GLOBAL PROTEOME RESPONSE OF HUMAN CANCER CELL LINES TO LOW DOSE eIF4E/eIF4G INHIBITION

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1.1 Abstract

Cachexia is a debilitating muscle wasting disease and co-morbidity strongly associated with chronic inflammatory conditions such as cancer, chronic heart failure, chronic obstructive pulmonary disease and sepsis. Cachexia has a strong negative impact on quality of life and research suggests that 20% of cancer patients will die of cachexia. Translation initiation is the most highly regulated step of protein synthesis and the eukaryotic initiation factor 4F (eIF4F) translation initiation complex is the gatekeeper of this process; the eIF4F complex is composed of eIFG, a scaffolding protein, eIF4E, an mRNA cap-recognition protein and eIF4A, an RNA helicase. Inhibition of eIF4A by pateamine A has been shown to rescue muscle wasting \emph{in vitro} and \emph{in vivo}, this result has been reproduced with other eIF4A inhibitors. Pateamine A is a sponge-derived natural product with nanomolar toxicity to cancer cells. Surprisingly, at doses well below its anti-neoplastic activity it exerts distinct effects on cachexia. The research in this thesis follows on from previous work in our laboratory with pateamine A in human cell lines. Work on the effects of pateamine A on the proteome suggests that not all the proteins changing in expression are explainable by stressing the translation initiation complex. A model by which motifs in the 5’ UTRs of transcripts are a recognised and removed from the system in a selective manner could help explain these effects. We aimed to target eIF4E, another component of the eIF4F system, with two compounds to see if a comparable dose of eIF4E inhibitors could elicit a pateamine-like response. DMSO, a solvent used extensively in this thesis, had unexpected effects on translation. We conclude that 4E1RCat, a compound developed as a selective inhibitor of eIF4E, is not likely to be useable in further work, due to its window of activity coinciding with an unacceptable concentration of DMSO. Ribavirin, our second compound, showed a proteomic response consistent with its classification as an eIF4E translation initiation inhibitor. The proteome response seen with our eIF4E inhibitors is consistent with disruption of translation initiation. However, the data for 4E1RCat was deemed untrustworthy in the wake of revelations that DMSO, the vehicle in which it is dissolved, exerts an almost identical response. From the results obtained, it was not possible to confidently test whether protein downregulation occurred in response to a 5’UTR sequence motif, as seen for inhibitors of eIF4A. Coupled with the uncertainty associated with the 4E1Rcat results, there were relatively few downregulated proteins from the treatments, and many of these could be explained by the direct biological response to the function of the compound in the treatment. All in all, we have obtained new insights into the effects of DMSO on the proteome which will aid further experimentation. This thesis has laid the groundwork for further investigation of the effects of eIF4F inhibition in the context of better understanding the remediation of cachexia through the eIF4F system.
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Lots of love,
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## 1.3 Abbreviations

| Abbreviation | Description                                                      |
|--------------|------------------------------------------------------------------|
| BMI          | Body mass index                                                  |
| CHCA         | α-Cyano-4-hydroxycinnamic acid                                   |
| CHX          | Cycloheximide                                                    |
| CID          | Collision-induced dissociation                                   |
| DMSO         | Dimethyl sulfoxide                                               |
| dH₂O         | Distilled water                                                 |
| eIF4(A/E/F/G)| Eukaryotic initiation factor 4 (A/E/F/G)                        |
| FBS          | Fetal bovine serum                                               |
| FDR          | False discovery rate/s                                           |
| GO           | Gene ontology                                                   |
| GOAT         | Gradient optimisation and analysis tool                          |
| iBAQ         | Intensity based absolute quantification                         |
| LBM          | Lean body mass                                                  |
| LC           | Liquid chromatography                                            |
| LTQ          | Linear trap quadrupole                                          |
| MALDI-TOF    | Matrix-assisted laser desorption/ionisation – time of flight    |
| MS           | Mass spectrometer/spectrometry                                   |
| MS/MS        | Tandem mass spectrometry                                        |
| MTT          | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide    |
| PatA         | Pateamine A                                                      |
| PBS          | Phosphate buffered saline                                       |
| PEG          | Polyethylene glycol                                              |
| PSM          | Peptide spectrum match                                           |
| RBV          | Ribavirin                                                        |
| RIPA buffer  | Radioimmunoprecipitation assay buffer                            |
| SDC          | Sodium deoxycholate                                              |
| SDS          | Sodium dodecyl sulfate                                           |
| STAT3        | Signal transducer and activator of transcription 3              |
| TIC          | Total ion current                                               |
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1 Introduction

