had on FN expression in IEC-6 cells. FN expression was significantly reduced by 50 % in IEC-6 cells immediately following HS (P< .05) and by 85 % at 3 h post-HS (P< .001). GLN treatment (10 mM) prevented FN expression directly after HS (P< .05) and 3 h post-HS (P< .001) (Fig. 6A and B). GLN-mediated preservation of FN expression was associated with a reduction of cleaved caspase-3 levels, indicating a reduction in cellular apoptosis (Fig. 6D and E). To demonstrate that FN expression is important in GLN-mediated protection of IEC-6 cells, cells were transfected with 20 nM FN siRNA. Fig. 6C shows a 50 % decrease in FN expression after FN siRNA treatment (P< .001). Attenuation of FN expression via siRNA led to a significant decrease of GLN-mediated protection shown by increases of cleaved caspase-3 levels following HS (P< .001) (Fig. 6D and E). This indicates GLN-mediated protection against apoptosis after HS was lost when FN expression was attenuated.

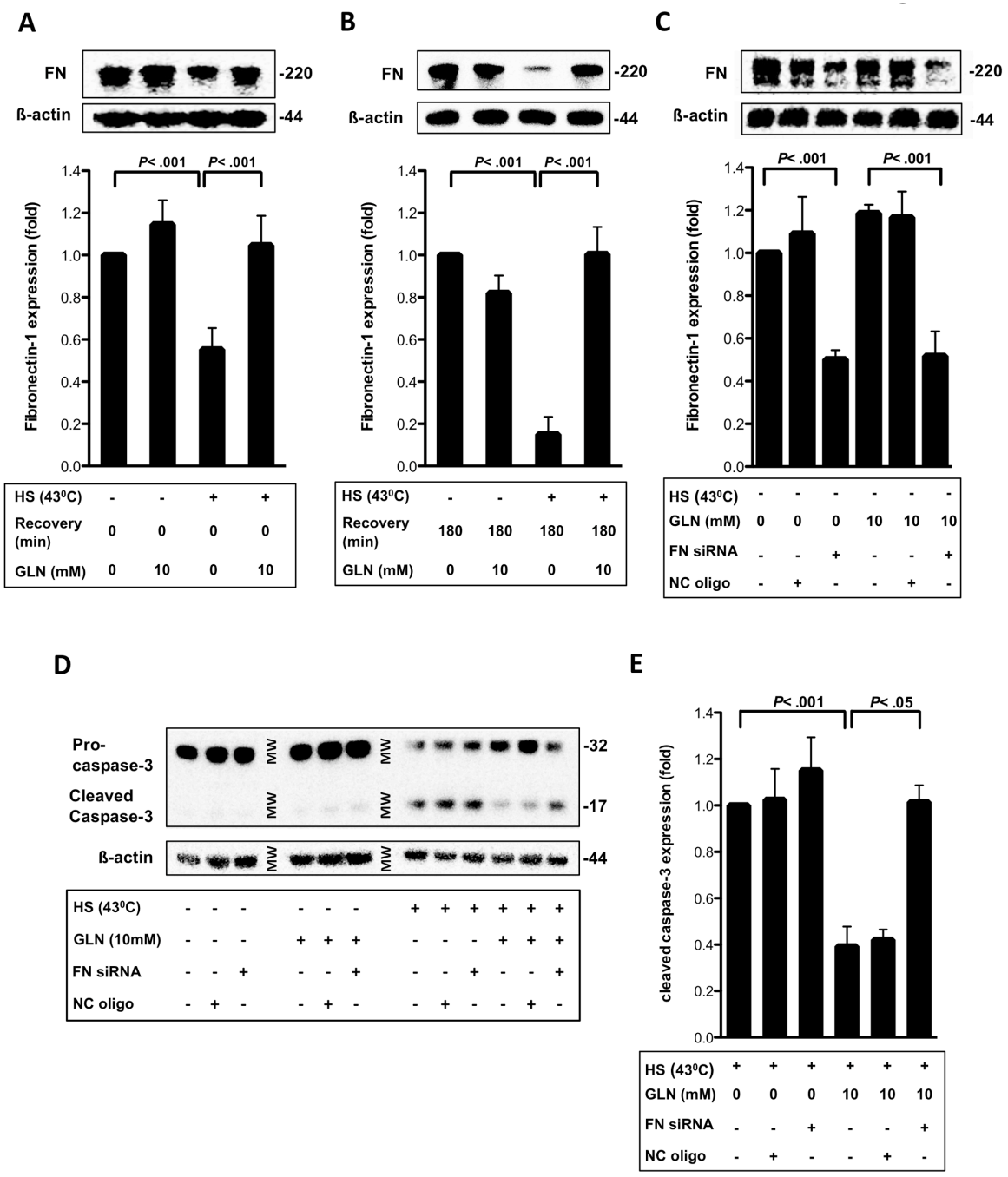
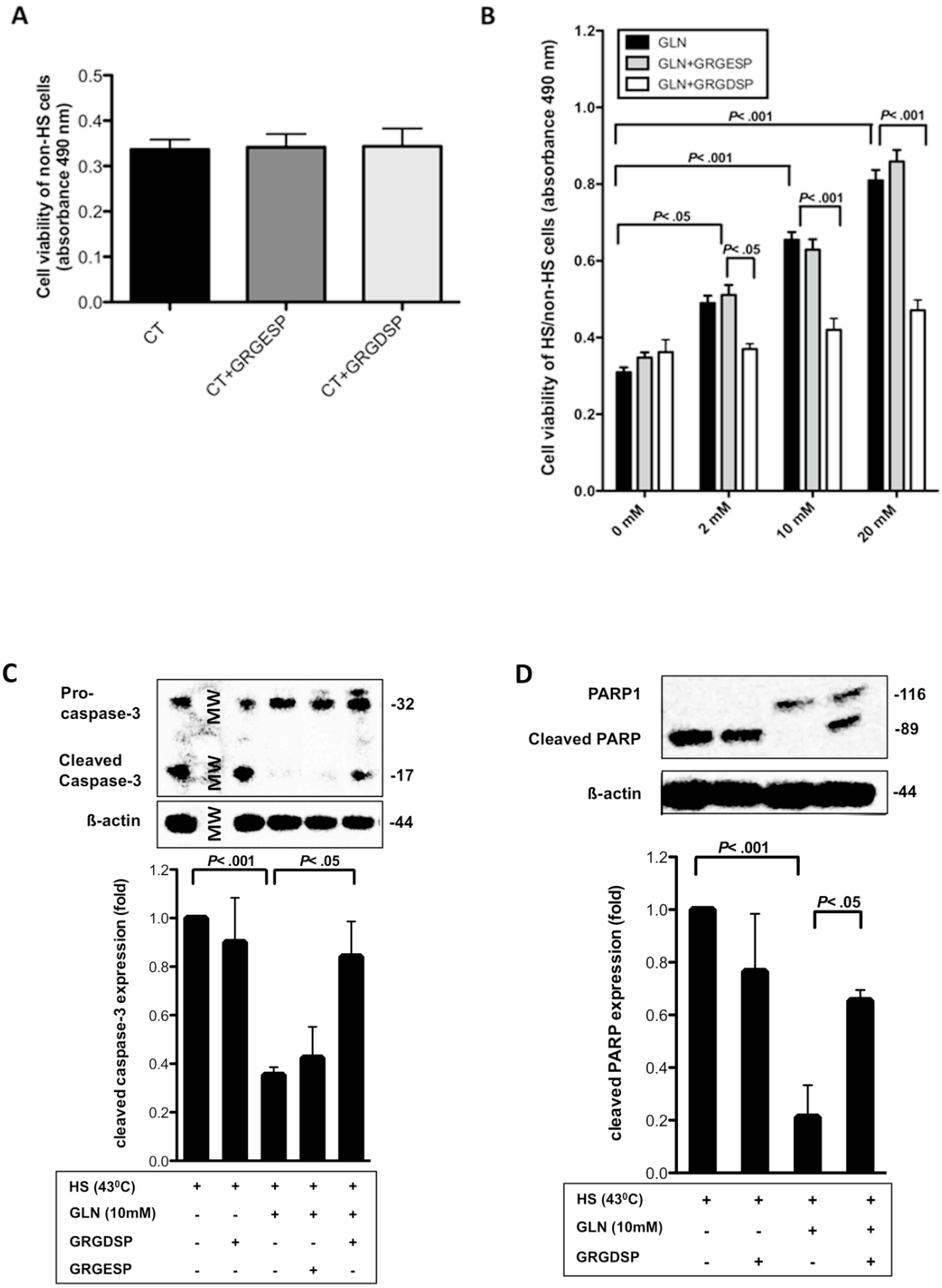


Fig. 6. GLN is protective by preventing FN expression after HS. A) FN levels in IEC-6 cells with or without GLN (10 mM) treatment under unstressed (37°C) or stressed conditions (43°C) were determined by Western blot with ß-actin as loading control after 0 h recovery. Densitometric analysis of FN expression is presented as mean fold change relative to 0 mM GLN cells±SEM (n=4). B) Same treatment as described in Fig. 6A. Western blot and densitometric analysis of FN after 3 h recovery is shown (n=4). C) FN expression of transfected IEC-6 cells with no siRNA, NC siRNA (20 nM) or FN siRNA (20 nM) with or without GLN (10 mM) treatment are presented (n=3). D) Caspase-3 and cleaved caspase-3 expression were determined via Western blot under baseline (37°C) and HS conditions (43°C) after IEC-6 cells were transfected with no siRNA, NC siRNA (20 nM) or FN siRNA (20 nM) (n=3). E) Treatment as in Fig. 6D described. Densitometric analysis of cleaved caspase-3 levels is shown as mean fold change relative to HS 0 mM GLN cells ±SEM (n=3).

1.2 Involvement of FN-Integrin interaction in GLN-mediated protection

Since FN contains an Arg-Gly-Asp (RGD) attachment site which can bind to integrin receptors, forming an important recognition system for cell adhesion and survival signaling pathways [131, 149], I investigated in my master thesis whether the FN-Integrin interaction inhibitor GRGDSP had an effect on GLN-mediated cellular protection following lethal HS. Control groups were added to measure any possible toxicity of the inhibitor reagents. In my master thesis, it could be shown that low doses of 1 µM and 10 µM GRGDSP were sufficient to attenuate GLN's protection in IEC-6 cells. Increasing concentrations of GRGDSP (20 µM and 50 µM) further inhibited GLN-mediated protection. Concentrations higher than 75 µM appeared to be toxic in IEC-6 control cells (data not shown). In the following experiments, a concentration of 50 µM GRGDSP was chosen as it showed an optimal inhibitory effect on GLN-mediated protection in IEC-6 cells. As shown in Fig. 7A, GRGDSP alone did not affect cell viability. GLN increased cell survival in HS cells in a dose-dependent manner ($P < .001$). Importantly, GRGDSP completely attenuated GLN's protection at a concentration of 2 mM GLN ($P < .05$). At higher GLN concentrations (10 mM and 20 mM), GRGDSP decreased GLN's protective effect by ~ 85 % ($P < .001$). The control peptide GRGESP did not affect GLN`s protective effect in either group (Fig. 7B). MTS cell survival assays measured mitochondrial dehydrogenases activities, which may be preserved in cells with compromised barriers. In addition, key apoptotic pathway markers were evaluated via Western blotting, confirming MTS cell survival results. HS increased apoptotic markers such as cleaved Caspase-3, Bax/bcl-2 ratio, and cleaved PARP levels ($P < .001$). GLN supplementation attenuated markers of apoptosis significantly after HS, indicating that GLN can improve cell survival and prevent apoptosis during HS (Fig. 7C-E). Specifically, GLN decreased Bax/bcl-2 ratio levels by 45 % ($P < .05$), cleaved PARP levels by 79 % ($P < .001$), and cleaved Caspase-3 levels by 70 % ($P < .001$). However, GRGDSP treatment decreased GLN-mediated anti-apoptotic effects as shown by increased Bax/bcl-2 ratio ($P < .001$), cleaved PARP levels ($P < .05$), and cleaved Caspase-3 levels ($P < .05$) (Fig. 7C-E). This data suggests that in the absence of FN-Integrin signaling, GLN's cellular protection against hyperthermia is lost.


A
Cell viability of non-HS cells (absorbance 490 nm)
0.0
0.1
0.2
0.3
0.4
0.5
CT
CT+GRGESP
CT+GRGDSP
B
Cell viability of HS/non-HS cells (absorbance 490 nm)
GLN
GLN+GRGESP
GLN+GRGDSP
P< .001
P< .001
P< .001
P< .05
P< .05
0 mM
2 mM
10 mM
20 mM
C
Pro-caspase-3
Cleaved Caspase-3
ß-actin
MW
-32
-17
-44
P< .001
P< .05
cleaved caspase-3 expression (fold)
HS (43⁰C)
GLN (10mM)
GRGDSP
GRGESP
D
PARP1
Cleaved PARP
ß-actin
-116
-89
-44
P< .001
P< .05
cleaved PARP expression (fold)
HS (43⁰C)
GLN (10mM)
GRGDSP

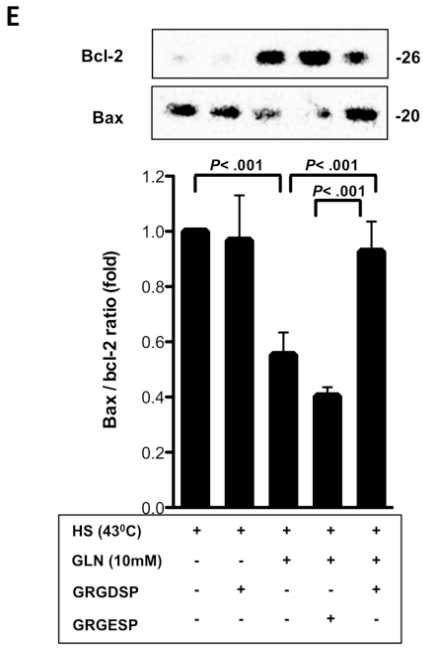


Fig. 7. GLN's intestinal protection is inhibited by GRGDSP. A) IEC-6 cells were treated for 1 h with or without GRGDSP (50 µM) or GRGESP (50 µM). After 24 h incubation at 37°C, cell viability was measured and is presented as mean±SEM (absorbance 490 nm) (n=4). B) IEC-6 cells were treated for 1 h with either media, GRGDSP (50 µM) or GRGESP (50 µM) before the cells were treated with 0, 2, 10 or 20 mM GLN. Cell survival, following lethal HS (44°C), was measured via MTS assay. All groups were normalized to their non-HS controls (37°C) to account for differences in cell growth. Assays were carried out in triplicate, experiments were performed 4 times and are shown as mean±SEM. C) Cells were treated for 1 h with either media, GRGDSP (50 µM) or GRGESP (50 µM) before the cells were treated with 0 mM or 10 mM GLN under stressed conditions (43°C). Procaspase-3, cleaved caspase-3 levels, and ß-actin are shown by Western blot and cleaved Caspase-3 levels are presented as fold change±SEM (n=3). D) IEC-6 cells were treated as described in Fig. 7C. Representative Western blot of PARP1 and cleaved PARP and densitometric analysis of cleaved PARP relative to HS 0 mM GLN are displayed and presented as fold change±SEM (n=4). E) IEC-6 cells were treated as described in Fig. 7C. After 3 h recovery at 37°C, Bax and Bcl-2 levels were measured by Western blot. Bax/Bcl-2 ratio was calculated and is shown as fold change ±SEM (n=4).

1.3 GRGDSP inhibits GLN-mediated increases in cell area size, disrupts F-actin organization, but has no impact on intracellular GLN-concentrations

Since FN-Integrin signaling appeared to be involved in GLN´s mechanism of protection, and integrins are known to be osmosensors [68] linked to the cytoskeleton [9, 157], the effects of

GRGDSP on cell area size and the F-actin cytoskeleton in IEC-6 cells were assessed during my master thesis at the University of Colorado. Fluorescence microscopy was utilized to visualize GLN-mediated cell swelling and cytoskeletal stabilization in the presence and absence of GRGDSP. We analyzed the cell area size of a large number of cells in an attempt to maximize accuracy of findings (50 cells per group in each of the 3-4 experiments). Each cell was masked using Slidebook 3.0 software and total cell area size statistics were exported into GraphPad Prism 5.0b. Based on these cell area measurements, we were able to confirm that GLN treatment (2 mM and 10 mM) caused cell swelling, which was inhibited after GRGDSP treatment in a robust fashion in IEC-6 cells. GLN increased cell area size in non-HS cells by 1.3-fold in the 2 mM GLN group and 1.5-fold in the 10 mM GLN group ($P < .001$). In HS cells, GLN increased cell area size by 1.4-fold in the 2 mM GLN group ($P < .05$) and 1.7-fold in the 10 mM GLN group ($P < .001$). Treatment with GRGDSP fully inhibited GLN-mediated cell swelling in all groups with a slight decrease in cell area size observed in the CT groups ($P < .05$). Control peptide GRGESP had no effect on GLN-mediated cell area size changes (Fig. 8A and B). As reduced intracellular GLN leads to cell shrinkage [113], we next investigated if GRGDSP treatment led to decreased intracellular GLN concentrations in basal (37°C) and stressed conditions. LC-MS/MS results, shown in Fig. 8C, demonstrated that GRGDSP treatment had no impact on intracellular GLN content under baseline (37°C) or stressed conditions ($P < .001$). Besides cell size, F-actin morphology and structure were of crucial interest, because GRGDSP inhibits integrins' binding to the ECM, which seemed to be important for cytoskeletal stabilization. Cytoskeletal structures, such as F-actin, play an important role in inflammation and in determining cell shape and cell size [50, 81]. To further investigate possible causes of cell-shrinkage following GRGDSP treatment, fluorescent staining of F-actin cytoskeleton by confocal microscopy revealed its intracellular disruption after HS and after GRGDSP treatment. In unstressed cells with 0 mM GLN and 10 mM GLN and GLN-treated cells post-HS F-actin was intact, continuous, and smooth. In contrast, after GRGDSP treatment in 0 mM and 10 mM GLN treated groups, F-actin appeared to be fragmented, disrupted, disorganized, and collapsed (arrows) (Fig. 8D).

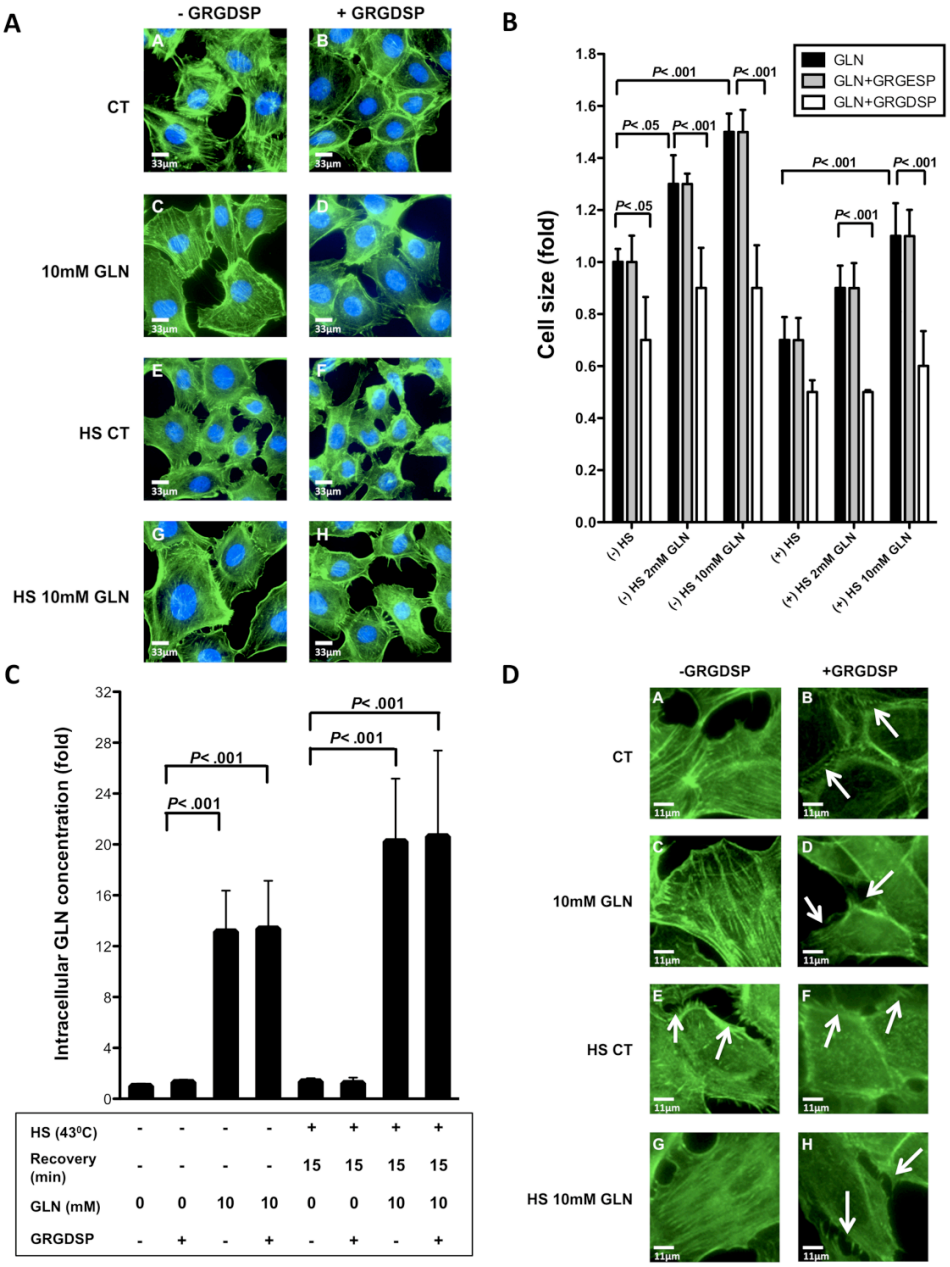


Fig. 8. GRGDSP's effect on cell area size, F-actin morphology, and intracellular GLN concentrations. A) Cell area size is shown by fluorescence microscopy in IEC-6 cells under 0 mM GLN conditions and 10 mM GLN treatment without inhibitor GRGDSP (50 µM) (Images A, C, E, G) or after 1 h prior treatment with GRGDSP (50 µM) (Images B, D, F, H) in basal (37°C) and stressed conditions (43°C). F-actin is shown in green and nuclei in blue. All images were taken 15 min following exposure of cells to GLN (10 mM) in non-HS cells and 15 min post-HS in stressed cells. All images were acquired using the same exposure times and were normalized for optical comparison (n=4). Scalebars = 33 µm. B) Cell area size data are shown in fold increase under non-HS (37°C) and HS (43°C) conditions (n=3-4). C) IEC-6 cells were treated as described in Fig. 8A. GLN levels in cell extracts were quantified using LC-MS/MS and are shown as fold change±SEM as compared to the 0 mM GLN group (n=6-7). D) Fluorescence microscopy was used for the morphological analysis of the distribution of F-actin. Microfilaments were visualized with Alexa Flour 488 phalloidin antibody. Representative results are shown from 4 independent fluorescence microscopy experiments for each condition. Scalebars: 11 µm.