Cachexia is a wasting disorder that affects millions of people world-wide and is generally associated with chronic inflammatory conditions. Research suggests it could be responsible for as many as 1 in 5 deaths in cancer patients. Systemic inflammation is central to the development of cachexia, and pro-inflammatory cytokines are a feature of all forms of cachexia. Translation initiation is controlled by the eIF4F complex and is the most highly regulated step in protein expression and is the gateway to cellular production of protein. The three-part eIF4F complex consists of a scaffolding protein, elf4G, a cap-recognition protein, elf4E, and the helicase elf4A, responsible for melting secondary structure and giving the ribosome access to mRNAs that might otherwise have their translation hindered. Inhibition of elf4A, by the marine natural product pateamine A (PatA) has recently been demonstrated to reverse cachexia in vitro, in lab-grown muscle fibres and in vivo in mice. Pateamine A is an exceptionally potent anti-cancer compound isolated from marine sponges in New Zealand and has a unique method of disrupting translation. PatA has been shown to have applications outside the realm of cancer treatment, at doses well-below its anti-cancer effects. PatA is unfortunately in short supply, and despite being the first therapy to address the cachexia at the molecular level, the transition of PatA from pre-clinical to clinical trials has been delayed. Efforts are being made to find a commercially viable method of procuring PatA, however, in the meantime lessons can be learned from the molecular effects of PatA on the elf4F system. The precise mechanism by which PatA rescues cachexia has not been elucidated and it is not known whether inhibiting elf4A is solely responsible, or if the elf4F complex is also involved. Our lab group has previously observed the effects of PatA on the proteome of a selection of human cell lines, the work in this thesis is done in parallel and mirrors the treatment conditions and cell lines used. This research aims, in part, to establish whether elf4F has a role in the anti-cachectic effects of elf4A inhibition. By partially disabling cap-recognition by inhibiting elf4E in human cell lines and observing the effects on the proteome, we hope to see whether there are shared outcomes between the cells subjected to inhibition of elf4E and elf4A.

1.1 Cachexia

Cachexia is a metabolic wasting disorder that results in the involuntary progressive loss of weight through atrophy of muscle and fat tissues. It is co-morbid with many chronic diseases, and its etiology is grounded in inflammatory processes, anorexia, and muscle and fat tissue catabolism (Argilés, Busquets, Stemmler, & López-Soriano, 2014). In the United States alone approximately 27 million people suffer from cachexia with around 5.5 million requiring treatment (Morley, Thomas, & Wilson, 2006). There are two clinical consensus definitions of cachexia. Fearon (2011) focus on weight loss, BMI, and sarcopenia, whilst Evans (2008) definition is based on decreased muscle strength, fatigue, anorexia, low fat-free mass index and abnormal biochemistry (an increase in inflammatory markers; anemia; and low serum albumin. The Evans definition has been shown to be especially effective (Vanhoutte et al., 2016). The Evans’ cohort identified chronic heart failure, chronic obstructive pulmonary disease (COPD), chronic kidney disease, chronic infection, sepsis and cancer as a set of disease states associated strongly with cachectic wasting. Loss of muscle, loss of fat, and anemia are key identifiers of cachexia (Evans et al., 2008).

1.1.1 Effects on survival prospects and quality of life

The most significant contributor to cachectic disease is COPD with 16 million sufferers, followed by heart failure with 4.8 million and rheumatoid arthritis at 2.1 million (Morley et al., 2006). In contrast, cancer cachexia at 1.4 million, ranks below even nursing home sufferers of cachexia at 1.6 million. The literature tends to focus heavily on cancer cachexia despite only about 6% of cachexia sufferers...
being in this group. Decreases in lean body mass (LBM) and functional impairment lead to a decreased quality of life. Along with this is increased risk of mortality, as shown in a study from 1932, documenting the cause of death in 500 cases of cancer with cachexia. In the study, fully one fifth of the cancer deaths were attributed to cachexia (Warren S, 1932).