1.4 GLN is protective by activating ERK1/2 via FN-Integrin pathway

To explore alternative signaling pathways known to be vital to HS-mediated survival pathways and affected by integrin signaling [133], we examined GLN's effect on ERK1/2 activation. I investigated whether the two ERK1/2 kinase inhibitors PD98059 and UO126 had an effect on GLN-mediated protection in heat-stressed IEC-6 cells. Control groups were added to measure any possible toxicity of the inhibitor reagents. Dose response experiments were performed and showed that 50 μM PD98059 and 30 μM UO126 had an optimal inhibitory effect on GLN-mediated protection in IEC-6 cells (data not shown). After proving that 50 μM PD98059 inhibited ERK1/2 activation (Fig. 9A) and was not toxic to non-HS cells (Fig. 9B), it could be demonstrated that inactivation of ERK1/2 inhibited GLN-mediated protection (Fig. 9C). By using another well-known ERK1/2 inhibitor, UO126 (30 μM), which was not toxic to non-HS cells (Fig. 9B), the same MTS results were obtained (Fig. 9C).

By inhibiting ERK1/2 kinase with 50 μM PD98059, we confirmed in this study via evaluation of cleaved caspase-3 and cleaved PARP that ERK1/2 activation is important in GLN's protective mechanism (Fig. 9D and E). GLN supplementation decreased cleaved caspase-3 ($P < .001$) and cleaved PARP levels after HS. However, PD98059 (50 μM) treatment attenuated GLN-mediated anti-apoptotic effects as demonstrated by increased cleaved caspase-3 ($P < .05$) and cleaved PARP levels (Fig. 9D and E).

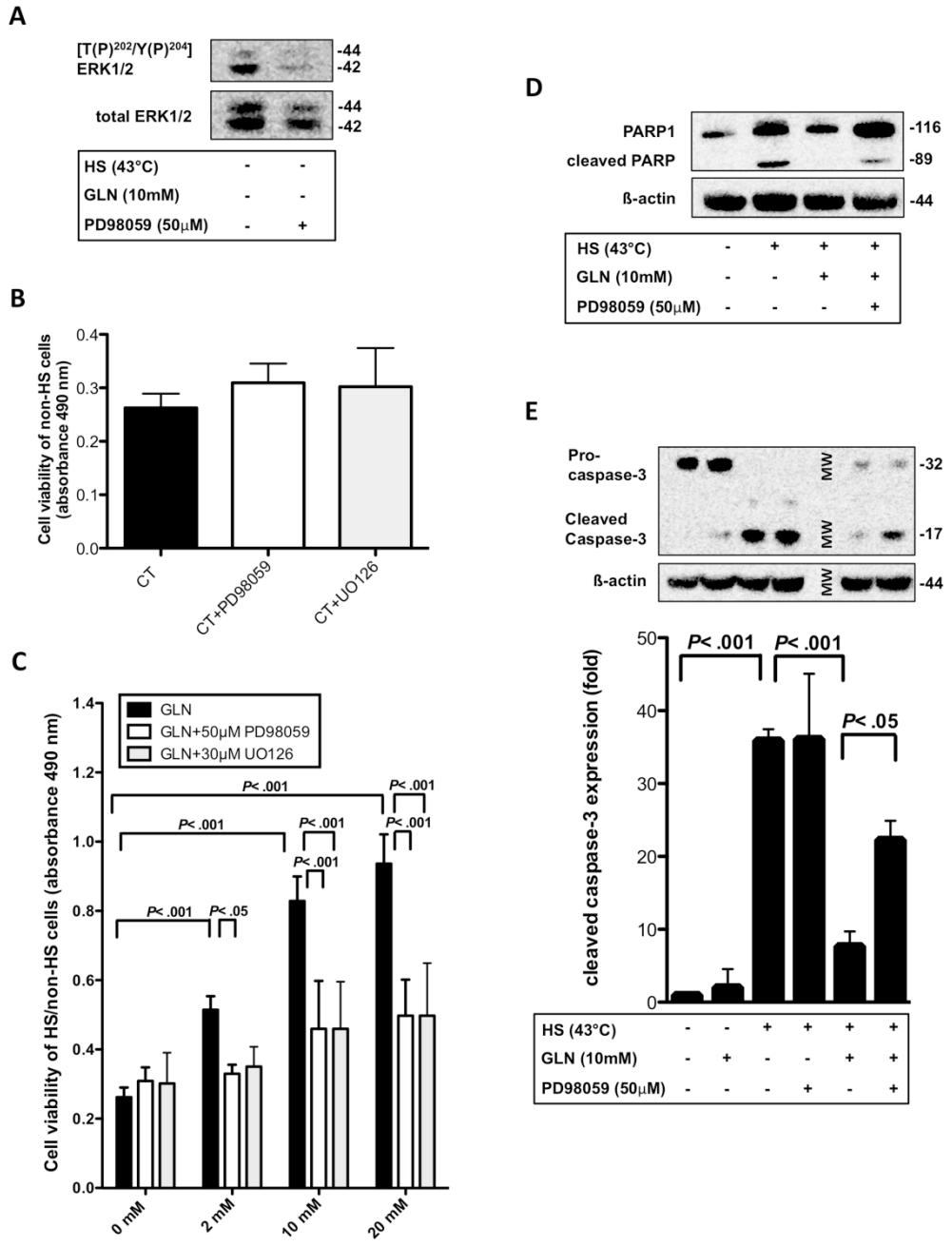


Fig. 9. ERK1/2 activation is involved in GLN's protective mechanism. A) IEC-6 cells were treated either with the ERK1/2 kinase inhibitor PD98059 (50μM) for 1 h or simply DMEM media without GLN under basal conditions (37°C). The inhibitory effect of PD98059 (50 μM) is represented in a Western blot of [T(P)^{202}/Y(P)^{204}]ERK1/2 and total ERK1/2 levels. B) Cells were treated for 1 h with either PD98059 (50 μM) or UO126 (30 μM). After 24 h incubation time at 37°C, cell viability was measured and represented as mean±SEM (absorbance 490 nm) (n=3). C) IEC-6 cells were treated with different concentrations of GLN (0, 2, 10, and 20 mM) with or without 1 h PD98059 (50 μM) or UO126 (30 μM) pre-treatment. Cell survival was measured via MTS assay. Results are shown as mean±SEM (n=3). D) IEC-6 cells were treated with 0 mM or 10 mM GLN with or without PD98059 (50 μM) 1 h pre-treatment under baseline (37°C) and stressed conditions (43°C). Representative Western blots show PARP1 and cleaved PARP levels (n=2). E) Cells were treated as described in Fig. 9D. Representative Western blots from 3 independent experiments show Procaspase-3, cleaved caspase-3 levels and densitometric analysis of cleaved caspase-3 (n=3).

Furthermore, Western blots of [T(P)^{202}/Y(P)^{204}]ERK1/2 relative to total ERK1/2 showed that HS increased phosphorylated ERK1/2 by 3-fold. GLN treatment further elevated ERK1/2 phosphorylation by 6-fold after HS (*P*< .001). Importantly, this GLN-mediated increase in ERK1/2 phosphorylation was inhibited after GRGDSP treatment (*P*< .05) (Fig. 10).

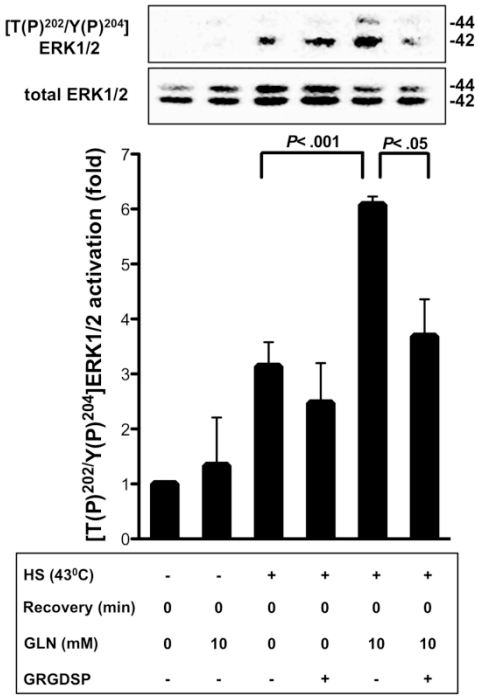


Fig. 10. GRGDSP attenuates GLN-mediated ERK1/2 activation after HS. Cells were treated for 1 h with either media, or GRGDSP (50 μM) before the cells were treated with 0 mM or 10 mM GLN under baseline (37°C) and stressed conditions (43°C). [T(P)^{202}/Y(P)^{204}]ERK1/2 and total ERK1/2 levels were determined by Western blot analysis after non-stressed (37°C) and stressed conditions (43°C) without recovery. ERK1/2 activation is shown as mean fold change relative to total ERK1/2±SEM and graphed relative to 0 mM GLN (n=3).

1.5 GLN-mediated HSF-1 activation and expression is dependent on FN-Integrin/ERK1/2 signaling

Since both ERK1/2 and HSF-1 phosphorylation are important in cell survival after HS [104, 177], we investigated if ERK1/2 and FN-Integrin signaling played a role in HSF-1 activation and expression. We initially looked at total HSF-1 expression and found that GLN could enhance total HSF-1 expression (P< .001). GLN-mediated increases in HSF-1 expression after HS and 3 h post-recovery were attenuated after GRGDSP (50 μM) (P< .05), but not by GRGESP (50 μM) (Fig. 11C). Next, we investigated HSF-1 phosphorylation at the phosphorylation site serine 303 after GRGDSP and PD98059 (50 μM) treatment since previous research indicated that ERK1/2 may participate in the activation of HSF-1, leading

to the induction of HSP expression [79]. HSF-1, when phosphorylated at serine 303, is repressed and found as a monomer. Upon activation, HSF-1 forms trimers, gains DNA binding activity and translocates to the nucleus [71]. In non-HS cells and HS cells treated with GLN or GLN+negative control GRGESP, HSF-1 is not phosphorylated at serine 303. However, in HS cells without GLN, and HS cells treated with GLN+GRGDSP and GLN+PD98059, HSF-1 undergoes inhibitory phosphorylation at serine 303 (P< .05) (Fig. 11A and B).

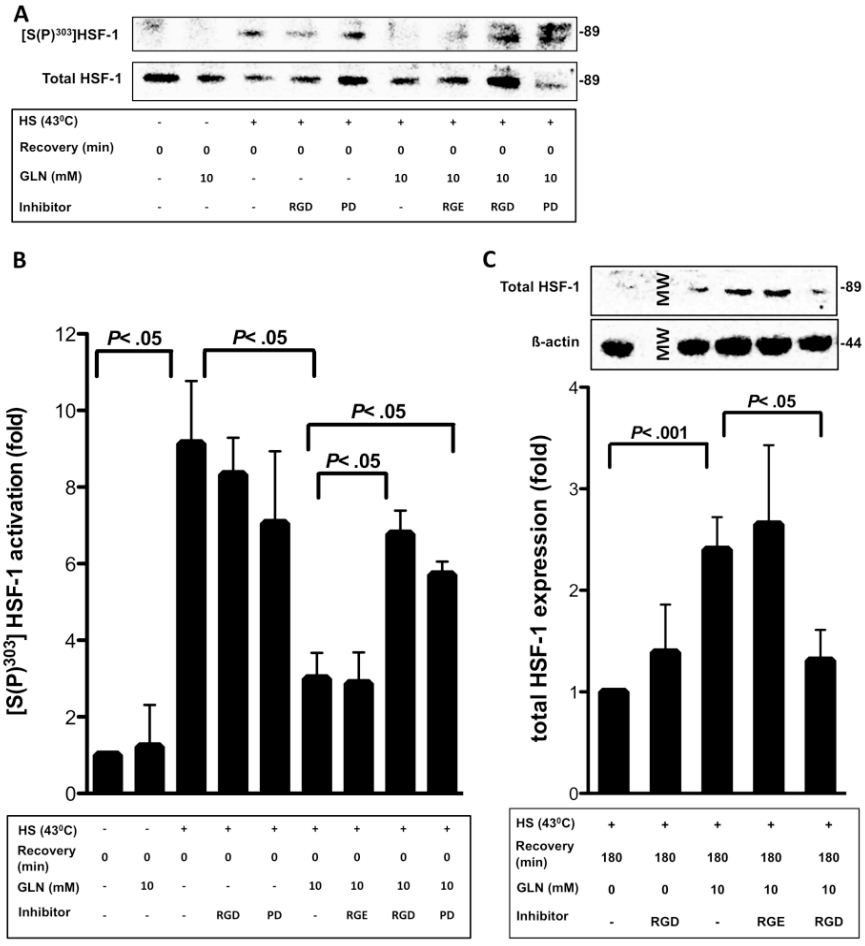


Fig. 11. FN-Integrin signaling inhibitor GRGDSP and ERK1/2 kinase inhibitor PD98059 attenuate GLN-mediated activation of HSF-1. A) IEC-6 cells were treated with 0 mM or 10 mM GLN with or without PD98059 (50 µM), GRGDSP (50 µM) or GRGESP (50 µM) 1 h pre-treatment. A representative Western blot from 3 independent experiments shows [S(P)^{303}]HSF-1 and total HSF-1 levels. B) Densitometric analysis of HSF-1 phosphorylation at serine 303 was measured as mean fold change relative to total HSF-1±SEM (n=3). C) Cells were treated for 1 h with either media, GRGDSP (50 µM) or GRGESP (50 µM) before the cells were treated with 0 mM or 10 mM GLN under stressed conditions (43°C). Total HSF-1 levels were measured after 3 h recovery after non-lethal HS (43°C). Results are displayed as fold change±SEM relative to HS 0 mM GLN groups (n=3).

1.6 GRGDSP attenuates increases in GLN-mediated HSP expression

Enhanced nuclear translocation of HSF-1 is required for augmented Hsp70 expression [60]. Our laboratory has shown that GLN's cytoprotective effect is, at least in part, mediated by increased Hsp70 expression [170]. To determine the effect of GRGDSP (50 μM) and PD98059 (50 μM) on GLN-mediated Hsp70 expression, we examined the expression of Hsp70 after HS in IEC-6 cells. As shown in Fig. 12A and B, IEC-6 cells treated with GLN significantly increased Hsp70 expression after HS ($P < .05$). Cells treated with GRGDSP (50 μM) and PD98059 (50 μM), but not GRGESP (50 μM), showed a significant decrease in GLN-mediated Hsp70 expression ($P < .05$).

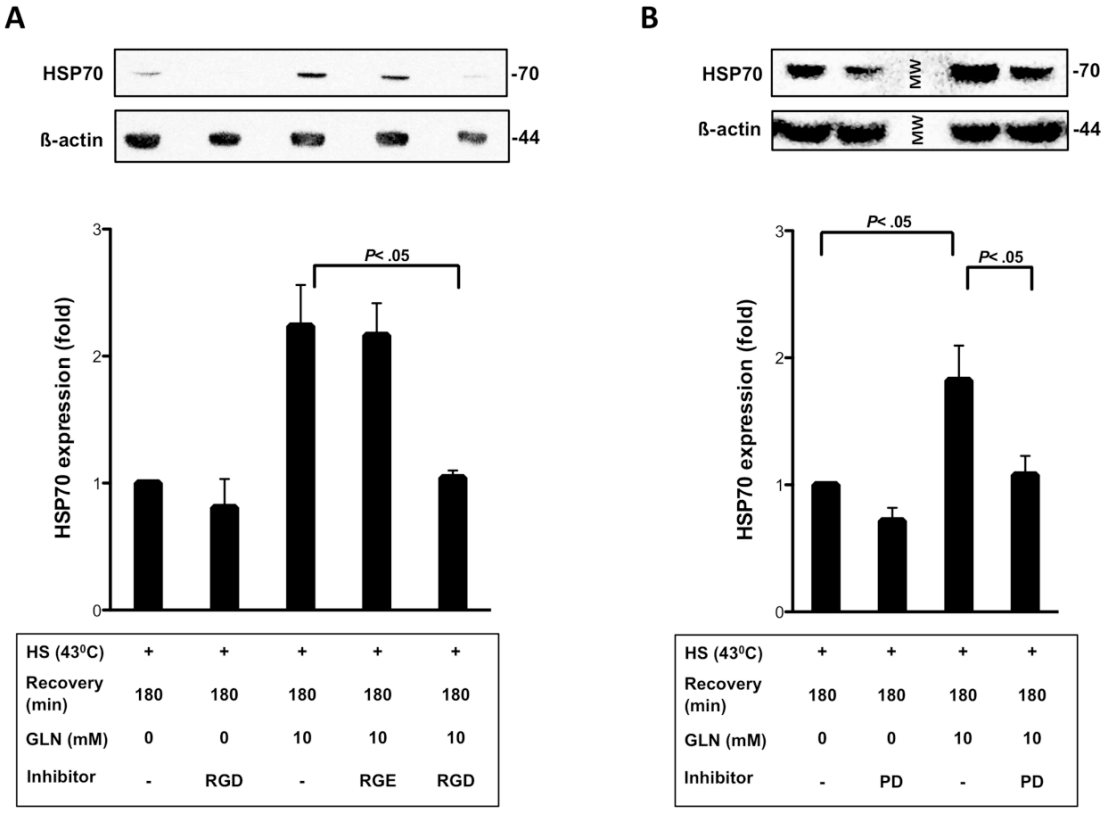


Fig. 12. GRGDSP and PD98059 affect GLN-mediated increases in Hsp70 expression. A) IEC-6 cells were treated for 1 h with either media, GRGDSP (50 μM) or GRGESP (50 μM) before the cells were treated with 0 mM or 10 mM GLN under stressed conditions (43°C). Hsp70 expression was determined by Western blot analysis. In addition, ß-actin was monitored to normalize total blotted protein. Data are shown as mean fold change relative to HS 0 mM GLN±SEM (n=5). B) Western blot of Hsp70 and ß-actin are shown after PD98059 (50 μM) treatment and HS (43°C). Results are presented relative to HS 0 mM GLN groups and represent mean±SEM (n=3).

Another recently discovered cytoprotective effect of increased cellular GLN concentration is the induction of Hsp32 expression [151]. Here, we show that GLN increased Hsp32 expression by 4-fold after HS. GRGDSP (50 µM), but not GRGESP (50 µM), decreased this enhancement in Hsp32 expression by 70 % (*P*< .05) (Fig. 13).

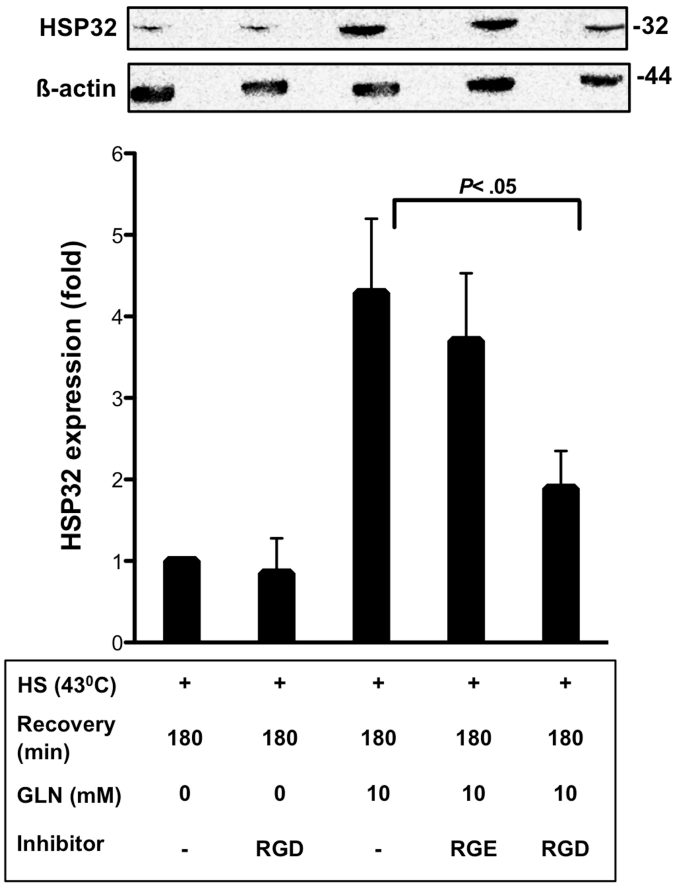


Fig. 13. GRGDSP affects GLN-mediated increases in Hsp32 expression. IEC-6 cells were treated with 0 mM or 10 mM GLN with or without 1 h GRGDSP (50 µM) or GRGESP (50 µM) pre-treatment. Cells then underwent non-lethal HS (43°C). Western blot of Hsp32 and ß-actin are shown. Results are shown relative to HS 0 mM GLN groups and represent mean±SEM (n=3).

1.7 GLN-mediated dephosphorylation of p38MAPK via FN-Integrin pathway

P38MAPK is considered to be a stress-related kinase. However, since p38MAPK's pro- or anti-apoptotic functions appear to be dependent on the cell type and cellular content [88, 99], we examined its role in GLN's protective mechanism in IEC-6 cells after hyperthermia. Lower doses of 10 μM SB203580 were already inhibiting p38MAPK phosphorylation (data not shown), however, doses starting at 10 μM were able to increase cell survival in heat-stressed control groups ($P < .05$) (Fig. 14B). Therefore, 10 μM SB203580 was used in the following experiments.

After proving that 10 μM SB203580 was inhibiting p38MAPK phosphorylation (Fig. 14A), MTS assays in IEC-6 cells revealed that 10 μM SB203580 increased cell survival in heat-stressed control groups ($P < .05$) (Fig. 14B). Furthermore, GLN was still protective when IEC-6 cells were treated with GLN+10 μM SB203580 (Fig. 14C), suggesting that dephosphorylation of p38MAPK regulates cell survival.

FN-Integrin-ERK1/2-Hsp70 osmosignaling was shown to be essential in GLN-mediated cellular protection in IEC-6 cells after HS in the experiments above and in our recent PLoS One publication [108]. In this study, I was interested in as to whether p38MAPK signaling was regulated via GLN-mediated FN-Integrin signaling as well. Thus, I investigated if FN-Integrin signaling is essential in GLN-mediated p38MAPK dephosphorylation. I wanted to see if GRGDSP was able to increase p38MAPK activation after GLN (10 mM) treatment. The results shown in Fig. 14D revealed that HS increased p38MAPK phosphorylation ($P < .05$) and 10 mM GLN significantly attenuated its phosphorylation ($P < .05$). GRGDSP (50 μM) altered GLN-mediated p38MAPK phosphorylation ($P < .05$), indicating involvement of FN-Integrin signaling in GLN-mediated p38MAPK signaling (Fig. 14D).

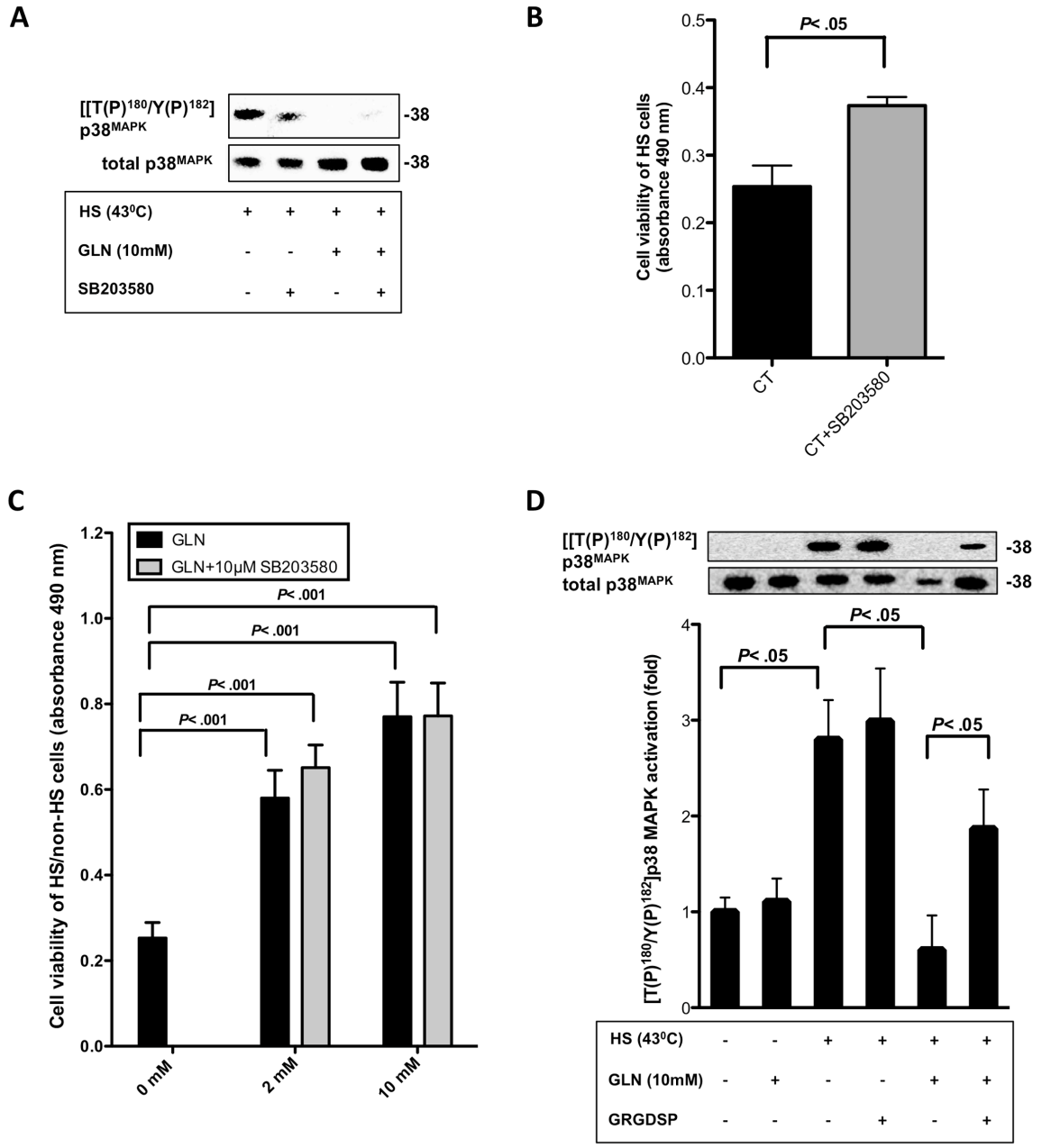


Fig. 14. GLN is protective by dephosphorylating p38MAPK via FN-Integrin signaling. A) Cells were treated with 0 mM or 10 mM GLN with or without SB203580 (10 µM) 1 h prior treatment under stressed conditions (43°C). A representative Western blot of [T(P)^{180}/Y(P)^{182}]p38MAPK and total p38MAPK levels is shown. B) IEC-6 cells were treated with/without 1 h SB203580 (10 µM) pre-treatment under stressed conditions (44°C). Cell survival was measured via MTS assay. Results are shown as mean±SEM (n=4). C) Cells were treated with different concentrations of GLN (0, 2, and 10 mM) with or without 1 h prior SB203580 (10 µM) treatment under basal (37°C) and stressed conditions (44°C). Cell survival was measured via MTS assay. Results are shown as mean±SEM (n=4). D) A representative Western blot of [T(P)^{180}/Y(P)^{182}]p38MAPK and total p38MAPK levels is shown after GRGDSP (50 µM) and GLN (10 mM) treatment under baseline (37°C) and stressed conditions (43°C). P38MAPK phosphorylation is presented as mean fold change relative to total p38MAPK±SEM and relative to 0 mM GLN (n=4).

1.8 PI3-K/Akt is involved in GLN's protection independent of FN-Integrin signaling

PI3-K phosphorylation activates Akt, reducing apoptosis and allowing proliferation [90]. By using the PI3-K inhibitor LY294002 (25 μM), we confirmed that PI3-K signaling activates Akt in IEC-6 cells after hyperthermia in CT groups and in GLN treated groups (Fig. 15A). After proving that concentrations of 50 μM and 100 μM of LY294002 were toxic to non-heat-stressed cells (data not shown), a concentration of 25 μM LY294002 was chosen, which was not toxic in IEC-6 cells under non-stressed conditions (37°C) (Fig. 15B) and showed optimal inhibitory effects on GLN-mediated cellular protection. MTS assays revealed that PI3-K/Akt signaling was involved in GLN's protective mechanism: GLN increased cell survival in a dose-dependent manner in IEC-6 ($P < .001$) (Fig. 15C). However, LY294002 (25 μM) attenuated GLN's protection significantly ($P < .001$) (Fig. 15C). Western blots of cleaved Caspase-3 and cleaved PARP expression strengthened the MTS results that the PI3-K pathway is essential in GLN's protective mechanism (Fig. 15D+E). LY294002 (25 μM) treatment significantly attenuated GLN's reduction of apoptosis, as measured by cleaved PARP levels (Fig. 15D) and cleaved Caspase-3 levels (Fig. 15E) after hyperthermia. HS increased cleaved PARP levels by 60-fold ($P < .001$) and cleaved Caspase-3 levels by 30-fold ($P < .001$). GLN (10 mM) supplementation attenuated them significantly ($P < .001$). However, GLN+LY294002 increased cleaved PARP levels by 40-fold after non-lethal HS ($P < .001$) and cleaved Caspase-3 levels by 30-fold ($P < .001$) (Fig. 15D and E).

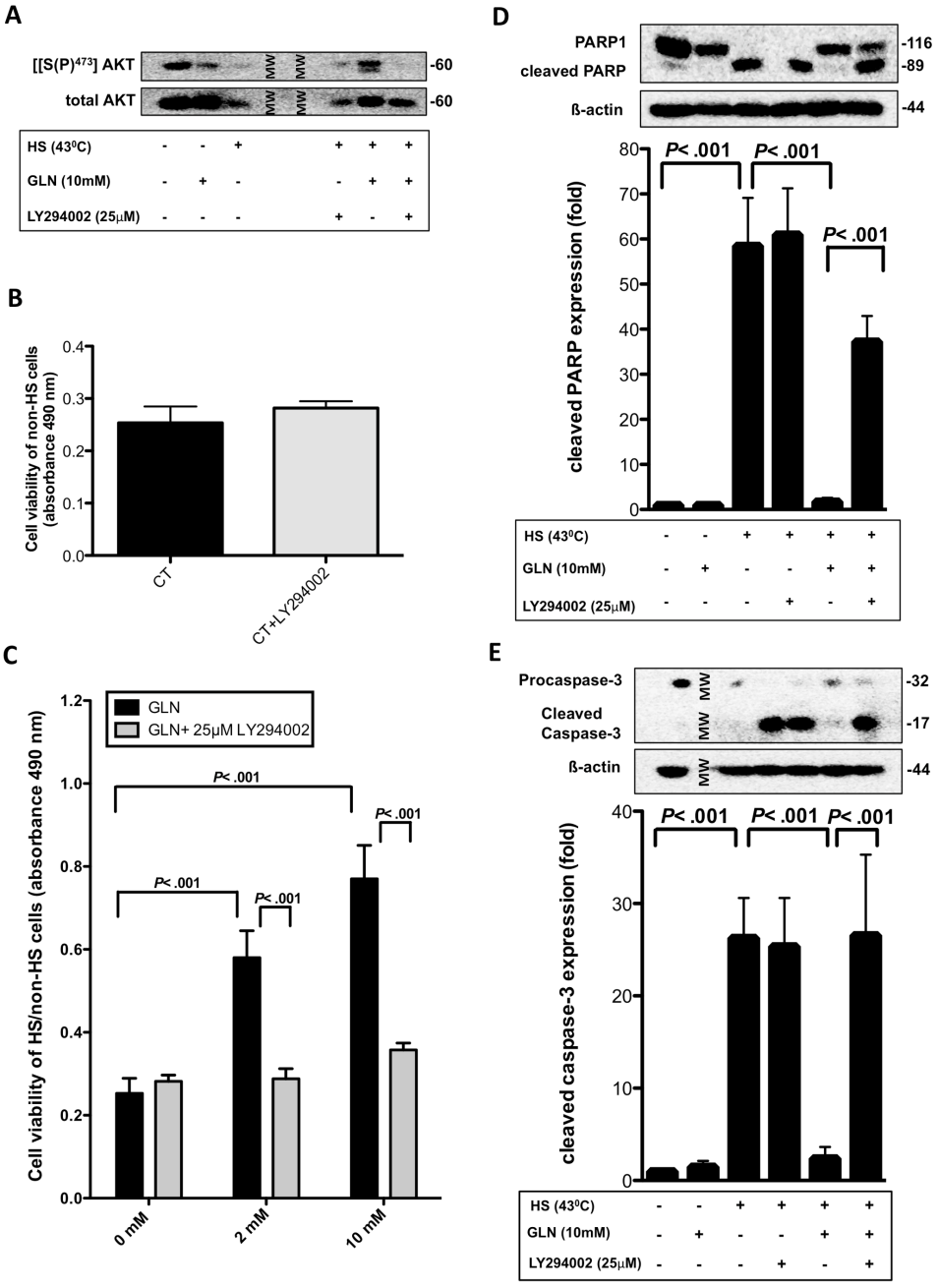


Fig. 15. GLN is protective via PI3-K signaling after HS. A) IEC-6 cells were treated with 0 mM or 10 mM GLN with or without 1 h LY294002 (25 μM) pre-treatment under basal (37°C) and stressed conditions (43°C). A representative Western blot of total Akt and [S(P)^{473}]Akt is shown. B) Cells were treated with/without 1 h LY294002 (25 μM) pre-treatment under non-stressed conditions (37°C). Cell survival was measured via MTS assay. Results are shown as mean±SEM (n=4). C) IEC-6 cells were treated for 1 h with either media, or LY294002 (25 μM) before they were treated with 0, 2, or 10 mM GLN. Cell survival, following lethal HS (44°C), was measured via MTS assay. All groups were normalized to their non-HS controls (37°C) to account for differences in cell growth. Assays were carried out in triplicate, experiments were performed 4 times and are shown as mean±SEM. D) Cells were treated as described in Fig. 15A. A representative Western blot of PARP1 is shown and cleaved PARP is presented as fold change±SEM relative to 0 mM GLN (n=4). E) Cells were treated as described in Fig. 15A. A representative Western blot of Caspase-3 is shown. Cleaved Caspase-3 was analyzed via densitometry and is presented as fold change±SEM relative to 0 mM GLN (n=4).

Our laboratory has shown in previous studies that GLN's cytoprotective effect is, at least in part, mediated by increased Hsp70 expression [170]. To determine the effect of LY294002 (25 µM) on GLN-mediated Hsp70 expression, we examined the expression of Hsp70 after HS in IEC-6 cells. Cells treated with 10 mM GLN increased Hsp70 levels after HS revealed by Western blot (P< .001) (Fig. 16A) and ELISA experiments (P< .05) (Fig. 16B). IEC-6 cells treated with LY294002 (25 µM), however, showed a significant decrease in GLN-mediated Hsp70 levels in both, Western blots (P< .001) (Fig. 16A) and Hsp70 ELISA experiments (P< .001) (Fig. 16B).

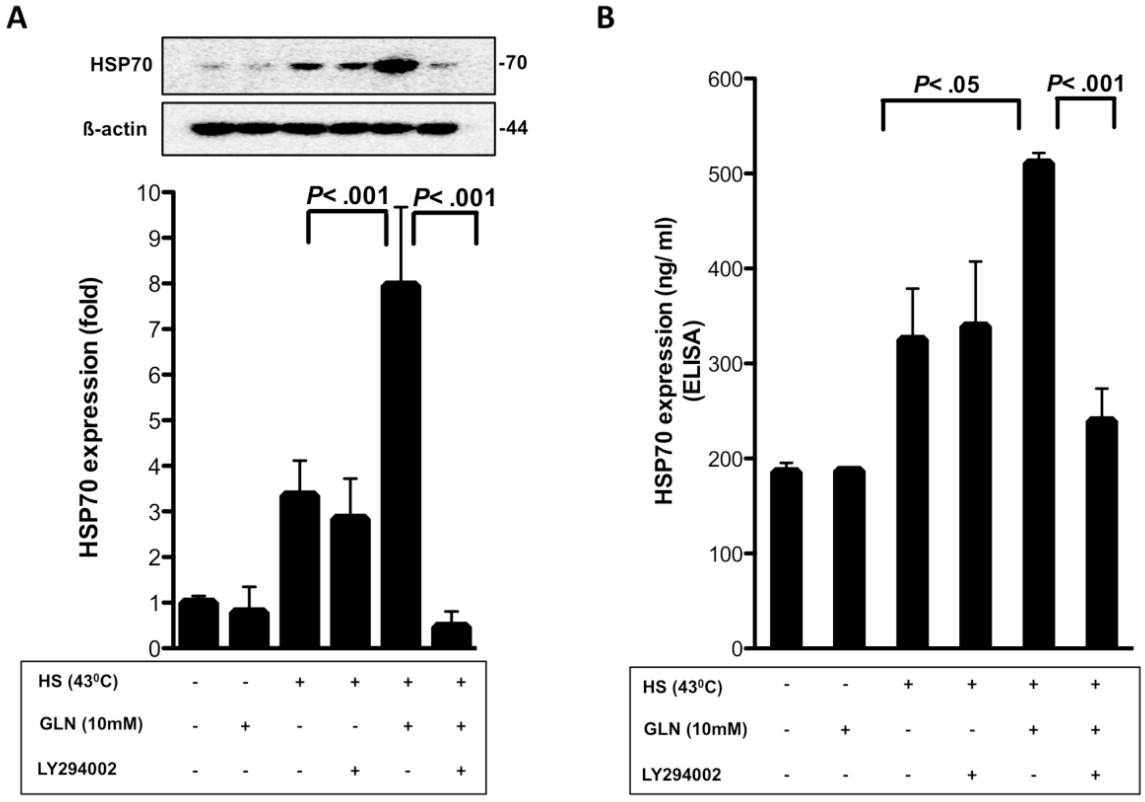


Fig. 16. LY294002 affects GLN-mediated increases in Hsp70 expression A) IEC-6 cells were treated with 0 mM or 10 mM GLN with or without 1 h LY294002 (25 µM) pre-treatment under non-stressed (37°C) and stressed conditions (43°C). Hsp70 expression was determined by Western blot analysis. In addition, ß-actin was monitored to normalize total blotted protein. Data are shown as mean fold change relative to 0 mM GLN±SEM (n=4). B) The treatment was the same as described in Fig. 16A. Hsp70 expression is shown in ng/ml±SEM (n=4) as measured by ELISA.

It is well known that Akt is essential for the “inside-out” activation of integrins, which in turn mediate matrix assembly in fibroblasts, such as FN [141]. Therefore, I investigated the role of GLN-mediated PI3-K/Akt signaling in conjunction with FN-Integrin signaling. I studied the ratio of phosphorylated Akt and total Akt after GLN (10 mM) and GRGDSP (50 μM) treatment in heat-stressed IEC-6 cells. The first thing that was noticed in Western blots of total and phosphorylated Akt levels in Fig. 17 was the decreased total Akt levels after hyperthermia. However, GLN returned total Akt protein levels to normal after thermal injury (Fig. 17). In addition, it was demonstrated that HS increased phosphorylated Akt by 2-fold and 10 mM GLN supplementation by 3-fold after HS ($P < .05$). However, adding GRGDSP (50 μM) to the GLN-treated group did not change the 3-fold increase in GLN-mediated Akt phosphorylation (Fig. 17), suggesting that PI3-K/Akt signaled before or in parallel of FN-Integrin signaling.

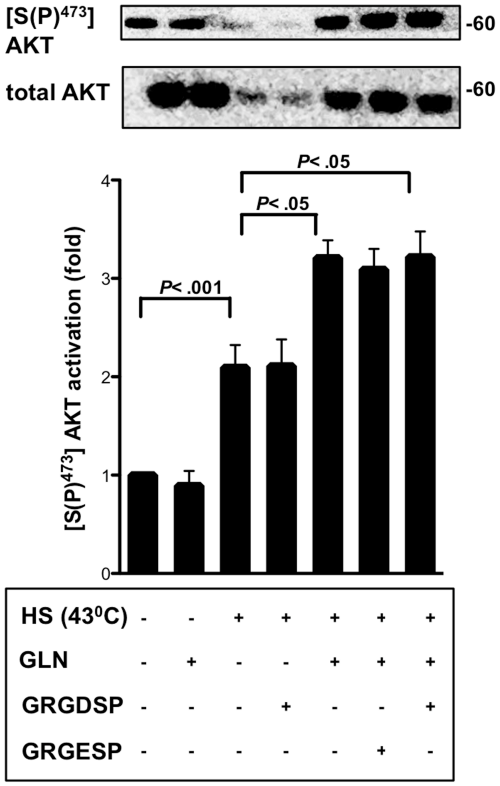


Fig. 17. PI3-K signaling occurs independent from FN-Integrin signaling. IEC-6 cells were treated for 1 h with either media, GRGDSP (50 μM) or GRGESP (50 μM) before the cells were treated with 0 mM or 10 mM GLN under basal (37°C) and stressed conditions (43°C). Representative Western blots of 4 independent experiments of [S(P)^{473}]Akt and total Akt are shown as mean fold change relative to total Akt±SEM and relative to 0 mM GLN.

1.9 PI3-K/Akt regulates FN expression

FN expression is important to regulate cell survival [61] and to interact with integrins for osmosignaling. Degradation of FN led to less interaction with integrins, reducing osmosignaling (Fig. 6). HS decreased FN expression significantly (P< .001). However, GLN inhibited this decrease after HS, as already shown in Fig. 6 (P< .001). In this experiment, we added PI3-K/Akt inhibitor LY294002 (25 µM) to the GLN-treated groups and showed that GLN was not able to prevent FN degradation after LY294002 (25 µM) was added (P< .001) (Fig. 18).

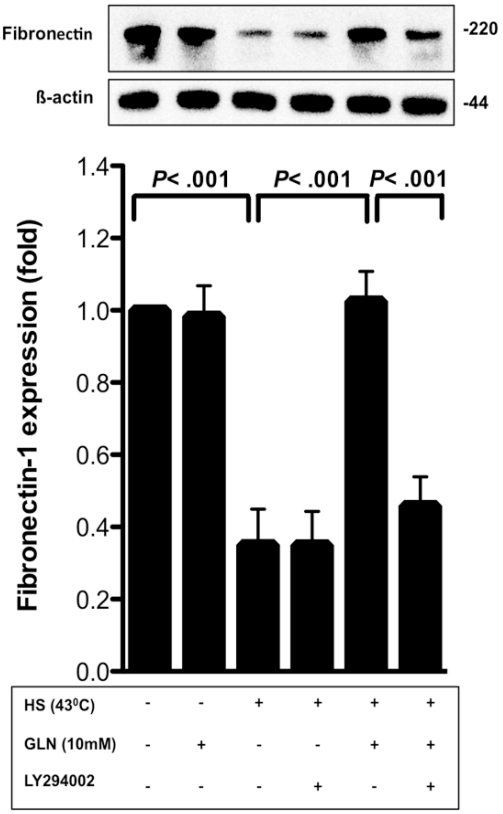


Fig. 18. LY294002 attenuated GLN-mediated increases in FN expression after HS. FN levels from IEC-6 cells with or without GLN (10 mM) and LY294002 (25 µM) treatment under unstressed (37°C) or stressed conditions (43°C) were determined by Western blot with ß-actin as loading control after 3 h recovery. Densitometric analysis of FN expression is presented as mean fold change relative to 0 mM GLN cells±SEM (n=3-8).

1.10 Involvement of Src-kinases in GLN-mediated protection

Vom Dahl and colleagues attested that integrins and Src-kinases are involved in osmosignaling. They demonstrated that hypoosmotic hepatocyte swelling led to an activation of Src, which was prevented by the integrin inhibitory hexapeptide GRGDSP, but not by GRGESP, its inactive analogue peptide [157]. Having observed that GLN promotes IEC-6 cell survival via FN-Integrin osmosensing (Fig. 7), it was of particular interest to know whether Src-kinase activity is involved in GLN-mediated cellular protection and whether GLN activates Src-kinases via FN-Integrin signaling.

Although I was not able to detect Src-Tyr^{418} kinase phosphorylation in cell lysates via Western blots, I could show by using Src-kinase inhibitor PP2 that GLN-mediated cellular protection was attenuated after PP2 treatment in heat-stressed cells (Fig. 19A).

It was shown by our laboratory that HS increased HSP expression [168, 170]. Here, we investigated PP2's influence on HSP expression. GLN (10 mM) increased Hsp70 expression by 3-fold ($P < .05$) after HS injury, and cells treated with PP2 (75 µM) showed a decrease in GLN-mediated Hsp70 expression ($P < .05$) (Fig. 19B).

In addition to Hsp70, we were interested in as to whether PP2 also had an attenuating effect on Hsp32 expression. Therefore, Western blot membranes were probed for Hsp32 protein expression in 4 independent experiments. GLN increased Hsp32 expression by 3-fold vs. HS CT and PP2 attenuated GLN-mediated increases in Hsp32 significantly ($P < .05$) (Fig. 19C).