1.1.2 The molecular basis of cachectic disease
Despite the complex and convoluted etiology of cachexia, research has implicated several molecular mechanisms and pathways of interest which are thought to account for the loss of lean muscle and fat tissues. Skeletal muscle accounts for 40% of an individual's body mass and its loss is an obvious, visual sign of disease. However, focusing too heavily on skeletal muscle loss is misleading and does not address the nuances inherent in this disease state. A correct assessment of cachexia involves acknowledgement of the syndrome as multi-organ, multi-factorial and typified by abnormal biochemistry (Argilés et al., 2014). Cancers’ meagre contribution to the overall number of cachexia sufferers’ contrasts with the extensive insights the study of cancer cachexia has bestowed upon the field. ‘Adipose browning’ or conversion of white adipose tissue to brown adipose is driven by tumour secreted molecules including IL-6 from the inflammatory response, and parathyroid-hormone-related protein (PTHrP) generally secreted by tumour cells. Neutralising PTHrP caused a decrease in both white adipose tissue browning and the loss of muscle mass in mice (Serkan Kir et al., 2014).

UCP1, an uncoupling protein commonly known as thermogenin, causes increased levels of thermogenesis and is responsible for fat-mass loss and contributes to the wasting process. The expression of thermogenin in brown adipose is caused by conversion of white adipose to brown adipose by IL-6 and PTHrP. Weight loss is a common symptom of cachexia, as a function of a negative tilt on an individual’s energy balance and homeostasis of muscle tissue caused by increased thermogenesis and increased levels of inflammatory cytokines. Commonly associated with cachexia is a loss of appetite, also indicating effects on the brain and hypothalamus and providing more support for the theory of cachexia as a multi-organ syndrome (Argilés et al., 2014). Decreased food intake is common in cancer patients and speeds up the decline of body mass. In these patients, weight loss is also driven by the high protein, glucose, and fat demands of tumours. Activated immune cells and tumours are the main producers of inflammatory cytokines in cancer cachexia. One of these cytokines, TNF-α, is known to have direct catabolic effects on skeletal muscle and adipose tissue (Reid & Li, 2001).

1.1.3 Cachexia management and current therapeutic approaches
The systemic inflammatory response seems to be the connection between the major pathologies that cause cachexia, and as such anti-inflammatory drugs are a logical way to counteract cachexia by reducing systemic inflammation. The most common way to achieve this is by suppression of pro-inflammatory cytokine production. TNF-α is a pro-inflammatory and pro-catabolic cytokine, in 2005 a randomised placebo controlled trial showed that thalidomide was well-tolerated and effective at attenuating weight loss, and LBM loss in pancreatic cancer patients suffering from cachexia (Gordon et al., 2005). Another suggested therapeutic option for addressing weight loss in cancer cachexia on a molecular level is β-adrenergic blockade. In a recent trial in severe chronic heart failure patients, carvedilol attenuated the development of and partially reversed cachexia (Clark et al., 2017). However, most research into cachexia treatments is aimed at management through symptom-focused therapies. Appetite stimulant therapies are a direct way of addressing loss of appetite often seen in cancer patients, as well as the weight loss associated with reduced food intake. These stimulants are therapeutic interventions that do not directly address cachexia on the molecular level but address the loss of fat and muscle tissue indirectly by addressing the weight loss associated with cachectic wasting. Megestrol acetate, an orally accessible synthetic progesterone, and L-carnitine have, in combination and individually, shown promise at helping with the loss of weight and LBM.
Megestrol acetate has also shown promise in dealing with cachectic weight loss in children suffering from cancer and the weight loss associated with chemotherapy; there was a mean increase in LBM of 19.7% in comparison to the placebo group with a loss of 1.2% over the course of the 90 day trial period (Frey & Davis, 2016). In terms of sarcopenia, nutritional support and exercise have marked effects on muscle loss; however, the effects of these measures are less clear in cachexia. A Cochrane meta-analysis of nutritional support and mortality in elderly people at risk of malnutrition indicates small benefits such as increased weight gain and a potential to lower the risk of complications in hospital conditions. The authors state that nutritional support may lower the risk of mortality in people who are malnourished (Milne, Potter, & Avenell, 2005). Since cachexia is in many ways a catabolic syndrome, the use of anabolic steroids may seem like a logical way to counteract the disease. Unfortunately, anabolic steroid therapies are limited to 2-week treatment periods due to concern about side effects (Von Haehling & Anker, 2015; Yu et al., 2014). In response, clinical trials of enobosarm, a selective androgen receptor modulator, in 2013 were completed and suggested some benefits (Dobs, 2013). However, no new clinical data has recently been reported.