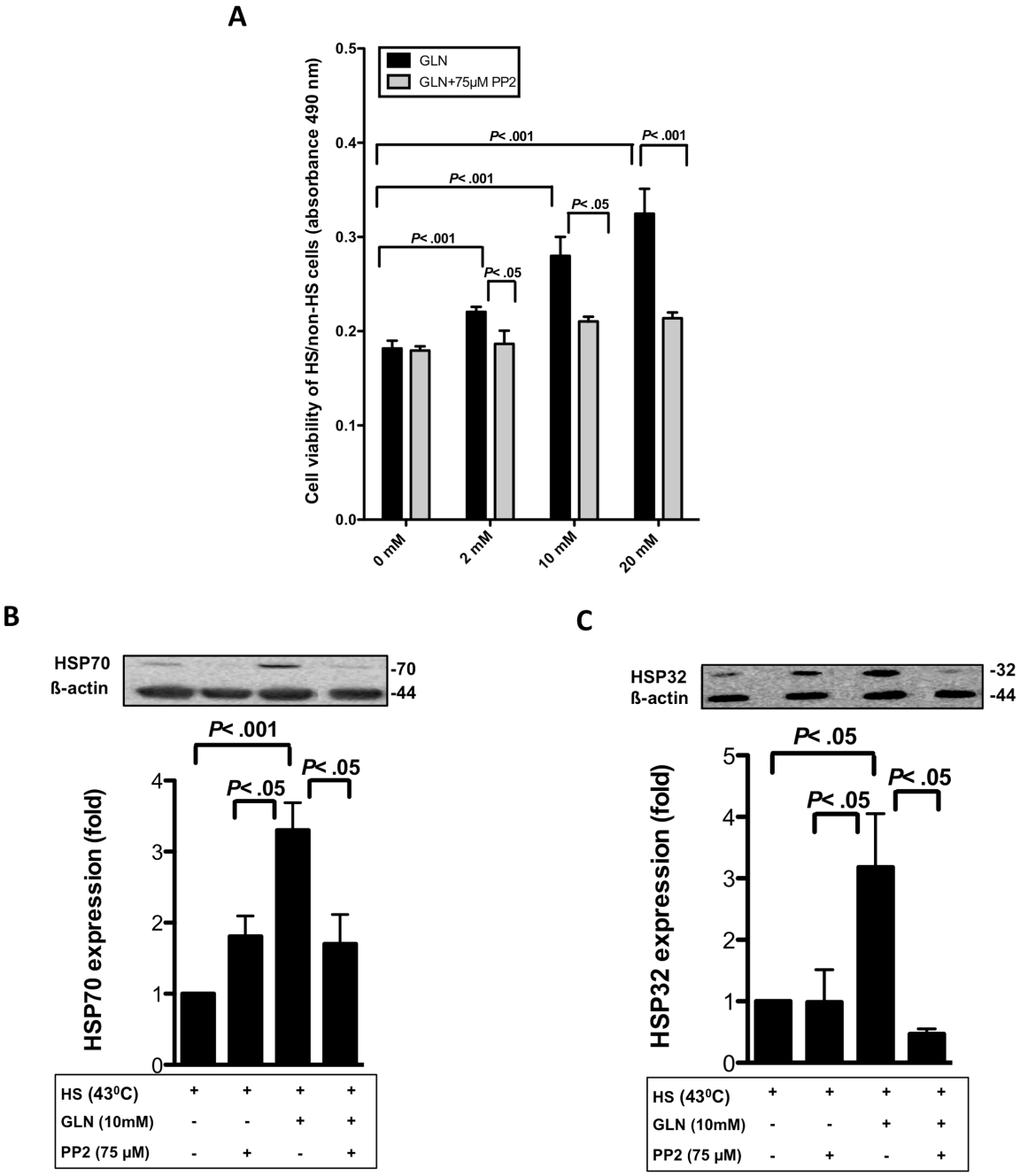


Fig. 19. Src-kinase is involved in GLN's protective cellular mechanism. A) IEC-6 cells were treated for 1 h with either media or PP2 (75 μM) before the cells were treated with 0, 2, 10 or 20 mM GLN. Cell survival, following lethal HS (44°C), was measured via MTS assay. All groups were normalized to their non-HS controls (37°C) to account for differences in cell growth. Assays were carried out in triplicate, experiments were performed 4 times and shown as mean±SEM. B) IEC-6 cells were treated for 1 h with either media or PP2 (75 μM) before the cells were treated with 0 mM or 10 mM GLN under stressed conditions (43°C). Hsp70 expression was determined by Western blot analysis. In addition, ß-actin was monitored to normalize total blotted protein. Data are shown as mean fold change relative to HS 0 mM GLN±SEM (n=3). C) The treatment was the same as described in Fig. 19B. Western blot of Hsp32 and ß-actin are shown. Results are shown relative to HS 0 mM GLN groups and represent means±SEM (n=3).

2 Involvement of Ntn-1 expression in GLN's protective mechanism

Ntn-1 is another well-known protective ECM protein in the intestine next to FN, which can interact with integrins [2]. Thus, I investigated if Ntn-1 potentially plays an important role in GLN's protective mechanism in IEC-6 cells after HS.

2.1 GLN prevents decreases in HS-mediated Ntn-1 expression

In the present study, I could show that Ntn-1 levels decreased by approximately 80 % after HS. GLN prevented this decrease after HS completely (Fig. 20A and B). Moreover, an increase in Ntn-1 expression is seen in the non-HS cells after 2 mM GLN. Whether GLN has the potential to increase Ntn-1 levels needs to be investigated in further studies.

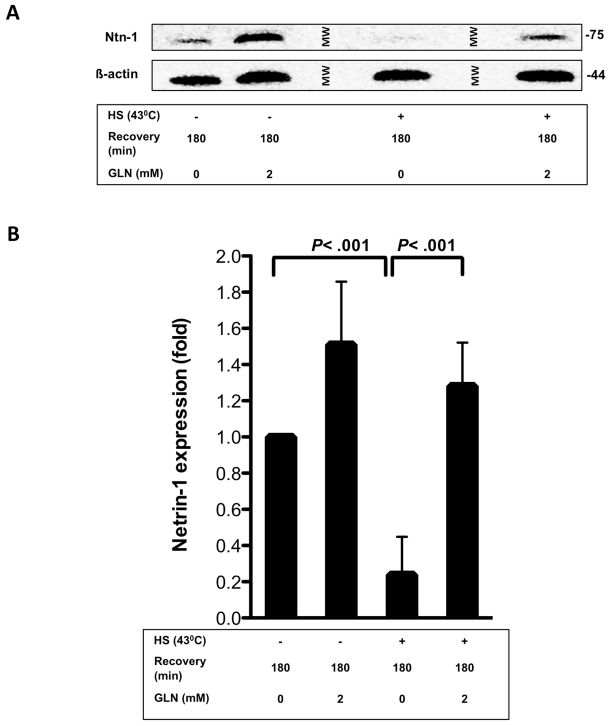


Fig. 20. GLN prevents Ntn-1 expression after HS. A) Ntn-1 levels from IEC-6 cells, with or without GLN (2 mM) treatment under unstressed (37°C) or stressed conditions (43°C) were determined by Western blot with ß-actin as loading control after 3 h recovery. B) Densitometric analysis of Ntn-1 expression is presented as mean fold change relative to 0 mM GLN cells±SEM (n=4).

2.2 Ntn-1 expression is essential in GLN's protective mechanism

To investigate whether Ntn-1 expression is essential in GLN's protective mechanism of IEC-6 cells, cells were transfected with 20 nM Ntn-1 siRNA. After proving that Ntn-1 expression was knocked down by 20 nM Ntn-1 siRNA as shown in Fig. 21A, I probed for cleaved Caspase-3 levels (Fig. 21B and C). Attenuation of Ntn-1 expression via siRNA (20 nM) led to a significant decrease of GLN-mediated protection following HS (Fig. 21). This indicated that GLN-mediated protection against apoptosis after HS was lost when Ntn-1 expression was attenuated.

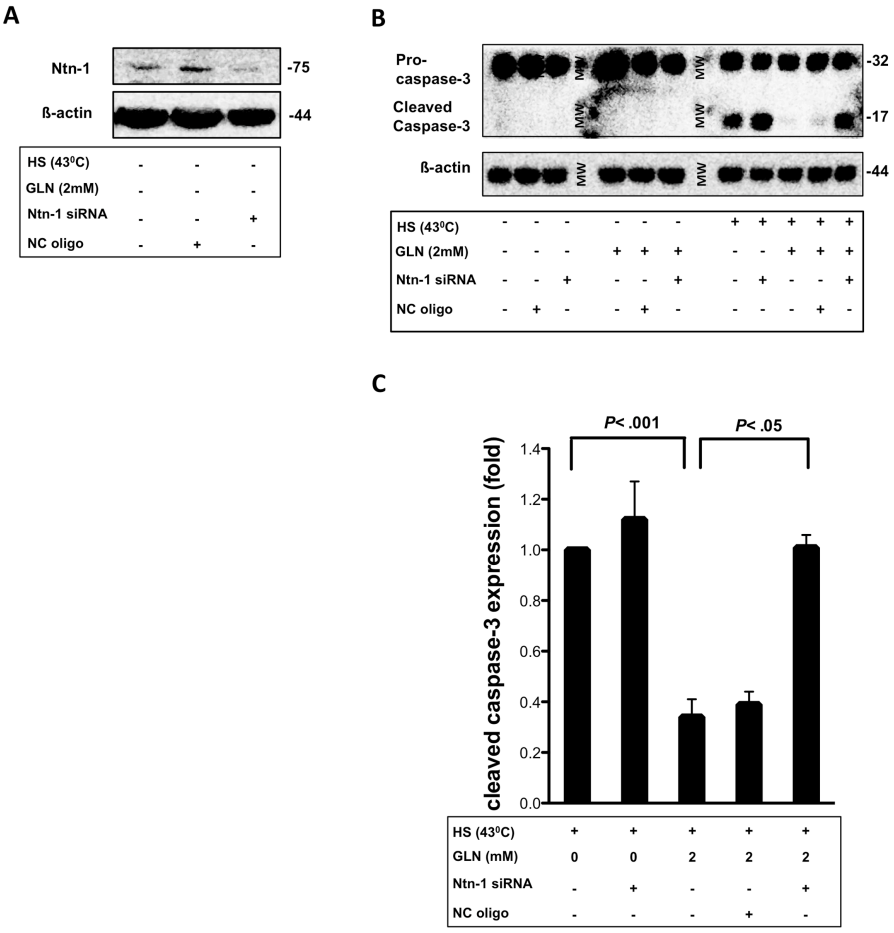


Fig. 21. GLN is protective by expressing Ntn-1 after HS. A) Ntn-1 expression of transfected IEC-6 cells without siRNA, with NC siRNA (20 nM) or Ntn-1 siRNA (20 nM) without GLN treatment are presented under baseline conditions (37°C). B) Caspase-3 and cleaved caspase-3 expression were determined via Western blot under baseline (37°C) and HS conditions (43°C) after IEC-6 cells were transfected without siRNA, NC siRNA (20 nM) or Ntn-1 siRNA (20 nM) following 0 mM and 2 mM GLN treatment (n=3). C) Treatment as described in Fig. 21B. Densitometric analysis of cleaved caspase-3 levels after HS (43°C) is shown as mean fold change relative to HS 0 mM GLN cells ±SEM (n=3).

3 Involvement of EGFR expression and signaling in GLN's protective mechanism after HS

It has been reported that EGFR is another upstream sensor and transducer of cell volume changes next to integrins [68]. Therefore, I investigated EGFR's role in GLN's protective mechanism and wanted to study whether the survival signaling pathways controlled by integrins are also regulated by EGFRs. The following data about the involvement of EGFR expression and signaling in GLN's protective mechanism were published in *AJP-Gastrointestinal and Liver Physiology* [107].

3.1 GLN is protective by preventing decreases in EGFR expression

The EGFR has been shown to be important in overall intestinal homeostasis, which regulates cell survival and cell growth [116, 119, 139, 160, 173, 174]. Thus, we investigated its role in GLN's protective mechanism. First we looked at its expression in IEC-6 cells. Total EGFR expression was significantly reduced in IEC-6 cells immediately following HS (43°C) and at 3 h post-HS. GLN treatment (10 mM) prevented this decrease in EGFR expression after HS (Fig. 22A and B). Since recent literature showed that EGFR is internalized to the nucleus and that nuclear EGFR is crucial for DNA repair and cell survival [42, 93], we looked at cytoplasmic and nuclear levels of EGFR after HS (43°C) and GLN (10 mM) treatment, in order to examine if GLN accumulates EGFR in the nucleus after HS. However, data showed that GLN-supplementation inhibited decreases in EGFR expression caused by HS in both the nucleus and cytoplasm (Fig. 22C).

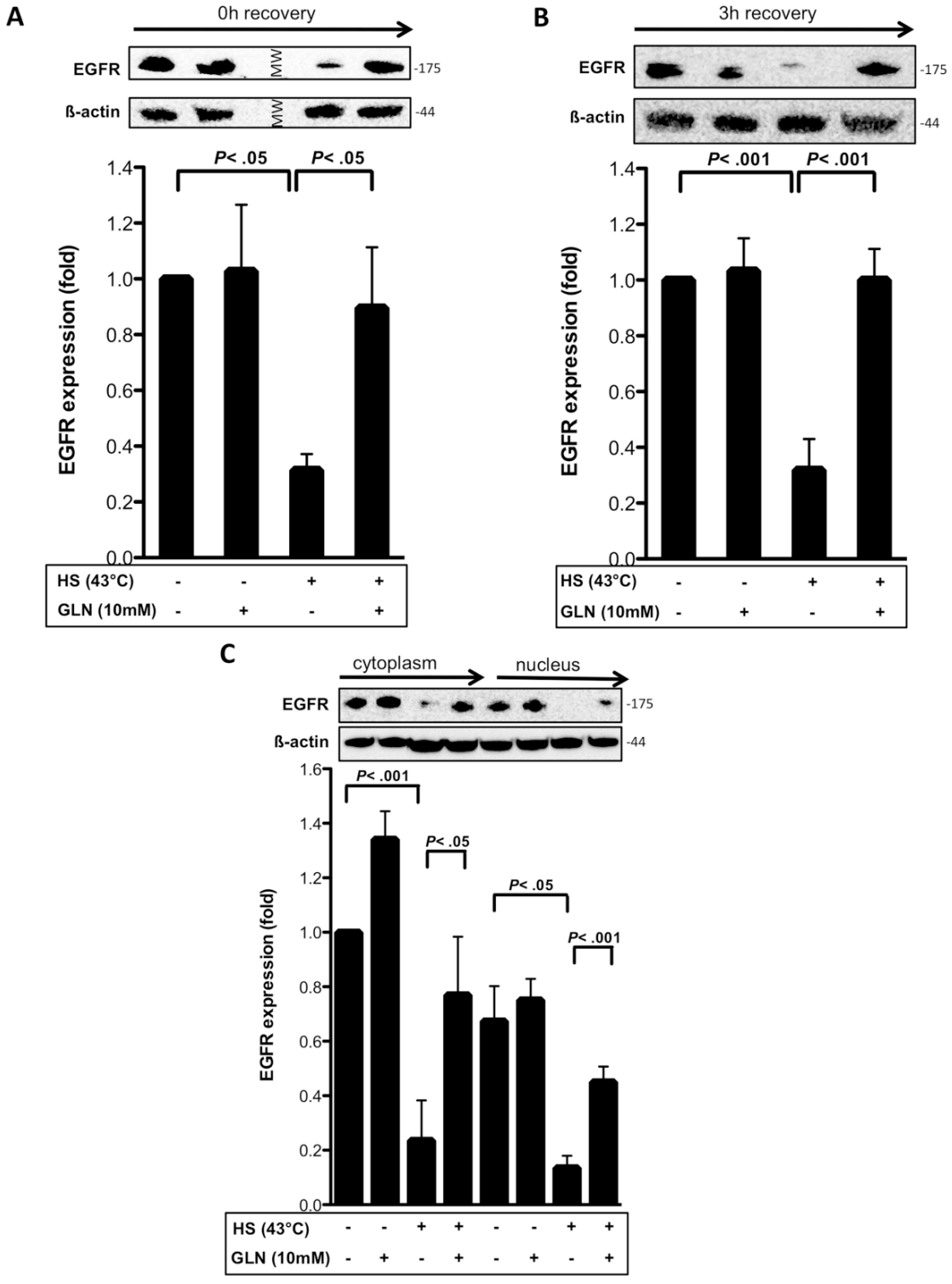


Fig. 22. GLN prevents HS-mediated decreases in EGFR levels. A) Total EGFR levels from IEC-6 cells with or without GLN (10 mM) treatment under unstressed (37°C) or stressed conditions (43°C) were determined by Western blot with ß-actin as loading control after 0 h recovery. Densitometric analysis of total EGFR expression as mean fold change relative to 0 mM GLN cells±SEM (n=3). B) Cells were treated as described in Fig. 22A and allowed to recover for 3 h. Western blot and densitometric analysis of EGFR after 3 h recovery is shown (n=4). C) A representative Western blot and densitometric analysis of cytoplasmic and nuclear EGFR levels after 3 h recovery are shown (n=6).

To demonstrate that EGFR expression is important in GLN's protective mechanism of IEC-6 cells, cells were transfected with 40 nM EGFR siRNA. EGFR knockdown, as shown in Fig. 23A, did not affect cell viability in non-HS cells (Fig. 23B). Attenuation of EGFR expression via siRNA (40 nM) led to a significant decrease of GLN-mediated protection following HS shown via MTS assay (Fig. 23C). This indicated that GLN-mediated protection against apoptosis after HS was lost when EGFR expression was attenuated.

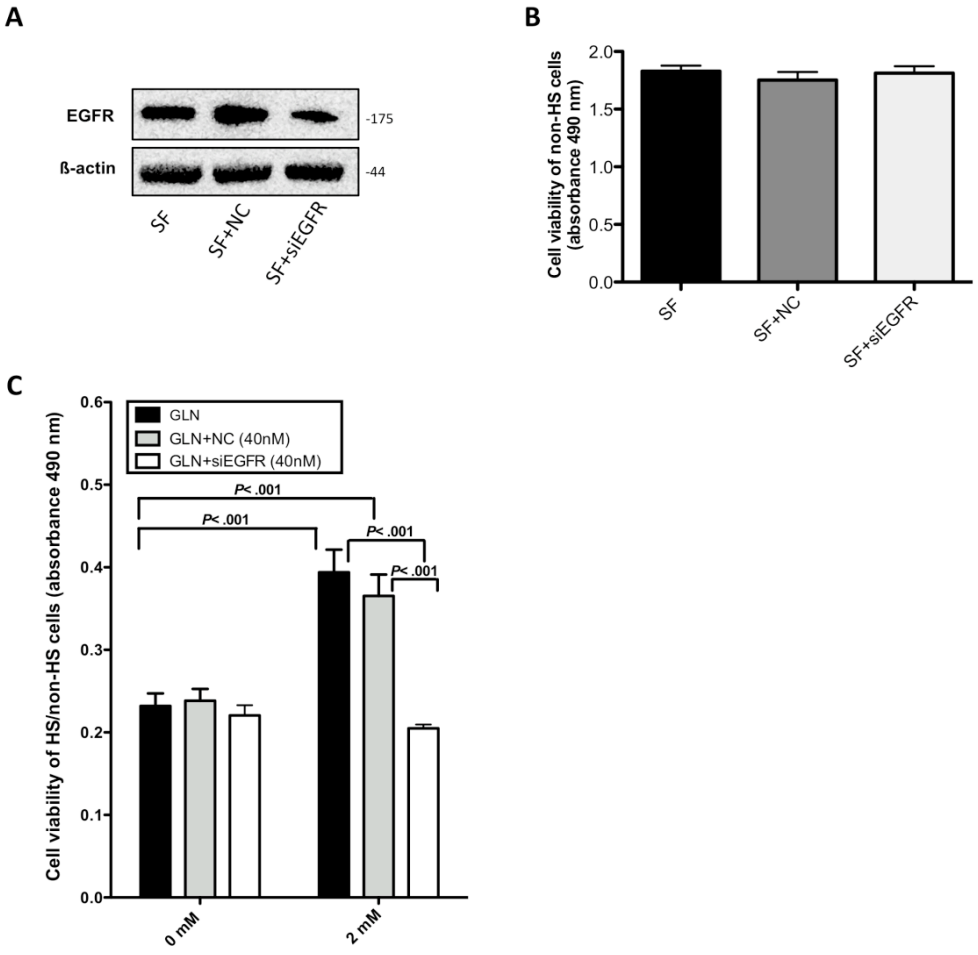


Fig. 23. GLN is protective by preserving EGFR expression after HS. A) Starved IEC-6 cells were transfected without siRNA, with NC siRNA (40 nM) or EGFR siRNA (40 nM) for 48 h. EGFR expression of transfected IEC-6 cells without siRNA, with NC siRNA or EGFR siRNA was assessed using Western blot. B) Cells were treated as described in Fig. 23A and cell viability was measured via MTS assay (n=3). C) Cell survival was determined via MTS assay following lethal HS (44°C) or baseline conditions (37°C) after IEC-6 cells had been transfected without siRNA, with NC siRNA (40 nM) or EGFR siRNA (40 nM) and treated with either 0 mM or 2 mM GLN. All HS groups were normalized to their non-HS controls to account for differences in cell growth. Assays were carried out in triplicate, experiments were performed 3 times and shown as mean±SEM.

3.2 Involvement of EGFR signaling in GLN-mediated cellular protection

Seth et al. showed that GLN induces a rapid increase in the tyrosine phosphorylation of EGFR and that GLN's protective effect is prevented by AG1478, the EGFR tyrosine kinase inhibitor, in Caco-2 cells [137]. Therefore, we investigated whether AG1478 had the same effect on GLN-mediated cellular protection following HS in a non-transformed intestinal epithelial cell line (IEC-6) (Fig. 24). First, I determined whether AG1478 inhibits EGFR phosphorylation. Unfortunately, I was not able to detect EGFR phosphorylation by Western blot. Three different EGFR phosphorylation site antibodies were used from Cell Signaling (Phospho-HER2/ErbB2 (Tyr1248)/ EGFR (Tyr 1173), Phospho-EGF Receptor (Tyr845), Phospho-EGF Receptor (Tyr1086)), but no phosphorylation could be detected. Although I was not able to detect EGFR phosphorylation by Western blot, I still performed EGFR tyrosine kinase inhibitor experiments with various concentrations of AG1478 (0, 5, 10, 20 μM). This allowed for measuring potential toxicity of the inhibitor reagents after addition of AG1478 (0, 5, 10, 20 μM) to non-HS groups. As shown in Fig. 24A, AG1478 (0, 5, 10, 20 μM) alone did not affect cell viability at any of the 4 concentrations. Heat-stressed plates (44°C) were normalized to their non-HS control groups (Fig. 24B) to account for differences in cell growth. GLN increased cell survival in HS cells in a dose dependent manner. Importantly, AG1478 (0, 5, 10, 20 μM) completely attenuated GLN's protection at concentrations of 2 mM and 10 mM GLN when used at all 4 concentrations (Fig. 24B). Evaluation of key apoptotic pathway markers confirmed MTS cell survival results. HS increased apoptotic markers such as cleaved PARP and cleaved caspase-3 levels. 10 mM GLN supplementation decreased markers of apoptosis after HS, indicating that 10 mM GLN can improve cell survival and prevents apoptosis during HS (Fig. 24C and D). However, AG1478 (20 μM) treatment decreased GLN-mediated anti-apoptotic effects as shown by increased cleaved PARP and cleaved caspase-3 levels (Fig. 24C and D). This data suggested that in the absence of EGFR signaling, GLN's cellular protection against hyperthermia was lost.

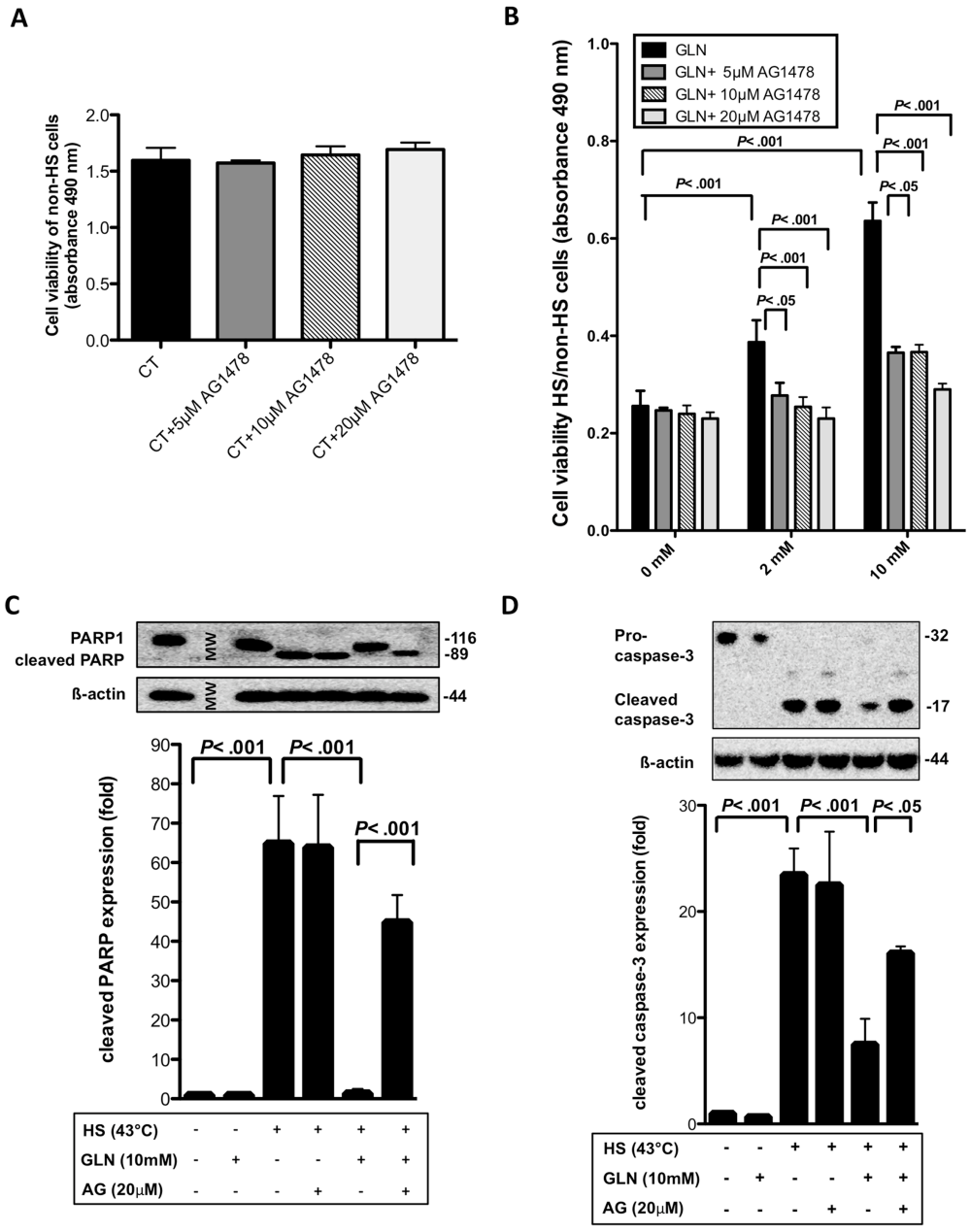


Fig. 24. GLN's protective signaling is inhibited by EGFR kinase activity inhibitor AG1478. A) IEC-6 cells were treated for 1 h with various concentrations of AG1478 (0, 5, 10, 20 μM). After 24 h incubation at 37°C, cell viability was measured and is presented as mean±SEM (absorbance 490 nm) (n=3). B) IEC-6 cells were treated as described in Fig. 24A. One plate recovered at 37°C for 24 h, meanwhile the other plate (treated the same way) underwent lethal HS at 44°C for 50 min following 24 h recovery at 37°C. Cell viability was measured via MTS assay. All groups were normalized to their non-HS controls to account for differences in cell growth. Assays were carried out in triplicates; experiments were performed 3 times and shown as mean±SEM (HS/non-HS cells (absorbance 490 nm). C) IEC-6 cells were treated with 0 mM or 10 mM GLN with or without 1 h AG1478 (20 μM) pre-treatment under basal (37°C) and stressed conditions (43°C). PARP1 and cleaved PARP were determined by Western blot. In addition, ß-actin was monitored to normalize total blotted protein. Cleaved PARP levels are presented as fold change±SEM (n=3). D) Procaspase-3, cleaved Caspase-3 levels, and ß-actin are shown by Western blot and cleaved Caspase-3 levels are presented as fold change±SEM (n=3).

3.3 GLN is protective by activating ERK1/2 via EGFR signaling

It is well established that ERK1/2 is activated by a number of key growth factors for cell survival [84, 135]. It was also shown that ERK1/2 is involved in GLN's protective mechanism via FN-Integrin signaling (Fig. 9 and Fig. 10). Thus, I investigated whether EGFR tyrosine kinase activity may play an initial role in GLN's activation of ERK1/2. Western blots of [T(P)^{202}/Y(P)^{204}]ERK1/2 relative to total ERK1/2 showed that HS increased phosphorylated ERK1/2 by 3-fold. GLN treatment further elevated ERK1/2 phosphorylation by 6-fold after HS. Importantly, this GLN-mediated increase in ERK1/2 phosphorylation was inhibited after AG1478 (20 µM) treatment (Fig. 25).

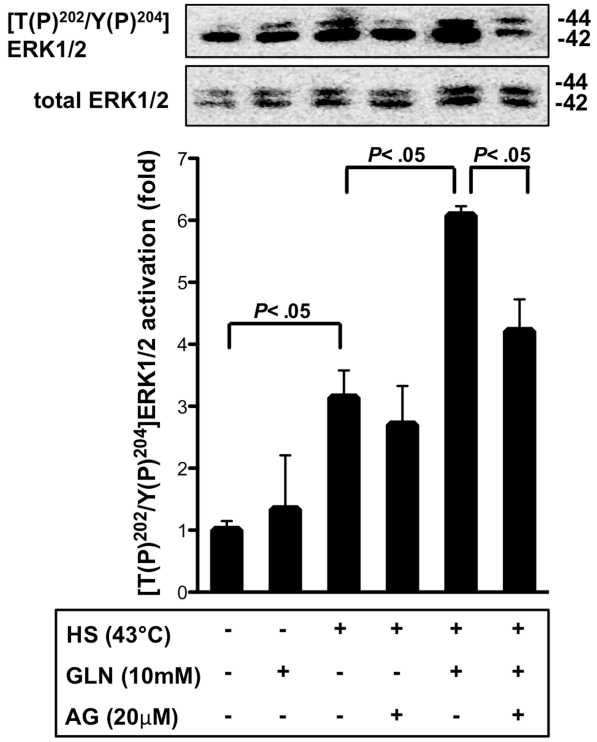


Fig. 25. GLN-mediated ERK1/2 activation after HS is regulated via EGFR signaling. IEC-6 cells were treated with 0 mM or 10 mM GLN with or without 1 h AG1478 (20 µM) pre-treatment. [T(P)^{202}/Y(P)^{204}]ERK1/2 and total ERK1/2 levels were determined by Western blot analysis after basal (37°C) and heat-stressed (43°C) conditions without recovery. ERK1/2 activation is shown as mean fold change relative to total ERK1/2±SEM and relative to 0 mM GLN (n=3).

3.4 GLN is protective by dephosphorylating p38MAPK via EGFR signaling

Since it was shown that GLN-mediated p38MAPK dephosphorylation was involved in GLN-mediated protection via FN-Integrin osmosignaling (Fig. 14), I investigated it's role in EGFR signaling. To see whether EGFR signaling was essential in GLN-mediated p38MAPK dephosphorylation, AG1478 (20 µM) was added to the heat-stressed GLN groups and demonstrated that HS+GLN+AG1478 treatment phosphorylated p38MAPK (Fig. 26), indicating that EGFR signaling was involved in GLN-mediated dephoshorylation of p38MAPK after HS.

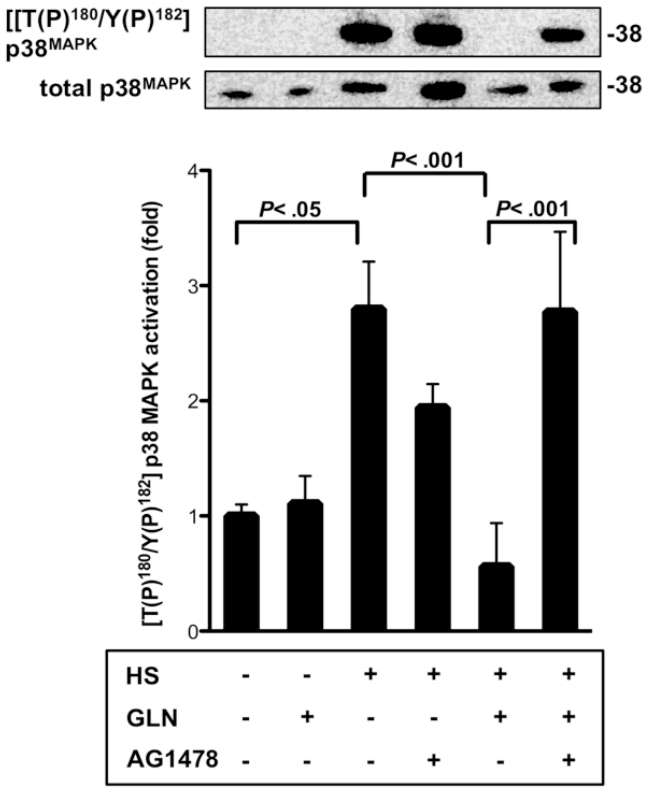


Fig. 26. GLN is protective by dephosphorylating p38MAPK via EGFR signaling. IEC-6 cells were treated with 0 mM or 10 mM GLN with or without 1 h AG1478 (20 µM) pre-treatment. A representative Western blot of [T(P)^{180}/Y(P)^{182}]p38MAPK and total p38MAPK levels is shown. P38MAPK phosphorylation is presented as mean fold change relative to total p38MAPK±SEM and relative to 0 mM GLN (n=4).

3.5 EGFR signaling has no effect on GLN-mediated Akt activation

Another essential cell survival signaling pathway is the PI3-K pathway [90]. To evaluate if EGFR signaling is involved in GLN-mediated PI3-K signaling, I studied total Akt protein levels and Akt activation after GLN (10 mM) and AG1478 (20 µM) treatment in heat-stressed IEC-6 cells. As already shown in Fig. 17, total Akt was significantly decreased after HS. However, GLN returned Akt protein levels to normal after thermal injury to prevent cell death (Fig. 27). After normalizing phosphorylated Akt relative to total Akt levels, HS increased phosphorylated Akt by 2-fold and 10 mM GLN supplementation increased phosphorylated Akt by 3-fold after HS. Addition of AG1478 (20 µM) to the GLN-treated group did not change the GLN-mediated increase in Akt phosphorylation (Fig. 27).

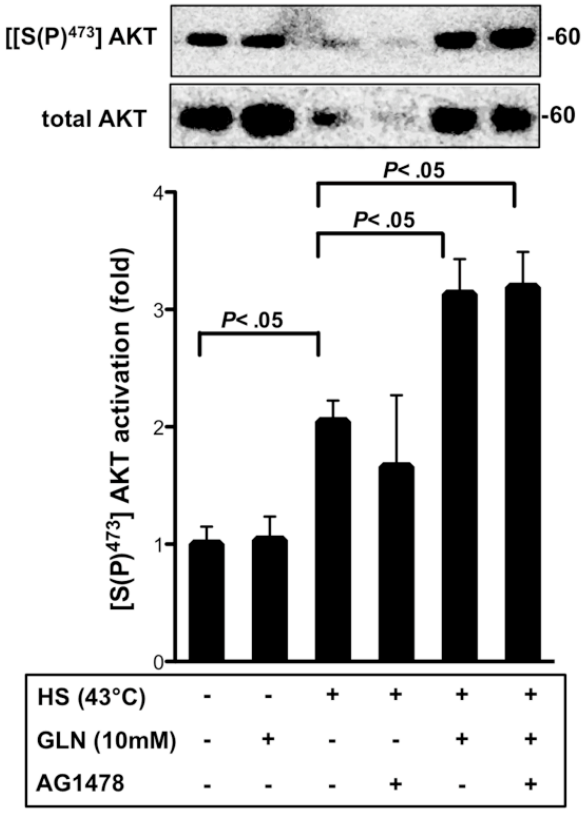


Fig. 27. PI3-K signaling is involved in GLN's protective mechanism independently from EGFR signaling. IEC-6 cells were treated with 0 mM or 10 mM GLN with or without 1 h AG1478 (20 µM) pre-treatment under non-stressed (37°C) and stressed conditions (43°C). A representative Western blot of 3 independent experiments of [S(P)^{473}]Akt and total Akt is shown as mean fold change relative to total Akt±SEM and relative to 0 mM GLN (n=4).

D Discussion

Following intestinal injury, GLN depletion leads to ongoing tissue injury, apoptosis, and failure of cellular repair [78]. However, despite multiple experimental and clinical studies demonstrating GLN's beneficial effects in the intestine [53, 82, 142, 178, 179], the underlying molecular mechanism of GLN still remains unclear. My findings provide novel mechanistic insights into the anti-apoptotic effects of GLN in the intestine after injury.

1 Involvement of FN expression and FN-Integrin signaling via ERK1/2 in GLN's protective mechanism after intestinal injury

FN is a vital structural component of the basement membranes in the crypts of Lieberkuehn in the gut [55, 122]. The expression of this key basement membrane protein is essential to the maintenance of cellular integrity and to the intestinal epithelial cell's response to injury [85]. Utilizing IEC-6 cells, a small intestinal crypt epithelial cell line, I showed that under non-stress conditions intestinal epithelial cells produce substantial amounts of FN. Immediately after HS injury, FN levels were reduced by 50 % and by 85 % at 3 h post-injury (Fig. 6A and B). Multiple bands scattered across 200–250 kDa were typically observed, consistent with the expected molecular weight range for FN fragments [136]. It is interesting to note that the appearances of these multiple bands were attenuated following HS. On the other hand, GLN-treatment in HS cells preserved this double or multiple band appearance (Fig. 6A-C). As integrin fragments have been hypothesized to play a key role in integrin binding and cellular adhesion, migration, and repair, the apparent loss of these fragments following HS may be an important contributor to the disrupted ECM interactions following stress that may contribute to impaired cellular repair [153]. Future studies will hopefully give more mechanistic insights into this complex mechanism of intestinal protection.

HS-mediated reductions of FN levels were associated with significant apoptosis. GLN was

able to preserve epithelial cell FN levels following HS to pre-injury levels. The preservation of FN levels seemed to correlate with reduced apoptosis (Fig. 6D and E). Here, I have shown for the first time, that GLN can rescue intestinal epithelial cells *in vitro* from cell death by preventing a decrease in FN expression after HS injury. Silencing FN inhibited GLN's protection (Fig. 6D and E), suggesting that FN expression is essential in GLN's protective mechanism. Both insufficient and excessive FN expression impair gastrointestinal function and can lead to worsening of disease in patients with Crohn's disease [125]. Our results showed that GLN prevented the decrease in FN expression, yet it did not lead to excessive expression of FN (Fig. 6A and B). Less FN may lead to reduced FN-Integrin interaction, following altered FN-Integrin osmosignaling, causing apoptosis. What effect attenuation of FN levels has on cell volume, ERK1/2, HSF-1, and Hsp70 expression still needs to be investigated in future studies. Here, evidence is presented that its expression plays an essential role in GLN's protective mechanism to prevent apoptosis shown via cleaved caspase-3 levels (Fig. 6D-E).

ECM protein stabilization and amino acid-mediated cell hydration changes can modulate many cellular pathways, including cell survival pathways, by initiating signal transduction [55, 57, 157]. I showed that both the preservation of total FN levels as well as cellular attachment to integrin receptors is important in GLN's protective mechanism after intestinal injury by using the FN-Integrin interaction inhibitor GRGDSP and its negative control GRGESP (Fig. 6 and Fig. 7). When FN-Integrin signaling was interrupted, GLN was incapable of cellular protection via ERK1/2, HSF-1, and Hsp70 (Fig. 10, Fig. 11, Fig. 12).

At the beginning of the present study, the initial steps in GLN's cytoprotective molecular mechanism were largely unknown. In this study, evidence is presented that integrins may play a key initial role in GLN-induced cell swelling by preserving the anchorage to the ECM. Therefore, I quantified cell size changes caused by GLN by measuring the total cell area size in IEC-6 cells using confocal fluorescence microscopy. I was interested in the possibility of GRGDSP affecting cell size via attachment to the ECM. GLN caused cell swelling in IEC-6 cells in a dose-dependent manner. However, inhibition of FN-Integrin signaling via GRGDSP

almost completely decreased GLN-mediated cell swelling, but not its inactive negative control GRGESP (Fig. 8A and B). One explanation for this decrease in cell area size may be that after the integrin inhibitor GRGDSP blocks the binding sites for the ECM proteins, signal transduction is reduced which leads to cell shrinkage despite the presence of sufficient GLN. My data agrees with literature showing that, prior to and during apoptosis, loss of cell volume can be observed [22, 80]. Volume decrease and actin disruption have been shown in previous studies to be correlated with apoptosis [50, 81]. In the present study, I could show that HS and the interruption of the FN-Integrin interaction by GRGDSP led to cell shrinkage. GLN, however, was able to protect IEC-6 cells by volume increase and actin stabilization via FN-Integrin signaling (Fig. 8). Based on these results, I hypothesized that GRGDSP may reduce cell size and attenuate GLN-mediated protection by decreasing intracellular GLN concentration. As GRGDSP significantly reduced GLN-mediated cell size increases, it seemed likely that GRGDSP treatment would lead to reduced intracellular GLN content. Surprisingly, using LC-MS/MS I found that GRGDSP had no impact on intracellular GLN concentrations in basal (37°C) or HS conditions (Fig. 8C). In our model of intestinal injury, increased intracellular GLN concentrations do not seem to be required to initiate cell size changes and signaling via ERK1/2 to initiate cellular protection pathways such as the HSP response. Thus, GLN appears to induce cell size changes via FN-Integrin signaling and potentially enhances HSP pathway activation in a manner that does not require increased intracellular GLN concentrations. Whether or not this osmosensing pathway via FN-Integrin interaction is specific to GLN needs be investigated in future studies, as other cell swelling inducing amino acids may function via the same osmosensing pathway.

Since F-actin assembly plays an important role in inflammation and cell size regulation [50, 81] and is regulated via FN-Integrin signaling [23], one possible explanation is that GLN increases cell size via F-actin stabilization by FN-Integrin signaling, which activates the protective ERK1/2 and HSP response (Fig. 8D). When FN-Integrin interaction is interrupted by GRGDSP, the adhesion complex is not intact and cytoskeletal rearrangement may be inhibited, causing fragmented, disrupted, disorganized, and collapsed actin (Fig. 8D). If the

adhesion complex is not functioning, baso-lateral actin may be disorganized, leading to cell adherence disruption. HS might cause disorganization of the cytoskeleton as FN expression is attenuated, leading to less FN-Integrin interaction and signaling, and subsequently causing apoptosis.

Next, we examined potential cell protective signaling pathways that could serve as downstream mediators of GLN-mediated FN-Integrin signaling. The ERK1/2 pathway is known to be activated by FN-Integrin signaling and can induce protective HSP expression following injury [133]. MTS assay data revealed by using two different specific ERK1/2 kinase inhibitors PD98059 and UO126 that ERK1/2 signaling is involved in GLN's protective mechanism in IEC-6 cells after HS (Fig. 9). Furthermore, the present study showed for the first time that GLN activates ERK1/2 via the FN-Integrin signaling pathway (Fig. 10). FN-Integrin signaling via ERK1/2 assists in mediating transactivation of HSF-1 leading to increases in Hsp70 expression, which is known to improve cell survival and to attenuate apoptosis after thermal injury (Fig. 11 and Fig. 12).

This data may indicate that the recently shown protective effect of fever in states such as human sepsis may depend not only on elevation of body temperature to initiate HSP expression (and other cell protective/immune response pathways), but also on sufficient GLN concentrations being available in the plasma or extracellular fluid at the time of injury. This hypothesis is consistent with data showing that deficient plasma GLN levels at ICU admission are associated with a significant increase in mortality [114].

Specific to the intestine, our data indicates that under conditions of GLN depletion, intestinal epithelial cells are unable to mount an appropriate cytoprotective response to HS, resulting in apoptosis (Fig. 7). Further, GLN induces cell survival during physiological stress, potentially independent of changes in intracellular GLN concentration via activation of the FN-Integrin osmosensing pathway. The results of the present study provided evidence that GLN`s mechanism of cellular protection in IEC-6 cells is mediated by two related pathways: 1) Via FN stabilization and 2) by the activation of the FN-Integrin cell volume sensing pathways via ERK1/2, HSF-1, and HSPs, which together lead to prevention of apoptosis. Fig. 28 shows

the proposed pathway by which GLN protects against intestinal epithelial cell injury via the FN-Integrin signaling pathway.

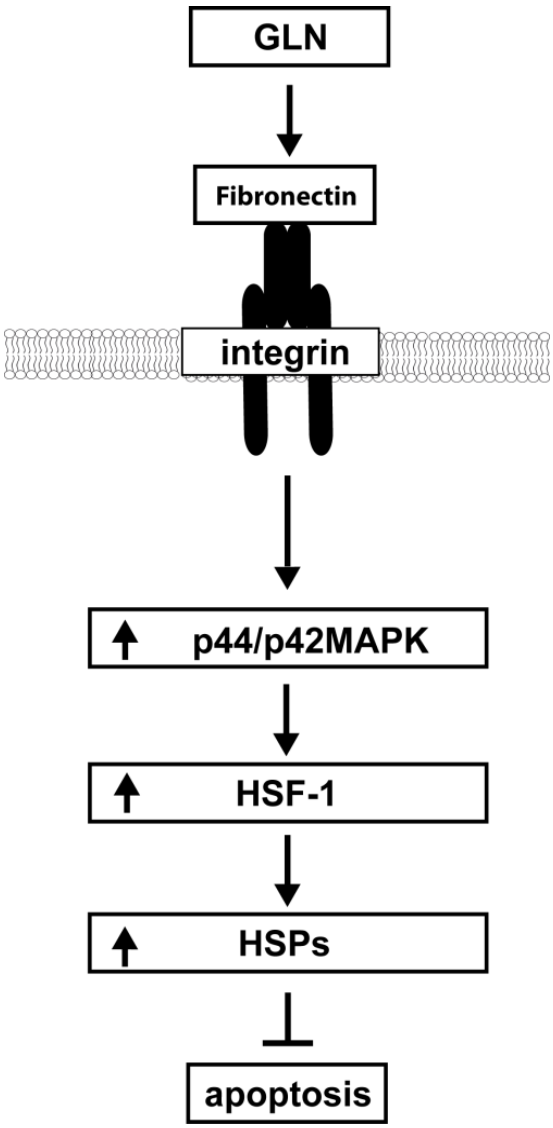


Fig. 28. Proposed signaling pathway of GLN's protective effects via the FN-Integrin signaling pathway. GLN is protective in the intestine by preventing FN expression after thermal injury, as well as by activating the protective FN-Integrin signaling pathway. GLN phosphorylates ERK1/2 (p44/p42MAPK) via the FN-Integrin pathway leading to HSF-1 activation, which enhances HSP expression to prevent apoptosis.