1.1.4 The lack of progress in the realm of cachexia treatment is a driver of our research
The lack of a cachexia therapeutics is not a symptom of the biomedical science field’s failure to explore therapeutic options. The effective treatment of cachexia is littered with the bodies of clinical trials showing modest to marginal improvements in cachexia endpoints. These endpoints include hand grip strength, performance in the 6-minute walk test, LBM and quality of life. Anamorelin, an appetite stimulant and anabolic therapeutic, showed marginal improvements in LBM, with no improvement in hand-grip strength or quality of life (Katakami et al., 2018; Von Haehling & Anker, 2015). On 14 September 2017, the European Medicines Agency confirmed the refusal of marketing authorisation for amorelin in cachexia treatment. Frustratingly, reading between the lines is necessary with therapeutics in clinical trials, it takes a significant amount of time to work out if some of the promising clinical agents have progressed in the cachexia therapy space. This may be economically motivated as there is no incentive for a company to actively broadcast that a therapeutic in development has marginal effects on the disease it has been formulated to treat. Enobosarm seems to have stalled in clinical trials and may be awaiting regulatory approval to proceed. Taken together, the attempts at addressing cachexia have highlighted several therapeutic options for clinicians with modest improvements in symptomatic consequences of cachetic muscle wasting. Nonetheless, therapy addressing the underlying molecular cause of cachexia is still needed. Studies in murine models of cachexia and in vitro at the level of protein production using inhibitors of the translation initiation factor eIF4A are a promising avenue to explore. The next step is to better understand the mechanistic underpinning of these effects, which may ultimately lead to effective treatments for this disease.

1.2 eIF4F
The eukaryotic initiation factor 4F (eIF4F) has been described as a nexus for cancer therapy (Pelletier, Graff, Ruggero, & Sonenberg, 2015). The eIF4F complex is necessary for recruitment of mRNA to the ribosome in cap-dependent translation. It is composed of three components: eukaryotic initiation factors 4A, 4G, and 4E. eIF4A is an RNA helicase; eIF4E, an m7G cap recognition protein, and eIF4G a large scaffolding protein (Figure 1-1). Unlike other translation regulatory pathways, eIF4F, with the exception of translation mediated by internal ribosome entry sites (IRE5es), is an unavoidable ‘bottleneck’ that cancers have a hard time circumventing. Enzymes which regulate eIF4F-mediated translation by phosphorylation, such as Mnk1 and 2, encounter redundancy issues whereby cancer cells can subvert the regulatory nature of these pathways by finding another way to favourably regulate key proteins that promote oncogenesis or maintenance of the cancer
cell. For example, the eIF4F complex is typically indispensable for translation and if Mnk1/2 inhibitors are being used to prevent activating the eIF4F complex by phosphorylation, the cancer cell will find another way to phosphorylate and activate eIF4F to meet its protein requirements.