In conclusion, the present study showed that GLN stabilized intestinal epithelial cell architecture by preventing FN levels and actin destabilization after a mechanical injury, such as HS, via FN-Integrin signaling. Mechanical injury interrupts cell-cell adhesion. ECM and tissue organization is important to preserve the integrity of intestinal epithelial cells after gut

injury. Cells lacking the interactions with the ECM are more susceptible to stress and succumb more rapidly to apoptosis. Excessive trauma or injury to the tissue resulting in extensive ECM denaturation would similarly lead to disruption of the integrin-ECM interaction and cell death [147].

In the present study, it could be shown that GLN depletion led to cell death because of loss of ECM and cytoskeleton architecture after HS. However, addition of GLN could improve epithelial tissue organization via FN-Integrin-ERK1/2 signaling to prevent apoptosis after intestinal injury as cell-cell adhesion between neighbor cells remained intact. GLN-mediated signaling may help explain the long known phenomenon of GLN mobilization from muscle and other stores to the plasma (and ultimately to the gut) following injury and illness [125]. GLN mobilization and GLN-mediated cytoprotective signaling via the FN-Integrin pathway may be vital to intestinal cell survival and recovery from intestinal inflammation, CI, and other gut injuries**.** These data give the mechanistic rationale for further studies of GLN as a potential therapeutic agent to prevent and/or promote recovery from intestinal injury.

2 PI3-K, FN-Integrin-p38MAPK, and Src signaling are essential in GLN-mediated protection

I was interested in as to whether ERK1/2 is the only MAPK involved in GLN-mediated FN-Integrin signaling or whether p38MAPK and/or other well-known protective signaling pathways, such as PI3-K and Src, are engaged in GLN-mediated FN-Integrin signaling as well.

By using specific kinase inhibitors, MTS cell viability assays, and Western blots of apoptotic markers, I could show that PI3-K, p38MAPK, and Src signaling play essential roles in GLN-mediated cell survival signaling (Fig. 14, Fig. 15, and Fig. 19).

GLN was able to activate PI3-K/Akt signaling independently from FN-Integrin signaling after HS (Fig. 17). Furthermore, GLN-mediated PI3-K/Akt signaling could prevent FN degradation (Fig. 18), and was able to increase Hsp70 expression (Fig. 16) to prevent apoptosis.

As aforementioned, it had previously been shown that Akt1 is essential for the “inside-out” activation of integrins, which in turn mediates matrix assembly, such as FN in fibroblast cells [141]. Akt1 is involved in the activation of integrins, which is an essential key step necessary for adhesion in endothelial cells, regulating ECM assembly [24, 141]. Thus, it seemed reasonable to assume that PI3-K/Akt signaling regulates GLN-mediated FN expression and FN-Integrin signaling via “inside-out” signaling in intestinal epithelial cells to prevent cell death after intestinal injury.

In this study, I was able to show for the first time that PI3-K/Akt signaling, indeed, regulates GLN-mediated FN expression after hyperthermia (Fig. 18). FN expression is important for cell survival [61] and is essential in GLN’s protective mechanism as previously described. Degradation of FN leads to less interaction with integrins, which reduces osmosignaling. Here, evidence is presented that PI3-K/Akt signaling regulates GLN-mediated FN expression after HS, possibly via “inside-out” signaling to activate FN-Integrin interactions.

I showed that the FN-Integrin interaction inhibitor GRGDSP and the ERK1/2 kinase inhibitor PD98059 attenuated GLN-mediated increases in Hsp70 expression (Fig. 12). Thus, it was interesting to know if the PI3-K-inhibitor LY294002 was also able to decrease GLN-mediated Hsp70 expression. The results in Fig. 16 indicated that PI3-K signaling regulates GLN-mediated Hsp70 upregulation.

If my hypothesis is correct and PI3-K signaling is able to regulate both FN expression and the activation of FN-Integrin signaling as part of GLN’s protective effects, this would be in agreement with my results that both GRGDSP and LY294002 decrease GLN-mediated Hsp70 expression (Fig. 12 and Fig. 16). However, it could also be possible that PI3-K/Akt signals in parallel to FN-Integrin signaling to prevent apoptosis via increased Hsp70 expression. This key area of research still needs to be investigated in future studies. Future experiments with LY294002 or PI3-K siRNA in conjunction with ERK1/2 and p38MAPK activation could give additional mechanistic insights.

In a next step, I showed that GLN was able to rescue intestinal epithelial cells *in vitro* from cell death by dephosphorylating p38MAPK after HS injury (Fig. 14). Exposure of IEC-6 cells

to the FN-Integrin inhibitor GRGDSP showed that p38MAPK serves as downstream mediator of GLN-mediated FN-Integrin signaling (Fig. 14).

In conclusion, it could be shown in IEC-6 cells that both MAPKs, ERK1/2 and p38MAPK, are regulated via GLN-mediated FN-Integrin signaling after hyperthermia. However, GLN-mediated PI3-K signaling either happens before FN-Integrin signaling or simultaneously.

Our current working hypothesis suggests that GLN-mediated PI3-K/Akt signaling regulates FN expression and possibly FN-Integrin osmosignaling after thermal injury. This increases Hsp70 expression, which prevents apoptosis. The following figure gives an overview of our current work hypothesis (Fig. 29).

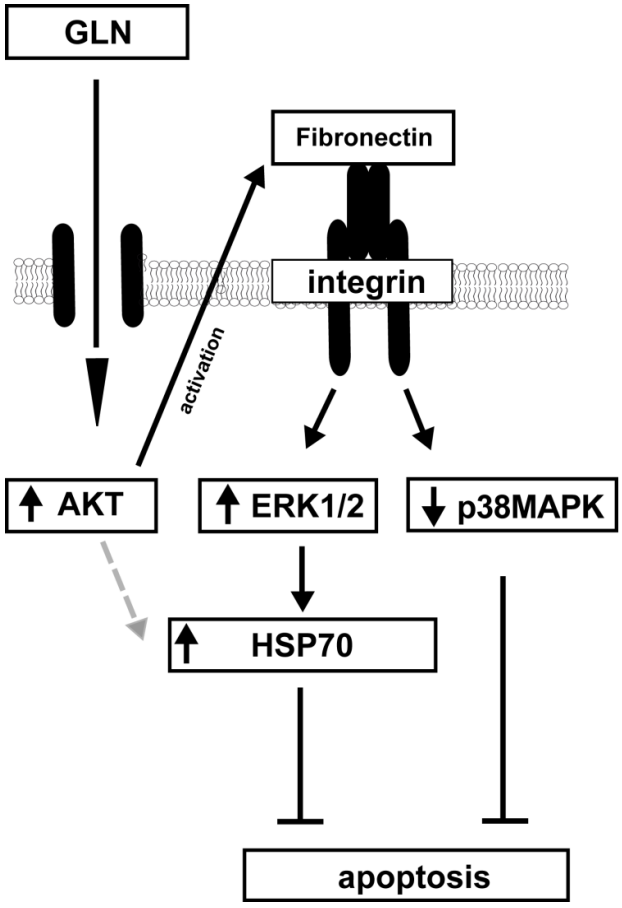


Fig. 29. Proposed signaling pathways underlying GLN's protective effects via PI3-K/Akt signaling. GLN is protective in the intestine by preventing FN expression and activating FN-Integrin-p38MAPK and FN-Integrin-ERK1/2 signaling after hyperthermia. GLN dephosphorylates p38MAPK via FN-Integrin signaling to prevent apoptosis. GLN is protective by activating the PI3-K pathway independently from FN-Integrin signaling. GLN-mediated PI3-K signaling regulates FN expression and may regulate FN-Integrin osmosignaling.

As already previously mentioned, Vom Dahl and his colleagues showed that Src-kinases are involved in integrin osmosensing pathways. They found that hypoosmotic hepatocyte swelling led to an activation of Src, which was prevented by GRGDSP, but not by GRGESP [157]. After showing that GLN promotes IEC-6 cell survival (Fig. 7) via FN-Integrin osmosignaling, I wanted to find out as to whether GLN also activated Src-kinases and whether GRGDSP could inhibit this activation.

I tried the Src-Tyr^{418} kinase phosphorylation antibody from two different sources (Cell Signaling and Invitrogen). Unfortunately, I was unable to show a proper band without 'a smear' with these currently available antibodies. Various parameters and conditions were tested to improve the results. I used different blocking solutions (5 % Milk in PBS-tween and 5 % BSA in PBS-tween), changed the incubation times and concentrations of blocking solutions and primary and secondary antibodies, but none of the changed parameters improved the detection of the [Y(P)^{418}]Src band. Hopefully, targeting different phosphorylation sites of Src or antibodies from new sources will make it possible to detect Src phosphorylation in the near future, since Src activation seemed to be involved in GLN-mediated protection after hyperthermia as indicated by Src kinase inhibitor PP2 in MTS assay experiments (Fig. 19A). Moreover, it was possible to show Hsp70 and Hsp32 expression in conjunction with GLN and PP2 treatment. GLN-mediated increases in Hsp70 and Hsp32 were attenuated after GLN+PP2 treatment, indicating the involvement of Src activation in GLN-mediated protection after HS (Fig. 19B and C).

As the mechanistic details of GLN-mediated PI3-K/Akt and FN-Integrin signaling become clearer, it should be possible to identify the exact molecular pathways by which GLN initiates cellular protection. Continued basic and clinical research regarding GLN as a potential therapeutic agent in gastrointestinal disease is essential, since GLN has dynamic effects on the gastrointestinal tract and remains an extremely promising nutrient for metabolic support of patients with intestinal disorders.

3 Involvement of EGFR expression and signaling in GLN's protection

Like integrins, EGFRs are known osmosensors [68], which work with integrins as a 'duet' with similar downstream signaling pathways [3]. Moreover, studies exist showing that ECM proteins, such as FN, are involved in EGFR activation, mediated by integrins [36]. How integrins, EGFR, and ECM proteins are integrated proximal to MAPK signaling is not well understood at present.

In the present study, I found that GLN, which is known to have growth factor-like signaling functions, can protect against intestinal injury by expressing EGFR after HS and activating EGFR signaling (Fig. 22, Fig. 23, and Fig. 24). Furthermore, I could show that the same kinases get activated/deactivated via EGFR signaling and FN-Integrin signaling.

First, I assessed total EGFR protein levels as well as cytoplasmic and nuclear fraction levels since literature had indicated that EGFR can be internalized and that nuclear EGFR is crucial for DNA repair because of its potential role as transcription factor [42, 93]. I was interested in as to whether there is an increase in nuclear EGFR levels after GLN treatment in comparison to the cytoplasmic EGFR fraction. The expression of EGFR is necessary for cellular integrity and response to injury [44, 95]. The results showed that under non-stressed conditions (37°C) IEC-6 cells produced significant amounts of EGFR. My data also revealed that following HS (43°C), total, cytoplasmic, and nuclear EGFR levels were significantly reduced at 0 h and 3 h post-injury (Fig. 22). This reduction in EGFR was associated with significant apoptosis (Fig. 23). GLN-supplementation was able to restore epithelial cellular EGFR levels following HS to pre-injury levels, both in the cytoplasm as well as in the nucleus. Thus, GLN did not just accumulate EGFR in the nucleus. The preservation of EGFR content seemed to correlate with reduced apoptosis and GLN was able to protect intestinal epithelial cells *in vitro* from cell death by preserved EGFR expression after HS injury (Fig. 23). Silencing EGFR inhibited GLN-mediated protection suggesting that EGFR expression is essential in GLN's protective mechanism. Insufficient EGFR expression deteriorates gastrointestinal

function leading to reduced survival of patients with gastrointestinal diseases [44]. Further studies will need to evaluate if GLN prevents EGFR degradation or if GLN induces EGFR expression after HS. Literature exists showing different possible EGFR degradation pathways (liposomal, proteosomal, other pathways such as EGFR cleavage by caspases) [6, 12]. Alwan et al. showed that ligand-induced polyubiquitination of EGFR is an essential factor in the down-regulation of activated EGFRs [6]. Bae et al. showed that caspases are capable of proteolytic cleavage of EGFRs [12]. The exact mechanism responsible for EGFR degradation following stress, however, still remains obscure and is an issue of debate. Thus, EGFR degradation pathway studies in conjunction with investigating GLN's prevention of EGFR degradation after HS is a key area of research for future studies.

In the present study, I showed that not only the preservation of EGFR expression is essential for GLN-mediated protection, but that also the activation of EGFR tryrosine kinase signaling via ERK1/2 activation and p38MAPK inactivation (Fig. 24, Fig. 25, and Fig. 26) play a key signaling role. Although I have made every attempt to show EGFR phosphorylation such as utilizing multiple antibodies with different phosphorylation sites from Cell Signaling (Phospho-HER2/ErbB2 (Tyr1248)/ EGFR (Tyr 1173) Antibody (#2244S), Phospho-EGF Receptor (Tyr845) Antibody (#2231S), and Phospho-EGF Receptor (Tyr1086) Antibody (#2220S)), I was unsuccessful in showing any signal or bands with these currently available antibodies. Personal communication with other research groups confirmed similar difficulties with these antibodies. Continued efforts by using different antibodies, or even a different approach such as immunoprecipitation will be made.

EGFR, MAPKs, and PI3-K signaling are important signal transduction pathways stimulated by growth factors affecting survival and apoptosis [90, 173, 174]. Therefore, I hypothesized that there may be a relationship between these pathways and GLN's protection in IEC-6 cells after thermal injury. The present study showed that EGFR, ERK1/2, p38MAPK, and PI3-K signaling play essential roles in GLN-mediated cell survival (Fig. 9, Fig. 14, Fig. 15, Fig. 24). However, only ERK1/2 and p38MAPK were involved in GLN-mediated EGFR signaling after HS (Fig. 25 and Fig. 26). GLN seemed to activate ERK1/2 phosphorylation and to

dephosphorylate p38MAPK after hyperthermia to prevent cell death. The potential interaction between these pathways will be an interesting area of investigation for future studies.

Moreover, I could confirm Galadari's study and show that Akt depletion is a prelude to cell death following HS [51]. GLN was able to preserve total Akt at pre-injury levels after HS and, thus, was able to prevent cell death. I also investigated Akt activation, since Akt activation plays an important role in survival signaling pathways [90]. Bang et al. showed that HS phosphorylates Akt, enhances the ability of cells to survive HS, and suppresses apoptosis [13]. I was interested in knowing as to whether GLN enhances Akt activation after hyperthermia to overcome cell death. Moreover, I wanted to investigate if GLN-mediated EGFR signaling regulates increases in Akt activation. In our study, I could show that GLN increased Akt activation by 3-fold (HS by 2-fold). However, not even at the highest concentration of AG1478 (20 μM) could I show any inhibitory effect on GLN-mediated increases in Akt phosphorylation, indicating that GLN-mediated PI3-K/Akt signaling happens before EGFR signaling or simultaneously. GLN seemed to activate Akt independently from EGFR signaling after thermal injury (Fig. 27D) as was observed in GLN-mediated FN-Integrin signaling (Fig. 17). The specific role and molecular mechanisms of PI3-K signaling in GLN's protective mechanism remain to be investigated.

Since EGFRs, like integrins [68], are known to be osmosensors, it will be important to study if PI3-K regulates EGFR expression and osmosensing as well as FN expression and FN-Integrin signaling in GLN's protective mechanism after HS.

As our previous data showed that GLN is protective via Fibronectin-Integrin signaling independent of intracellular GLN concentrations [108], and that integrins, EGFR, and ECM proteins are acting in concert [3, 36], it may be possible that EGFR expression and signaling is regulated via FN-Integrin signaling or vice-versa. As specific details of interactions between EGFRs, integrins, and ECM become clearer, GLN' s precise molecular pathway leading to prevention of cell death should completely be identified.

In summary, EGFR expression and kinase activity is involved in GLN's protective mechanism in IEC-6 cells after HS. The results of the present study suggested that GLN preserves

expression of EGFR after HS and activates EGFR tyrosine activity, which phosphorylates ERK1/2 and deactivates p38MAPK, preventing apoptosis independently from PI3-K signaling. However, GLN is also protective via activation of PI3-K signaling. Figure 25 shows the proposed pathway by which GLN mediates protection against intestinal epithelial cell injury via EGFR signaling.

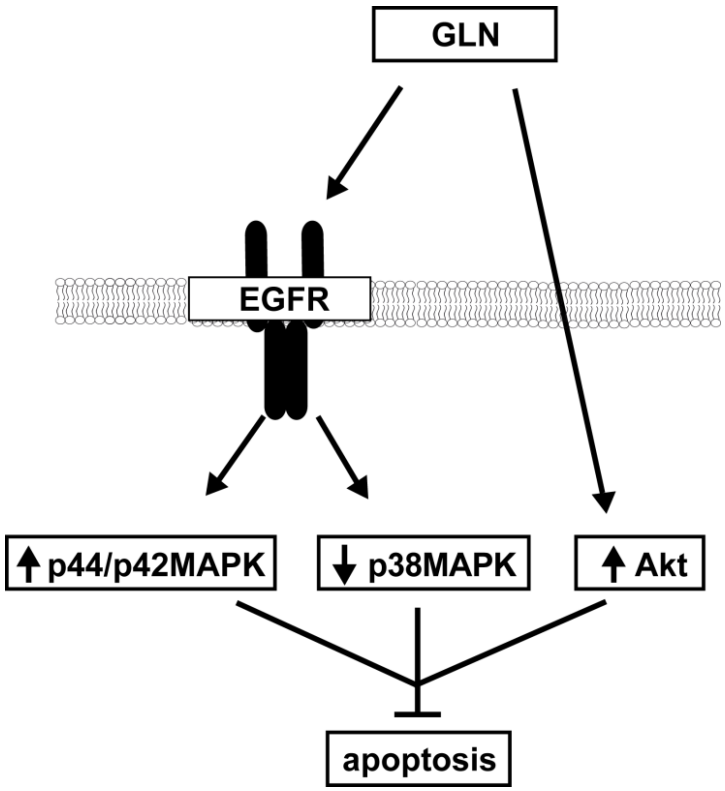


Fig. 30. Proposed pathways of GLN's protective effects after HS via EGFR signaling. GLN is protective in the intestine by preventing EGFR expression after HS, as well as by activating the protective EGFR signaling pathway. GLN phosphorylates ERK1/2 and inactivates p38MAPK via the EGFR tyrosine kinase signaling to prevent apoptosis. However, GLN is protective by activating the PI3-K pathway independently from EGFR signaling.

Whether GLN is protective via two distinct simultaneous pathways or whether PI3-K signaling has a unique effect on EGFR signaling will need to be investigated in future studies.

Many conditions of injury and stress, including inflammatory gut injury, lead to rapid GLN deficiency [115]. GLN deficiency may lead to a reduction of EGFR levels and tyrosine kinase

activity, which increases cell death because of insufficient ERK1/2 activation and p38MAPK dephosphorylation. If this hypothesis is correct, these data have particular relevance to the clinical care of human disease because of GLN's potential to optimize EGFR expression and signaling to protect tissues/organs, and ultimately improve clinical outcome.

4 Ntn-1 expression is important in GLN's protection in the intestine

The ECM anchors various diffusible proteins, such as Ntn-1, providing informational cues for several cellular functions including cell survival [31]. Recently, it has been shown that Ntn-1 plays an important protective role in non-neural cells, e.g. intestinal epithelial cells and that extracellular Ntn-1 treatment was able to rescue mice after DSS injury [2]. IBDs, encapsulating ulcerative colitis and Crohn's disease affect the gastrointestinal tract and disrupt the intestinal epithelial barrier. Approximately one million people suffer from ulcerative colitis and Crohn's disease and the disease progression is relentless since up to 70 % of IBD patients need to undergo surgery [2, 35]. There remains an urgent need for novel therapeutics to control the exaggerated inflammation within the intestine of the patients with IBD. Recent studies have implicated Ntn-1 in mediating tissue inflammatory responses [2, 98]. Moreover, Ntn-1 has been associated in dampening hypoxia-induced inflammation in IBD [2]. This notion coupled with an inhibitory effect on Ntn-1 on leukocyte transmigration [2] led to the hypothesis that Ntn-1 expression may mediate tissue protection after thermal intestinal injury. Based on these findings, GLN's effect on Ntn-1 expression after HS seemed to be an interesting field of investigation. Since GLN prevents FN expression and signals via integrins [31, 175] after thermal intestinal injury, I was interested in as to whether GLN may also prevent the expression of Ntn-1, another well-known protective ECM protein. Directly after HS, Ntn-1 levels were reduced by 80 % (Fig. 20). GLN-mediated preservation of Ntn-1 levels seemed to be associated with reduced apoptosis (Fig. 21). I was able to show, that GLN can protect intestinal epithelial cells *in vitro* from cell death by preserved Ntn-1

expression after HS injury (Fig. 21). Silencing Ntn-1 inhibited GLN's protection (Fig. 21), suggesting that Ntn-1 expression is important in GLN's protection against intestinal injury. My results showed that GLN was able to preserve Ntn-1 content after HS (Fig. 20). Taken together, my data revealed for the first time a protective role of GLN-mediated Ntn-1 expression after thermal intestinal injury. Future studies will need to determine the exact molecular mechanism by which GLN is protective via prevention of Ntn-1 content in the intestine after hyperthermia. Moreover, a more mechanistic understanding of Ntn-1 signaling events would set the stage for potentially targeting Ntn-1 in the treatment of patients suffering from IBDs. Studies with partial Ntn-1 deficient mice (*Ntn-1*$^{+/-}$) in conjunction with or without GLN treatment will be a key area of future research. Furthermore, PCR experiments will need to be performed to assess whether GLN increases Ntn-1 expression or prevents Ntn-1 degradation after HS.

E Summary - Zusammenfassung

Critical illness like sepsis, shock, and intestinal bowel disease are one of the leading causes of morbidity and mortality in the US and around the world. At present, studies to define new therapeutic interventions that can protect tissues and cells against injury and attenuate inflammation are fields of intense investigation. While research over the past decade has clearly identified GLN as a vital stress substrate facilitating cellular survival following injury, the initiation steps in GLN's cytoprotective molecular mechanism still remain elusive. Previously published work suggested that stabilization of ECM proteins and activation of ECM receptor osmosignaling may play a central role in the orchestration of many cellular pathways following stress.

Thus, I hypothesized that preservation of ECM protein and EGFR levels as well as ECM receptor signaling play key roles in the molecular mechanisms underlying GLN's protection against thermal injury in the intestine.