1.2.1 Eukaryotic initiation factor 4A

The eukaryotic initiation factor 4A (eIF4A), is a dumb-bell shaped protein with two major domains connected by a linker. It is an ATP-dependent DEAD-box RNA helicase of the eukaryotic initiation factor-4A family of proteins (Bordeleau et al., 2005). The two eIF4A domains move between an open and closed conformation; in the closed conformation, the two domains interact in a way that favours the binding of mRNA and ATP (Sun et al., 2014). eIF4A unwinds secondary structure in mRNA, and mRNAs with any secondary structure in the 5'UTR ultimately require this activity for translation. The helicase activity of eIF4A is increased as a by-product of stabilisation of the ‘closed’ conformation; the ATP binding pocket and mRNA binding region require both eIF4A domains to interact to be fully form in the closed conformer, this becomes clear when viewing the binding pocket and mRNA binding region in silico (Ho et al., 2009). The weak helicase activity of unbound eIF4A increases 20-fold when bound to the eIF4G scaffold (Oberer, Marintchev, & Wagner, 2005; Rozen et al., 1990).

eIF4A is vital because it is a requirement for 40S ribosomal subunit binding to the mRNA for translation, and by extension, protein synthesis. Some mRNA molecules are ready to be translated into protein with little or no requirement for the action of eIF4A. Other mRNAs have stable secondary structures in their 5’ untranslated regions (UTRs) like RNA duplexes, or even G-quadruplexes which require enzymatic activity to ‘unwind’. The degree of requirement for the RNA helicase eIF4A for translation is dependent on both the length and complexity of the transcript. Longer transcripts and those with greater secondary structure are more dependent on eIF4A for translation (Svitkin, Pause, Haghighat, & Pyronnet, 2001).

There are three isoforms of eIF4A in humans: 4AI, 4AII and 4AIII. 4AI and 4AII share 90-95% homology and in vitro assay work suggests they are functionally interchangeable (Yoder-Hill, Pause, Sonenberg, & Merrick, 1993) but differentially expressed (Merrick, 1992). 4AI is expressed in all growing tissues while 4AII preferentially binds to eIF4G and is expressed in organs with low proliferative capacity (Nielsen & Trachsel, 1988; Williams-Hill, Duncan, Nielsen, & Tahara, 1997). 4AIII shares 65% homology with 4AI and 4AII and is a core component of the exon junction complex.
involved in mRNA surveillance and nonsense mediated decay (Le Hir, Gatfield, Izaurralde, & Moore, 2001). Due to its different function, elf4AIII is not found in the elf4F complex.

1.2.2 Eukaryotic initiation factor 4E
The role of the eukaryotic initiation factor 4E (elf4E) component of the elf4F complex in eukaryotic translation initiation is to bind and recognise the 5' m7G cap, consisting of the sequence m7GpppN (where N is any nucleotide). There are three isoforms of elf4E in humans: 4EI, 4EII and 4EIII (Joshi, Lee, Maeder, & Jagus, 2005). The isoforms differ in their ability to engage in cap-binding, with isoforms II and III being 40-fold weaker binders of the m7G cap (Frydryskova et al., 2016). elf4EI is the main isoform involved in global cap-dependent translation, elf4EII is involved in translational repression of a subset of mRNAs through AU-rich sequences in the 3' UTR and involving the protein Bicoid (Bcd) (Cho, Osler, & Hg, 2008; Tao, 2015). Overexpression of elf4E is present in 30% of all cancers and is generally associated with a poor prognosis (Volpin et al., 2017). A hallmark of many cancers is over-expression of elf4E because unregulated growth increases demand for protein. In a given non-cancerous eukaryotic cell, protein synthesis is indirectly controlled by the stoichiometry of the elf4F components. Under normal conditions, the rate-limiting component of elf4F is elf4E, which is available at the lowest abundance in comparison to elf4A4 and elf4G. The activity of elf4E is regulated through elf4E by 4E binding proteins (4EBPs), which in turn are regulated by hyperphosphorylation. The addition of multiple phosphate groups decreases the 4EBP binding affinity for elf4E, freeing it to interact with elf4G thus permitting cap-recognition to take place and allowing the elf4F complex to assemble. 4EBPs and elf4G share a binding motif involved in elf4E binding, research shows that elf4G and elf4E interface at a single canonical alpha-helical motif (Zhao, Liu, Miller, & Goss, 2017). The elf4E canonical binding motif has been characterised as Tyr-X-X-Leu-φ where X is any amino-acid and φ is a hydrophobic residue (Marcotrigiano, Gingras, Sonenberg, & Burley, 1999). Awareness of this motif is important when considering how drugs target elf4E.

1.2.3 Eukaryotic initiation factor 4G
The eukaryotic initiation factor 4G (elf4G) is the scaffolding protein and core of the elf4F complex. At least 2 genes for elf4G exist in humans, elf4G1 and elf4G2. The N-terminal third binds to elf4E and poly-A binding proteins (PABPs), the middle third binds elf4A and elf3, and the C-terminal third represents the regulatory portion, containing a second elf4A binding site and a docking sequence for the ser/thr kinase Mnk1 (Morino, Imataka, Svitkin, Pestova, & Sonenberg, 2000). Mnk1 has been identified as the main kinase that acts on elf4E, and the phosphorylated form shows increased affinity for the m7G cap (Minich, Balastat, Gosst, & Rhoads, 1994). Thus, elf4G has two elf4A binding sites, one in the central domain and one in the carboxylic acid terminal domain (Korneeva, Lamphear, Hennigan, Merrick, & Rhoads, 2001). The association of elf4E with elf4G and is likely to cause conformational changes in the scaffold, modulating its capacity to interact with other elfs.

1.2.4 elf4F inhibitors
The elf4F complex has been identified as an excellent target for cancer therapy, as such an extensive number of drugs have been identified or synthesised that target components of the complex. Although the impetus for identifying elf4F inhibitors came from the fields interested in cancer therapy, inhibitors of the elf4F complex are also a useful tool to study the functional consequences of disturbing the translation initiation machinery. Several compounds exist that interact with the elf4F machinery in various mechanistically distinct ways. Known elf4A inhibitors include hippuristanol, pateamine A, elatol, the roaglate family drugs and include an elf4A aptamer (Cencic & Pelletier, 2016; Iwasaki, Floor, & Ingolia, 2016; James H. Matthews, Maass, Northcote, Atkinson, & Teesdale-Spittle, 2013; Oguro, Ohtsu, Svitkin, Sonenberg, & Nakamura, 2003; Peters et al., 2018).
eIF4E inhibitors include 3 commercially available inhibitors, 4EGI-1, 4E1RCat, and ribavirin. Two of these inhibitors, 4E1RCat and ribavirin, are the subject of this research, as their effects have not been extensively studied in the context of translation. These two compounds are described in more detail below. A short review of other eIF4F inhibitors and their mechanisms of inhibiting eIF4F components, and by extension translational output is also included.

1.2.4.1 4E1RCat
4E1RCat, is a small molecule inhibitor of eIF4E that functions by preventing 4EBP from binding eIF4E, as well as preventing eIF4E-eIF4G interaction. 4E1RCat has provided evidence that targeting the eIF4F machinery can have positive outcomes for cancer treatment. Mice bearing Pten^−/+Ep-Myc, and Tsc2^−/−Ep-Myc lymphomas showed similar regression patterns when treated with a 4E1RCat-doxorubicin combination in comparison to rapamycin-doxorubicin combination therapy (Cencic et al., 2010). 4E1RCat had no effect on tumour regression as a standalone treatment, whereas rapamycin and doxorubicin were individually capable of causing moderate tumour regression. (Cencic et al., 2010). In silico modelling of the molecular interaction of 4E1RCat with eIF4E suggests that the eIF4G/4EBP binding pocket is occupied by 4E1RCat preventing it from associating with eIF4G (Cencic et al., 2010). Disruption of the eIF4E/eIF4G interaction occurs at the canonical eIF4E binding motif previously mentioned. This mechanism is unlike 4EGI-1 which increases the binding affinity of eIF4E for 4EBP (Moerke et al., 2007), the effect of 4E1RCat prevents 4EBP from binding eIF4E as well as preventing eIF4E-eIF4G interaction signifying two molecular interactions to be aware of.