I was able to confirm via Western blotting and by using silencing RNA against FN, Ntn-1, EGFR, and their negative controls, that GLN-mediated preservation of FN, Ntn-1, and EGFR levels is critical in GLN's protection against hyperthermia in IEC-6 cells. By using a selective FN-Integrin interaction inhibitor GRGDSP, its negative control peptide GRGESP, and Src-kinase inhibitor PP2, I showed that FN-Integrin signaling and Src-kinase activation are essential in GLN-mediated protection in the intestine. This applied to EGFR signaling as demonstrated using the EGFR tyrosine kinase inhibitor AG1478. In addition to GRGDSP and AG1478, ERK1/2 inhibitors PD98059 and UO126 as well as the p38MAPK inhibitor SB203580 revealed that GLN is protective by activating ERK1/2 and dephosphorylating p38MAPK via FN-Integrin and EGFR signaling. However, GLN-mediated PI3-K/Akt/Hsp70 activation seems to occur independently of FN-Integrin and EGFR signaling as indicated by Western blots as well as experiments using the PI3-K inhibitor LY294002, GRGDSP, and AG1478. The results showed that GLN activates cell survival signaling pathways via integrins as well as EGFRs after hyperthermia. Moreover, I found that GLN-mediated preservation of

FN expression after HS is regulated via PI3-K signaling. Whether GLN-mediated PI3-K signaling happens simultaneously to FN-Integrin and EGFR signaling or whether PI3-K signaling coordinates FN-Integrin and EGFR signaling needs to be investigated in future studies. Further, experiments with PD98059 and GRGDSP revealed that ERK1/2 assists in mediating transactivation of HSF-1 following HS. This leads to increases in Hsp70 expression via FN-Integrin signaling, which is known to attenuate apoptosis after thermal injury. Fluorescence microscopy results indicated that HS and GLN regulate cell are size changes and the morphology of F-actin via FN-Integrin signaling. Experiments using GRGDSP and GRGESP showed that GLN enhances cellular survival via FN-Integrin signaling in a manner that does not require increased intracellular GLN concentrations (as quantified using LC-MS/MS).

In summary, my thesis work gives new and potentially clinically relevant mechanistic insights into GLN-mediated molecular cell survival pathways. These results warrant clinical translation to assess if clinical outcome of critically ill patients suffering from gastrointestinal diseases can be improved by GLN treatment and/or by targeting the molecular pathways found in my studies.

Zusammenfassung

Kritische Erkrankungen wie Sepsis, Schock und entzündliche Darmerkrankungen sind eine der häufigsten Todesursachen in den Vereinigten Staaten und dem Rest der Welt. Von großer Bedeutung sind deshalb Studien, die darauf abzielen, neue therapeutische Ansätze zu finden, die Gewebe und Zellen vor Verletzungen schützen und Entzündungen verhindern. Im letzten Jahrzehnt hat sich herausgestellt, dass Glutamin Schutz gegen die pathophysiologischen und pathobiochemischen Entgleisungen während solcher Erkrankungen vermitteln kann. Trotzdem ist es bisher nur sehr unvollständig gelungen, die zugrundeliegenden molekularen Mechanismen, die für diese zellulären Schutzfunktionen von Glutamin verantwortlich sind, aufzuklären. In diesem Zusammenhang gab es erste Hinweise darauf, dass die Stabilisierung von extrazellulären Matrixproteinen und die Aktivierung der

Signalwege extrazellulärer Matrixproteinrezeptoren eine wichtige Rolle spielen könnten.

Hierauf basierend habe ich die Hypothesen aufgestellt, dass die Expression von extrazellulären Matrixproteingehalten und von epidermalen Wachstumsfaktorrezeptoren (EGFR), sowie die Aktivierung von Integrin- und EGFR-Signalwegen Schlüsselrollen in den protektiven Mechanismen von Glutamin spielen.

Meine Arbeiten zeigten anhand von Western Blots und durch den Einsatz von Silencing RNA (gegen Fibronektin, Netrin-1, EGFR und deren Negativkontrollen), dass Glutamin extrazelluläre Matrixproteingehalte, wie Fibronektin und Netrin-1, und EGFR-Levels vor Hitzestress in Darmepithelzellen bewahrt, wodurch Apoptose verhindert wird. Außerdem konnte ich mit Hilfe des selektiven Inhibitors GRGDSP, seiner Negativkontrolle GRGESP und des Src-Kinase-Inhibitors PP2 nachweisen, dass auch die Aktivierung von Fibronektin-Integrin-Signalwegen und von Src-Kinasen an den Schutzmechanismen von Glutamin im Darm beteiligt sind. Wie mit Hilfe des spezifischen EGFR-Tyrosinkinase-Inhibitors AG1478 nachgewiesen werden konnte, trifft dies auch für die EGFR-Signalwege zu. Zusätzlich zu GRGDSP und AG1478 wurden auch die ERK1/2-Kinase-Inhibitoren PD98059 und UO126 und der p38MAPK-Inhibitor verwendet, um zu zeigen, dass Glutamin Darmepithelzellen durch die Aktivität von Fibronektin-Integrin- und EGFR-Signalwegen vor Hitzestress schützt, indem es EKR1/2 phosphoryliert und p38MAPK dephosphoryliert. PI-3K/Akt/Hsp70-Signalwege werden ebenfalls von Glutamin aktiviert, jedoch unabhängig von Fibronektin-Integrin- und EGFR-Signalwegen. Dies konnte anhand von Western Blot Experimenten und mit Hilfe von LY294002 (PI-3K-Inhibitor), GRGDSP und AG1478 bewiesen werden. Glutamin scheint die gleichen Signalwege sowohl mit Hilfe von Integrinen als auch von EGFR zu beeinflussen, um so den Zelltod in Darmepithelzellen nach Hitzestress zu verhindern. Desweiteren konnte gezeigt werden, dass Glutamin die Erhaltung von Fibronektinkonzentrationen nach Hitzestress durch den PI-3K-Signalweg reguliert. Ob Glutamin den PI-3K-Signalweg parallel zu den Integrin- und EGFR-Signalwegen aktiviert oder ob der von Glutamin ausgelöste PI-3K-Signalweg die Integrin- und EGFR-Signalwege steuert, muss noch weiter untersucht werden. Experimente mit PD98059 und GRGDSP

zeigten, dass ERK1/2 in der Gegenwart von Glutamin den Transkriptionsfaktor HSF-1 durch den Fibronektin-Integrin-Signalweg nach Hitzestress transaktiviert. Dadurch nimmt die Expression von Hsp70, welches den Zelltod nach Hitzeschock verhindert, zu. Mit Hilfe der Fluoreszenzmikroskopie konnte gezeigt werden, dass Hitzestress und Glutamin die Zellgröße und die Aktinmorphologie von Darmepithelzellen durch Fibronektin-Integrin-Signalwege steuern können. Außerdem ergaben Versuche mit GRGDSP und GRGESP, dass Glutamin unabhänging von seinen intrazellulären Konzentrationen das Überleben von Darmepithelzellen durch die Aktivierung von Fibronektin-Integrin-Signalwegen erhöhen kann. Hierbei wurden die Glutaminkonzentrationen mit LC-MS/MS gemessen.

Die Versuche im Rahmen meiner Dissertation konnten neue und potentiell klinisch relevante Signalwege der molekularen Schutzmechanismen von Glutamin aufdecken. Diese Ergebnisse können die Grundlage für translationale Studien darstellen, die weiter untersuchen, ob die Überlebensrate von Patienten mit entzündlichen Darmerkrankungen durch die Behandlung mit Glutamin oder durch gezielte Beeinflussung der hier untersuchten molekularen Mechanismen verbessert werden kann.

F References

1. Adams, J.C. and F.M. Watt, *Regulation of development and differentiation by the extracellular matrix.* Development, 1993. **117**(4): p. 1183-98.
2. Aherne, C.M., et al., *Neuronal guidance molecule netrin-1 attenuates inflammatory cell trafficking during acute experimental colitis.* Gut, 2012. **61**(5): p. 695-705.
3. Alam, N., et al., *The integrin-growth factor receptor duet.* J Cell Physiol, 2007. **213**(3): p. 649-53.
4. Alberts, B., et al., *Essential cell biology*. 2004, New York and London: Garland.
5. Alexander, R.J., et al., *Expression of growth factor receptor-encoded mRNA by colonic epithelial cells is altered in inflammatory bowel disease.* Dig Dis Sci, 1995. **40**(3): p. 485-94.
6. Alwan, H.A., E.J. van Zoelen, and J.E. van Leeuwen, *Ligand-induced lysosomal epidermal growth factor receptor (EGFR) degradation is preceded by proteasome-dependent EGFR de-ubiquitination.* J Biol Chem, 2003. **278**(37): p. 35781-90.
7. Anderson, R.A., et al., *Phosphatidylinositol phosphate kinases, a multifaceted family of signaling enzymes.* J Biol Chem, 1999. **274**(15): p. 9907-10.
8. Angus, D.C., et al., *Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care.* Crit Care Med, 2001. **29**(7): p. 1303-10.
9. Aplin, A.E., et al., *Signal transduction and signal modulation by cell adhesion receptors: the role of integrins, cadherins, immunoglobulin-cell adhesion molecules, and selectins.* Pharmacol Rev, 1998. **50**(2): p. 197-263.
10. Armstrong, M., K. Jonscher, and N.A. Reisdorph, *Analysis of 25 underivatized amino acids in human plasma using ion-pairing reversed-phase liquid chromatography/time-of-flight mass spectrometry.* Rapid Commun Mass Spectrom, 2007. **21**(16): p. 2717-26.
11. Avissar, N.E., H.C. Sax, and L. Toia, *In human entrocytes, GLN transport and ASCT2 surface expression induced by short-term EGF are MAPK, PI3K, and Rho-dependent.* Dig Dis Sci, 2008. **53**(8): p. 2113-25.
12. Bae, S.S., et al., *Proteolytic cleavage of epidermal growth factor receptor by caspases.* FEBS Lett, 2001. **491**(1-2): p. 16-20.
13. Bang, O.S., et al., *Activation of Akt is induced by heat shock and involved in suppression of heat-shock-induced apoptosis of NIH3T3 cells.* Biochem Biophys Res Commun, 2000. **278**(2): p. 306-11.
14. Beaulieu, J.F., *Integrins and human intestinal cell functions.* Front Biosci, 1999. **4**: p. D310-21.
15. Benjamin, I.J. and D.R. McMillan, *Stress (heat shock) proteins: molecular chaperones in cardiovascular biology and disease.* Circ Res, 1998. **83**(2): p. 117-32.
16. Berkes, J., et al., *Intestinal epithelial responses to enteric pathogens: effects on the tight junction barrier, ion transport, and inflammation.* Gut, 2003. **52**(3): p. 439-51.
17. Bernal, N.P., et al., *Epidermal growth factor receptor signaling regulates Bax and Bcl-w expression and apoptotic responses during intestinal adaptation in mice.* Gastroenterology, 2006. **130**(2): p. 412-23.
18. Bill, H.M., et al., *Epidermal growth factor receptor-dependent regulation of integrin-mediated signaling and cell cycle entry in epithelial cells.* Mol Cell Biol, 2004. **24**(19): p. 8586-99.
19. Biolo, G., et al., *Metabolic response to injury and sepsis: changes in protein metabolism.* Nutrition, 1997. **13**(9 Suppl): p. 52S-57S.
20. Boelens, P.G. and P.A. Van Leeuwen, *Glutamine-supplemented diets in enteral nutrition*, in *Metabolic and therapeutic aspects of amino acids in clinical nutrition*, L.A. Cynober, Editor. 2004, CRC Press: New York.

21. Bonni, A., et al., *Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms.* Science, 1999. **286**(5443): p. 1358-62.
22. Bortner, C.D. and J.A. Cidlowski, *Apoptotic volume decrease and the incredible shrinking cell.* Cell Death Differ, 2002. **9**(12): p. 1307-10.
23. Brakebusch, C. and R. Fassler, *The integrin-actin connection, an eternal love affair.* EMBO J, 2003. **22**(10): p. 2324-33.
24. Byzova, T.V., et al., *A mechanism for modulation of cellular responses to VEGF: activation of the integrins.* Mol Cell, 2000. **6**(4): p. 851-60.
25. Cantley, L.C., *The phosphoinositide 3-kinase pathway.* Science, 2002. **296**(5573): p. 1655-7.
26. Carpenter, C.L. and L.C. Cantley, *Phosphoinositide kinases.* Curr Opin Cell Biol, 1996. **8**(2): p. 153-8.
27. Carpenter, G., *The EGF receptor: a nexus for trafficking and signaling.* Bioessays, 2000. **22**(8): p. 697-707.
28. Carroll, P.V., *Protein metabolism and the use of growth hormone and insulin-like growth factor-I in the critically ill patient.* Growth Horm IGF Res, 1999. **9**(6): p. 400-13.
29. Charalabopoulos, K., et al., *Integrin receptors and extracellular matrix components in parotid neoplasms.* Exp Oncol, 2005. **27**(1): p. 2-6.
30. Chow, A. and R. Zhang, *Glutamine reduces heat shock-induced cell death in rat intestinal epithelial cells.* J Nutr, 1998. **128**(8): p. 1296-301.
31. Cirulli, V. and M. Yebra, *Netrins: beyond the brain.* Nat Rev Mol Cell Biol, 2007. **8**(4): p. 296-306.
32. Clark, J.A. and C.M. Coopersmith, *Intestinal crosstalk: a new paradigm for understanding the gut as the "motor" of critical illness.* Shock, 2007. **28**(4): p. 384-93.
33. Clark, J.A., et al., *Intestinal barrier failure during experimental necrotizing enterocolitis: protective effect of EGF treatment.* Am J Physiol Gastrointest Liver Physiol, 2006. **291**(5): p. G938-49.
34. Clark, J.A., et al., *Epidermal growth factor reduces intestinal apoptosis in an experimental model of necrotizing enterocolitis.* Am J Physiol Gastrointest Liver Physiol, 2005. **288**(4): p. G755-62.
35. Clark, M., et al., *American gastroenterological association consensus development conference on the use of biologics in the treatment of inflammatory bowel disease, June 21-23, 2006.* Gastroenterology, 2007. **133**(1): p. 312-39.
36. Cybulsky, A.V., A.J. McTavish, and M.D. Cyr, *Extracellular matrix modulates epidermal growth factor receptor activation in rat glomerular epithelial cells.* J Clin Invest, 1994. **94**(1): p. 68-78.
37. Cynober, L.A., *Metabolic and therapeutic aspects of amino acids in clinical nutrition*. 2004: CRC Press.
38. Danielsen, A.J. and N.J. Maihle, *The EGF/ErbB receptor family and apoptosis.* Growth Factors, 2002. **20**(1): p. 1-15.
39. Daugherty, A.L. and R.J. Mrsny, *Transcellular uptake mechanisms of the intestinal epithelial barrier Part one.* Pharm Sci Technolo Today, 1999. **4**(2): p. 144-151.
40. De Maio, A., *Heat shock proteins: facts, thoughts, and dreams.* Shock, 1999. **11**(1): p. 1-12.
41. Debaveye, Y. and G. Van den Berghe, *Risks and benefits of nutritional support during critical illness.* Annu Rev Nutr, 2006. **26**: p. 513-38.
42. Dittmann, K., et al., *Radiation-induced caveolin-1 associated EGFR internalization is linked with nuclear EGFR transport and activation of DNA-PK.* Mol Cancer, 2008. **7**: p. 69.
43. Dombrovskiy, V.Y., et al., *Rapid increase in hospitalization and mortality rates for severe sepsis in the United States: a trend analysis from 1993 to 2003.* Crit Care Med, 2007. **35**(5): p. 1244-50.
44. Dube, P.E., et al., *Epidermal growth factor receptor inhibits colitis-associated cancer in mice.* J Clin Invest, 2012. **122**(8): p. 2780-92.

45. Edinger, A.L. and C.B. Thompson, *Akt maintains cell size and survival by increasing mTOR-dependent nutrient uptake.* Mol Biol Cell, 2002. **13**(7): p. 2276-88.
46. Egger, B., et al., *Mice harboring a defective epidermal growth factor receptor (waved-2) have an increased susceptibility to acute dextran sulfate-induced colitis.* Scand J Gastroenterol, 2000. **35**(11): p. 1181-7.
47. Egger, B., et al., *Reduced susceptibility of mice overexpressing transforming growth factor alpha to dextran sodium sulphate induced colitis.* Gut, 1998. **43**(1): p. 64-70.
48. Egger, B., et al., *Mice lacking transforming growth factor alpha have an increased susceptibility to dextran sulfate-induced colitis.* Gastroenterology, 1997. **113**(3): p. 825-32.
49. Ehrenfried, J.A., et al., *Glutamine-mediated regulation of heat shock protein expression in intestinal cells.* Surgery, 1995. **118**(2): p. 352-6; discussion 356-7.
50. Fletcher, D.A. and R.D. Mullins, *Cell mechanics and the cytoskeleton.* Nature, 2010. **463**(7280): p. 485-92.
51. Galadari, S., et al., *Akt depletion is an important determinant of L929 cell death following heat stress.* Ann N Y Acad Sci, 2008. **1138**: p. 385-92.
52. Garcia-Lafuente, A., et al., *Modulation of colonic barrier function by the composition of the commensal flora in the rat.* Gut, 2001. **48**(4): p. 503-7.
53. Gianotti, L., et al., *Oral glutamine decreases bacterial translocation and improves survival in experimental gut-origin sepsis.* JPEN J Parenter Enteral Nutr, 1995. **19**(1): p. 69-74.
54. Gilcrease, M.Z., *Integrin signaling in epithelial cells.* Cancer Lett, 2007. **247**(1): p. 1-25.
55. Goke, M., A. Zuk, and D.K. Podolsky, *Regulation and function of extracellular matrix intestinal epithelial restitution in vitro.* Am J Physiol, 1996. **271**(5 Pt 1): p. G729-40.
56. Griffiths, R.D., C. Jones, and T.E. Palmer, *Six-month outcome of critically ill patients given glutamine-supplemented parenteral nutrition.* Nutrition, 1997. **13**(4): p. 295-302.
57. Gu, J., et al., *Laminin-10/11 and fibronectin differentially prevent apoptosis induced by serum removal via phosphatidylinositol 3-kinase/Akt- and MEK1/ERK-dependent pathways.* J Biol Chem, 2002. **277**(22): p. 19922-8.
58. Hallbrucker, C., et al., *Control of hepatic proteolysis by amino acids. The role of cell volume.* Eur J Biochem, 1991. **197**(3): p. 717-24.
59. Halpern, M.D., et al., *Ileal cytokine dysregulation in experimental necrotizing enterocolitis is reduced by epidermal growth factor.* J Pediatr Gastroenterol Nutr, 2003. **36**(1): p. 126-33.
60. Hamiel, C.R., et al., *Glutamine enhances heat shock protein 70 expression via increased hexosamine biosynthetic pathway activity.* American Journal of Physiology-Cell Physiology, 2009. **297**(6): p. C1509-C1519.
61. Han, S.W. and J. Roman, *Fibronectin induces cell proliferation and inhibits apoptosis in human bronchial epithelial cells: pro-oncogenic effects mediated by PI3-kinase and NF-kappa B.* Oncogene, 2006. **25**(31): p. 4341-9.
62. Harari, Y., N.W. Weisbrodt, and F.G. Moody, *Ileal mucosal response to bacterial toxin challenge.* J Trauma, 2000. **49**(2): p. 306-13.
63. Häussinger, D., D. Graf, and O.H. Weiergraber, *Glutamine and cell signaling in liver.* J Nutr, 2001. **131**(9 Suppl): p. 2509S-14S; discussion 2523S-4S.
64. Häussinger, D., et al., *Cell volume is a major determinant of proteolysis control in liver.* FEBS Lett, 1991. **283**(1): p. 70-2.
65. Häussinger, D., et al., *Cell swelling inhibits proteolysis in perfused rat liver.* Biochem J, 1990. **272**(1): p. 239-42.
66. Häussinger, D., et al., *Molecular aspects of medicine: from experimental to clinical hepatology.* Mol Aspects Med, 2004. **25**(3): p. 221-360.
67. Häussinger, D. and F. Lang, *Cell volume and hormone action.* Trends Pharmacol Sci, 1992. **13**(10): p. 371-3.
68. Hoffmann, E.K., I.H. Lambert, and S.F. Pedersen, *Physiology of cell volume regulation in vertebrates.* Physiol Rev, 2009. **89**(1): p. 193-277.

69. Houdijk, A.P., et al., *Randomised trial of glutamine-enriched enteral nutrition on infectious morbidity in patients with multiple trauma.* Lancet, 1998. **352**(9130): p. 772-6.
70. Huang, L.E., et al., *Rapid activation of the heat shock transcription factor, HSF1, by hypo-osmotic stress in mammalian cells.* Biochem J, 1995. **307 (Pt 2)**: p. 347-52.
71. Hung, J.J., et al., *Differential activation of p38 mitogen-activated protein kinase and extracellular signal-regulated protein kinases confers cadmium-induced HSP70 expression in 9L rat brain tumor cells.* J Biol Chem, 1998. **273**(48): p. 31924-31.
72. Hynes, R.O., *Integrins: versatility, modulation, and signaling in cell adhesion.* Cell, 1992. **69**(1): p. 11-25.
73. Hynes, R.O., *Integrins: bidirectional, allosteric signaling machines.* Cell, 2002. **110**(6): p. 673-87.
74. Jakob, U., et al., *Small heat shock proteins are molecular chaperones.* J Biol Chem, 1993. **268**(3): p. 1517-20.
75. Jorissen, R.N., et al., *Epidermal growth factor receptor: mechanisms of activation and signalling.* Exp Cell Res, 2003. **284**(1): p. 31-53.
76. Juliano, R.L. and S. Haskill, *Signal transduction from the extracellular matrix.* J Cell Biol, 1993. **120**(3): p. 577-85.
77. Junttila, M.R., S.P. Li, and J. Westermarck, *Phosphatase-mediated crosstalk between MAPK signaling pathways in the regulation of cell survival.* FASEB J, 2008. **22**(4): p. 954-65.
78. Kallweit, A.R., et al., *Glutamine Prevents Apoptosis in Intestinal Epithelial Cells and Induces Differential Protective Pathways in Heat and Oxidant Injury Models.* JPEN J Parenter Enteral Nutr, 2012.
79. Keller, J.M., J.F. Escara-Wilke, and E.T. Keller, *Heat stress-induced heat shock protein 70 expression is dependent on ERK activation in zebrafish (Danio rerio) cells.* Comp Biochem Physiol A Mol Integr Physiol, 2008. **150**(3): p. 307-14.
80. Kerr, J.F., A.H. Wyllie, and A.R. Currie, *Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics.* Br J Cancer, 1972. **26**(4): p. 239-57.
81. Keshavarzian, A., et al., *Increases in free radicals and cytoskeletal protein oxidation and nitration in the colon of patients with inflammatory bowel disease.* Gut, 2003. **52**(5): p. 720-8.
82. Klimberg, V.S., et al., *Oral glutamine accelerates healing of the small intestine and improves outcome after whole abdominal radiation.* Arch Surg, 1990. **125**(8): p. 1040-5.
83. Klimberg, V.S., et al., *Prophylactic glutamine protects the intestinal mucosa from radiation injury.* Cancer, 1990. **66**(1): p. 62-8.
84. Kohno, M. and J. Pouyssegur, *Pharmacological inhibitors of the ERK signaling pathway: application as anticancer drugs.* Prog Cell Cycle Res, 2003. **5**: p. 219-24.
85. Kolachala, V.L., et al., *Epithelial-derived fibronectin expression, signaling, and function in intestinal inflammation.* J Biol Chem, 2007. **282**(45): p. 32965-73.
86. Kovacevic, Z. and J.D. McGivan, *Mitochondrial metabolism of glutamine and glutamate and its physiological significance.* Physiol Rev, 1983. **63**(2): p. 547-605.
87. Kumar, V., A.K. Abbas, and F. Fausto, *Patholoolgic Basis of Disease*. 7th edition ed. 2004: Elsevier.
88. Kyriakis, J.M. and J. Avruch, *Protein kinase cascades activated by stress and inflammatory cytokines.* Bioessays, 1996. **18**(7): p. 567-77.
89. Lacey, J.M., et al., *The effects of glutamine-supplemented parenteral nutrition in premature infants.* JPEN J Parenter Enteral Nutr, 1996. **20**(1): p. 74-80.
90. Larson, S.D., et al., *Molecular mechanisms contributing to glutamine-mediated intestinal cell survival.* Am J Physiol Gastrointest Liver Physiol, 2007. **293**(6): p. G1262-71.
91. Lewin, B., et al., *Cells*. 2006: Jones and Bartlett.
92. Lin, C.Q. and M.J. Bissell, *Multi-faceted regulation of cell differentiation by extracellular matrix.* FASEB J, 1993. **7**(9): p. 737-43.

93. Lin, S.Y., et al., *Nuclear localization of EGF receptor and its potential new role as a transcription factor.* Nat Cell Biol, 2001. **3**(9): p. 802-8.
94. Liu, F., et al., *Heat-stress-induced damage to porcine small intestinal epithelium associated with downregulation of epithelial growth factor signaling.* J Anim Sci, 2009. **87**(6): p. 1941-9.
95. Liu, S., D.A. Calderwood, and M.H. Ginsberg, *Integrin cytoplasmic domain-binding proteins.* J Cell Sci, 2000. **113 (Pt 20)**: p. 3563-71.
96. Lodish, H., et al., *Molecular Cell Biology*. 2000: W.H. Freeman and Company.
97. Lord, L.M. and H.C. Sax, *The role of the gut in critical illness.* AACN Clin Issues Crit Care Nurs, 1994. **5**(4): p. 450-8.
98. Ly, N.P., et al., *Netrin-1 inhibits leukocyte migration in vitro and in vivo.* Proc Natl Acad Sci U S A, 2005. **102**(41): p. 14729-34.
99. Matsumoto, T., et al., *p38 MAP kinase negatively regulates endothelial cell survival, proliferation, and differentiation in FGF-2-stimulated angiogenesis.* J Cell Biol, 2002. **156**(1): p. 149-60.
100. Meisse, D., et al., *Cell swelling increased the alpha2-macroglobulin gene expression in cultured rat hepatocytes.* FEBS Lett, 1998. **422**(3): p. 346-8.
101. Mestril, R., et al., *Expression of inducible stress protein 70 in rat heart myogenic cells confers protection against simulated ischemia-induced injury.* J Clin Invest, 1994. **93**(2): p. 759-67.
102. Miranti, C.K. and J.S. Brugge, *Sensing the environment: a historical perspective on integrin signal transduction.* Nat Cell Biol, 2002. **4**(4): p. E83-90.
103. Morimoto, R., A. Tissieres, and C. Georgopoulos, *The stress response function of the proteins and perspectives*. Stress Proteins in Biology and Medicine. 1990, Cold Spring Harbour, NY: Cold Spring Harbour Laboratory Press.
104. Morrison, A.L., et al., *Glutamine's protection against cellular injury is dependent on heat shock factor-1.* Am J Physiol Cell Physiol, 2006. **290**(6): p. C1625-32.
105. Murphy, S.L., *Deaths: final data for 1998.* Natl Vital Stat Rep, 2000. **48**(11): p. 1-105.
106. Nair, R.R., B.B. Warner, and B.W. Warner, *Role of epidermal growth factor and other growth factors in the prevention of necrotizing enterocolitis.* Semin Perinatol, 2008. **32**(2): p. 107-13.
107. Niederlechner, S., et al., *Epidermal Growth Factor Receptor Expression and Signaling Is Essential in Glutamine's Cytoprotective Mechanism in Heat-Stressed Intestinal Epithelial-6 Cells.* Am J Physiol Gastrointest Liver Physiol, 2012.
108. Niederlechner, S., et al., *Fibronectin-Integrin Signaling Is Required for L-Glutamine's Protection against Gut Injury.* PLOS ONE, 2012. **7**(11): p. e50185.
109. Nissim, I., et al., *Effect of glutamine on heat-shock-induced mRNA and stress proteins.* J Cell Physiol, 1993. **157**(2): p. 313-8.
110. Novak, F., et al., *Glutamine supplementation in serious illness: a systematic review of the evidence.* Crit Care Med, 2002. **30**(9): p. 2022-9.
111. Nover, L., *Heat shock response*. 1st ed. 1991, Boston: CRC Press.
112. O'Toole, T.E., et al., *Modulation of the affinity of integrin alpha IIb beta 3 (GPIIb-IIIa) by the cytoplasmic domain of alpha IIb.* Science, 1991. **254**(5033): p. 845-7.
113. Oehler, R. and E. Roth, *Glutamine metabolism*. Metabolic and Therapeutic Aspects of Amino Acids in Clinical Nutrition, ed. L.A. Cynober. 2004, London, New York, Washington DC: CRC Press.
114. Oudemans-van Straaten, H.M., et al., *Plasma glutamine depletion and patient outcome in acute ICU admissions.* Intensive Care Med, 2001. **27**(1): p. 84-90.
115. Parry-Billings, M., et al., *Does glutamine contribute to immunosuppression after major burns?* Lancet, 1990. **336**(8714): p. 523-5.
116. Paul, G., et al., *Interferon-gamma alters downstream signaling originating from epidermal growth factor receptor in intestinal epithelial cells: functional consequences for ion transport.* J Biol Chem, 2012. **287**(3): p. 2144-55.
117. Pelham, H.R., *Speculations on the functions of the major heat shock and glucose-regulated proteins.* Cell, 1986. **46**(7): p. 959-61.

118. Piraud, M., et al., *ESI-MS/MS analysis of underivatised amino acids: a new tool for the diagnosis of inherited disorders of amino acid metabolism. Fragmentation study of 79 molecules of biological interest in positive and negative ionisation mode.* Rapid Commun Mass Spectrom, 2003. **17**(12): p. 1297-311.
119. Playford, R.J. and N.A. Wright, *Why is epidermal growth factor present in the gut lumen?* Gut, 1996. **38**(3): p. 303-5.
120. Plumier, J.C., et al., *Transgenic mice expressing the human heat shock protein 70 have improved post-ischemic myocardial recovery.* J Clin Invest, 1995. **95**(4): p. 1854-60.
121. Preiser, J.C. and J. Wernerman, *Glutamine, a life-saving nutrient, but why?* Crit Care Med, 2003. **31**(10): p. 2555-6.
122. Quaroni, A., K.J. Isselbacher, and E. Ruoslahti, *Fibronectin synthesis by epithelial crypt cells of rat small intestine.* Proc Natl Acad Sci U S A, 1978. **75**(11): p. 5548-52.
123. Quaroni, A., et al., *Epithelioid cell cultures from rat small intestine. Characterization by morphologic and immunologic criteria.* J Cell Biol, 1979. **80**(2): p. 248-65.
124. Rangel-Frausto, M.S., et al., *The natural history of the systemic inflammatory response syndrome (SIRS). A prospective study.* JAMA, 1995. **273**(2): p. 117-23.
125. Rieder, F., et al., *Wound healing and fibrosis in intestinal disease.* Gut, 2007. **56**(1): p. 130-9.
126. Ritossa, F., *Discovery of the heat shock response.* Cell Stress Chaperones, 1996. **1**(2): p. 97-8.
127. Roediger, W.E., *Utilization of nutrients by isolated epithelial cells of the rat colon.* Gastroenterology, 1982. **83**(2): p. 424-9.
128. Roskelley, C.D., A. Srebrow, and M.J. Bissell, *A hierarchy of ECM-mediated signalling regulates tissue-specific gene expression.* Curr Opin Cell Biol, 1995. **7**(5): p. 736-47.
129. Roth, E., et al., *Metabolic disorders in severe abdominal sepsis: glutamine deficiency in skeletal muscle.* Clin Nutr, 1982. **1**(1): p. 25-41.
130. Rothman, J.E., *Polypeptide chain binding proteins: catalysts of protein folding and related processes in cells.* Cell, 1989. **59**(4): p. 591-601.
131. Ruoslahti, E., *RGD and other recognition sequences for integrins.* Annu Rev Cell Dev Biol, 1996. **12**: p. 697-715.
132. Sakiyama, T., et al., *Glutamine increases autophagy under Basal and stressed conditions in intestinal epithelial cells.* Gastroenterology, 2009. **136**(3): p. 924-32.
133. Saleem, S., et al., *beta1 integrin/FAK/ERK signalling pathway is essential for human fetal islet cell differentiation and survival.* The Journal of pathology, 2009. **219**(2): p. 182-92.
134. Schliess, F., R. Reinehr, and D. Haussinger, *Osmosensing and signaling in the regulation of mammalian cell function.* FEBS J, 2007. **274**(22): p. 5799-803.
135. Seger, R. and E.G. Krebs, *The MAPK signaling cascade.* FASEB J, 1995. **9**(9): p. 726-35.
136. Sen, S., W.P. Ng, and S. Kumar, *Contractility dominates adhesive ligand density in regulating cellular de-adhesion and retraction kinetics.* Annals of biomedical engineering, 2011. **39**(4): p. 1163-73.
137. Seth, A., et al., *L-Glutamine ameliorates acetaldehyde-induced increase in paracellular permeability in Caco-2 cell monolayer.* Am J Physiol Gastrointest Liver Physiol, 2004. **287**(3): p. G510-7.
138. Shen, L. and J.R. Turner, *Role of epithelial cells in initiation and propagation of intestinal inflammation. Eliminating the static: tight junction dynamics exposed.* Am J Physiol Gastrointest Liver Physiol, 2006. **290**(4): p. G577-82.
139. Sheng, G., J. Guo, and B.W. Warner, *Epidermal growth factor receptor signaling modulates apoptosis via p38alpha MAPK-dependent activation of Bax in intestinal epithelial cells.* Am J Physiol Gastrointest Liver Physiol, 2007. **293**(3): p. G599-606.

140. Söderholm, J.D. and M.H. Perdue, *Stress and gastrointestinal tract. II. Stress and intestinal barrier function.* Am J Physiol Gastrointest Liver Physiol, 2001. **280**(1): p. G7-G13.
141. Somanath, P.R., et al., *Akt1 signaling regulates integrin activation, matrix recognition, and fibronectin assembly.* J Biol Chem, 2007. **282**(31): p. 22964-76.
142. Souba, W.W., *Glutamine: a key substrate for the splanchnic bed.* Annu Rev Nutr, 1991. **11**: p. 285-308.
143. Souba, W.W., et al., *Oral glutamine reduces bacterial translocation following abdominal radiation.* J Surg Res, 1990. **48**(1): p. 1-5.
144. Souba, W.W., R.J. Smith, and D.W. Wilmore, *Effects of glucocorticoids on glutamine metabolism in visceral organs.* Metabolism, 1985. **34**(5): p. 450-6.
145. Souba, W.W., R.J. Smith, and D.W. Wilmore, *Glutamine metabolism by the intestinal tract.* JPEN J Parenter Enteral Nutr, 1985. **9**(5): p. 608-17.
146. Streat, S.J., A.H. Beddoe, and G.L. Hill, *Aggressive nutritional support does not prevent protein loss despite fat gain in septic intensive care patients.* J Trauma, 1987. **27**(3): p. 262-6.
147. Stupack, D.G. and D.A. Cheresh, *Get a ligand, get a life: integrins, signaling and cell survival.* J Cell Sci, 2002. **115**(Pt 19): p. 3729-38.
148. Thomson, A.B., et al., *Small bowel review: Normal physiology, part 2.* Dig Dis Sci, 2003. **48**(8): p. 1565-81.
149. Todorovic, V., et al., *The matrix protein CCN1 (CYR61) induces apoptosis in fibroblasts.* J Cell Biol, 2005. **171**(3): p. 559-68.
150. Tozer, E.C., P.E. Hughes, and J.C. Loftus, *Ligand binding and affinity modulation of integrins.* Biochem Cell Biol, 1996. **74**(6): p. 785-98.
151. Uehara, K., et al., *The lower intestinal tract-specific induction of heme oxygenase-1 by glutamine protects against endotoxemic intestinal injury.* Critical Care Medicine, 2005. **33**(2): p. 381-390.
152. Urayama, S., et al., *Dexamethasone protection of rat intestinal epithelial cells against oxidant injury is mediated by induction of heat shock protein 72.* J Clin Invest, 1998. **102**(10): p. 1860-5.
153. Valenick, L.V., H.C. Hsia, and J.E. Schwarzbauer, *Fibronectin fragmentation promotes alpha4beta1 integrin-mediated contraction of a fibrin-fibronectin provisional matrix.* Experimental cell research, 2005. **309**(1): p. 48-55.
154. Van den Berghe, G., *Novel insights into the neuroendocrinology of critical illness.* Eur J Endocrinol, 2000. **143**(1): p. 1-13.
155. Van den Berghe, G., et al., *A paradoxical gender dissociation within the growth hormone/insulin-like growth factor I axis during protracted critical illness.* J Clin Endocrinol Metab, 2000. **85**(1): p. 183-92.
156. Vidrich, A., et al., *Physiology of Gatrointestinal Stem Cells*. Physiology of the Gastrointestinal Tract, ed. L.R. Johnson. 2006: Academic Press.
157. vom Dahl, S., et al., *Involvement of integrins in osmosensing and signaling toward autophagic proteolysis in rat liver.* J Biol Chem, 2003. **278**(29): p. 27088-95.
158. Weisel, J.W., et al., *Examination of the platelet membrane glycoprotein IIb-IIIa complex and its interaction with fibrinogen and other ligands by electron microscopy.* J Biol Chem, 1992. **267**(23): p. 16637-43.
159. Welbourne, T.C., *Interorgan glutamine flow in metabolic acidosis.* Am J Physiol, 1987. **253**(6 Pt 2): p. F1069-76.
160. Wells, A., *EGF receptor.* Int J Biochem Cell Biol, 1999. **31**(6): p. 637-43.
161. Wheeler, A.P. and G.R. Bernard, *Treating patients with severe sepsis.* N Engl J Med, 1999. **340**(3): p. 207-14.
162. Wilmore, D.W., *Catabolic illness. Strategies for enhancing recovery.* N Engl J Med, 1991. **325**(10): p. 695-702.
163. Windmueller, H.G. and A.E. Spaeth, *Uptake and metabolism of plasma glutamine by the small intestine.* J Biol Chem, 1974. **249**(16): p. 5070-9.

164. Windmueller, H.G. and A.E. Spaeth, *Intestinal metabolism of glutamine and glutamate from the lumen as compared to glutamine from blood.* Arch Biochem Biophys, 1975. **171**(2): p. 662-72.
165. Windmueller, H.G. and A.E. Spaeth, *Respiratory fuels and nitrogen metabolism in vivo in small intestine of fed rats. Quantitative importance of glutamine, glutamate, and aspartate.* J Biol Chem, 1980. **255**(1): p. 107-12.
166. Wischmeyer, P., *Mechanism of Heat Shock Protein Induction by Glutamine*. NIH NIGMS R01 GM078312, NIH NIGMS, 5/1/2008-5/1/2013.
167. Wischmeyer, P., J.H. Pemberton, and S.F. Phillips, *Chronic pouchitis after ileal pouch-anal anastomosis: responses to butyrate and glutamine suppositories in a pilot study.* Mayo Clin Proc, 1993. **68**(10): p. 978-81.
168. Wischmeyer, P.E., *Glutamine and heat shock protein expression.* Nutrition, 2002. **18**(3): p. 225-8.
169. Wischmeyer, P.E., et al., *Glutamine administration reduces Gram-negative bacteremia in severely burned patients: a prospective, randomized, double-blind trial versus isonitrogenous control.* Crit Care Med, 2001. **29**(11): p. 2075-80.
170. Wischmeyer, P.E., et al., *Glutamine protects intestinal epithelial cells: role of inducible HSP70.* Am J Physiol, 1997. **272**(4 Pt 1): p. G879-84.
171. Wu, C., *Heat shock transcription factors: structure and regulation.* Annu Rev Cell Dev Biol, 1995. **11**: p. 441-69.
172. Wurgler-Murphy, S.M. and H. Saito, *Two-component signal transducers and MAPK cascades.* Trends Biochem Sci, 1997. **22**(5): p. 172-6.
173. Yamaoka, T., et al., *Transactivation of EGF receptor and ErbB2 protects intestinal epithelial cells from TNF-induced apoptosis.* Proc Natl Acad Sci U S A, 2008. **105**(33): p. 11772-7.
174. Yan, F., et al., *Epidermal growth factor receptor activation protects gastric epithelial cells from Helicobacter pylori-induced apoptosis.* Gastroenterology, 2009. **136**(4): p. 1297-1307, e1-3.
175. Yebra, M., et al., *Recognition of the neural chemoattractant Netrin-1 by integrins alpha6beta4 and alpha3beta1 regulates epithelial cell adhesion and migration.* Dev Cell, 2003. **5**(5): p. 695-707.
176. Yee, K.L., V.M. Weaver, and D.A. Hammer, *Integrin-mediated signalling through the MAP-kinase pathway.* IET Syst Biol, 2008. **2**(1): p. 8-15.
177. Yu, J., et al., *Involvement of ERK1/2 signalling and growth-related molecules' expression in response to heat stress-induced damage in rat jejunum and IEC-6 cells.* Int J Hyperthermia, 2010. **26**(6): p. 538-55.
178. Zapata-Sirvent, R.L., et al., *Bacterial translocation in burned mice after administration of various diets including fiber- and glutamine-enriched enteral formulas.* Crit Care Med, 1994. **22**(4): p. 690-6.
179. Ziegler, T.R., et al., *Glutamine and the gastrointestinal tract.* Curr Opin Clin Nutr Metab Care, 2000. **3**(5): p. 355-62.
180. Ziegler, T.R., et al., *Clinical and metabolic efficacy of glutamine-supplemented parenteral nutrition after bone marrow transplantation. A randomized, double-blind, controlled study.* Ann Intern Med, 1992. **116**(10): p. 821-8.
181. Zimmermann, D., *Der Einfluß cyclischer RGD-Peptide auf die Wechselwirkung Fibronectin-Integrin alpha5 beta1*, in *Fakultät für Chemie*. 2003, Universität Bielefeld: Bielefeld.

G Acknowledgment

"Talent wins games,

but teamwork and intelligence

win championships."

Michael Jordan

First of all, I would like to thank Prof. Dr. Paul Wischmeyer, Prof. Dr. Uwe Christians, and Prof. Dr. Erhard Wischmeyer for the opportunity to conduct my doctoral thesis at the University of Colorado in the United States. Without them I would have never had the opportunity to join this extraordinary team. It was a great experience to work in Prof. Dr. Paul Wischmeyer's young, motivating, and aspiring laboratory team. I am thankful for his permanent scientific support as well as his ideas to progress my research in this work. He always took the time to answer open questions and to discuss resolutions to problems. Due to his natural character, his open communication, his understanding and his empathy I always felt accepted and respected in my research. His broad knowledge was absolutely admirable to me. His patience and support enabled me to finish this work. Thank you so much for the opportunity to attend many conferences where I had the opportunity to present my data and to get valuable feedback for my research project. Especially, I would like to thank Prof. Dr. Uwe Christians and Prof. Dr. Erhard Wischmeyer's, who believed in my research and who made my research project in Prof. Dr. Wischmeyer's laboratory possible. Special thanks go to Dr. Ann-Kathrin Riegel and Christine Hamiel for their teaching, their practical help, and their moral support. Furthermore, I would like to thank Dr. Jelena Klawitter for teaching me the LC-MS/MS technique and for achieving important results for my first publication. My very special thanks go to my family for their encouragement, patience, and immense support.

H Abbreviations

°C	degree Celsius
ATP	adenosine triphosphate
BCA	bicinchoninic acid protein assay
BSA	bovine serum albumin
CAD	collision gas
CE	collision energy
CI	critical illness
CIP	critically ill patients
CT	control (0 mM GLN)
CUR	curtain gas
d	day(s)
DCC	deleted in colorectal cancer
dH_2O	de-ionized water
DMEM	Dulbecco's modified Eagle's medium
DMSO	diethyl suffixed
DP	declustering potential
e.g.	exempli gratia (for example)
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetate
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
EP	entrance potential
ERK1/2	extracellular-signal regulated kinases 1 and 2
EXP	exit potential
F-actin	filamentous actin
FBS	fetal bovine serum
FN	fibronectin
g	g-force acceleration
g, mg, µg	gram, milligram (10-3), microgram (10-6)
G1	gas 1

G2	gas 2
GLN	glutamine
h	hour(s)
HNa_2O_4P	di-sodium hydrogen phosphate anhydrous
HPLC	high-performance liquid chromatography
HS	heat shock
HSF	heat shock factor
HSP	heat shock protein
Hsp70	heat shock protein 70
IBD	inflammatory bowel disease
ICU	intensive care unit
IgG	immunoglobulin G
IS	ionization voltage
KCl	potassium chloride
kDa	kilodalton
LC-MS/MS	liquid chromatography–mass spectrometry
M, mM, µM, nM	molar, millimolar (10-3), micromolar (10-6), nanomolar (10-9)
mA	milliampere
MAPK	mitogen-activated protein kinase
MEK	MAP kinase kinase
MetOH	methyl alcohol, CH3OH
min	minute(s)
M-PER	mammalian protein extraction reagent
MRM	multiple reaction monitoring
MS	mass spectrometry
MTS	(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)
NaCl	sodium chloride
NaH_2PO_4	sodium phosphate monobasic anhydrous
NaN_3	sodium azide
NDS	normal donkey serum
nm	nanometer(s)

Ntn-1	netrin-1
PAGE	polyacrylamide gel electrophoresis
PARP	poly (ADP-ribose) polymerase
PBS	phosphate buffered saline
PFA	paraformaldehyde
PI3-K	phosphoinositide 3-kinase
PMS	phenazine methosulfate
PVDF	polyvinylidene fluoride
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
s	second(s)
SDS	sodium dodecylsulfate
SF	silentfect
siRNA	small interfering RNA
TB	treatment buffer
TFA	trifluoroacetic acid
U	unit(s)
UV	ultra-violet
UVP	ultra-violet products
V	volt