1.2.4.2 Ribavirin
Ribavirin is an FDA approved drug of the nucleotide analogue variety, it is used primarily for the treatment of Hepatitis C viral infection. Ribavirin in combination with radiation therapy or temozolomide has shown increased efficacy in cancer treatment, in comparison to either treatment alone. This was demonstrated by an increase in the median survival of rats and mice with orthotopically implanted gliosarcoma, and stem-like glioma cells respectively (Volpin et al., 2017). Unlike 4E1RCat, which can be considered pharmacologically ‘clean’, ribavirin has a host of molecular interactions and despite being approved by the FDA in 1986 some of ribavirin’s interactions are still debated. Even the ability of ribvarin to inhibit eIF4E has been hotly contested, especially the notion that it is an m7G mimic (Kentsis et al., 2005; Kentsis, Topisirovic, Culjkovic, Shao, & Borden, 2004; Westman et al., 2005; Yan, Svitkin, Lee, Bisaillon, & Pelletier, 2005). In a letter to the editor Kentis et al, defend ribavirin as an eIF4E-cap interactor mimicking the m7G cap, citing the pleiotropic, concentration dependent effects of ribavirin and usage errors made in the papers contesting their findings. They also direct the attention to the low-micromolar dose range in which ribavirin has been shown to interact with eIF4E, as well as questioning the validity of the use of lysates as a tool to study a process as complex and highly regulated as translation initiation (Kentsis et al., 2005)

1.2.4.3 A brief mechanistic appraisal of other eIF4F inhibitors
The eIF4F complex is well characterised, and many inhibitors of the individual components have been identified using high-throughput screens and affinity assays. As eIF4E is the main eIF4F component investigated in this thesis, this section aims to briefly introduce other inhibitors and their mechanisms of (inter)action to demonstrate the range of inhibitory compounds available to biologists studying eIF4F. This section also aims to indicate the role and context of this research in the field.

Pateamine A (PatA) is perhaps the most well-known eIF4A inhibitor, it has an IC_{50} in the low-nanomolar range and is suspected to function by increasing the binding affinity of eIF4A for mRNA, ‘locking’ it onto a transcript, and disrupting protein-protein interactions with eIF4G, thereby
preventing translation initiation (Low, Dang, Bhat, Romo, & Liu, 2007). Hippuristanol, is a polyhydroxysteroid with an IC\textsubscript{50} in the high nanomolar range, it has a mechanistically distinct mode of action from PatA whereby the allosteric binding of the drug prevents association of mRNA with both free and eIF4F complex bound eIF4A (Waldron, Raza, & Le Quesne, 2018). Elatol is another natural product inhibitor of eIF4AI. Elatol’s IC\textsubscript{50} is in the low-micromolar range, it binds eIF4A1 with 2:1 (elatol:eIF4A1) stoichiometry and has been identified as a specific inhibitor of ATP hydrolysis (Peters et al., 2018). The rocaglate family a class of cyclopenta[b]benzofuran drugs including rocaglamide A (RocA), silvestrol and episilvestrol originate from Aglaia sp; these compounds tend to have IC\textsubscript{50} values in the mid-to-low nanomolar range. The mechanism of RocA interaction involves a binding interface created by the mRNA:eIF4A complex (Iwasaki et al., 2019), with an initial increase in helicase activity, but ultimately leading to eIF4A inhibition (Sadlish et al., 2014). Silvestrol and episilvestrol interact with eIF4AI/II but do not interact with any other molecular targets, and all molecular effects are traceable to their interaction with eIF4AI/II (Chambers et al., 2013). The use of RNA aptamers involved development of an eIF4A aptamer that ‘staples’ the two domains of eIF4A into a closed conformation preventing the conformational changes necessary for ATP hydrolysis and helicase activity (Oguro et al., 2003). Antisense oligonucleotides have been developed against the eIF4E transcript preventing production of the eIF4E subunit, and are in Phase I/II clinical trials in combination with the chemotherapy drug, irinotecan (Duffy, Makarova-Rusher, & Ulahannan, 2016).

1.3 The Pateamine A Story
Pateamine A is an immunosuppressive, cytotoxic, and anti-fungal inhibitor of eukaryotic translation initiation (Kuznetsov et al., 2009; Low et al., 2005; Northcote, Blunt, & Munro, 1991). It is a thiazole-containing macrodiolide. This small molecule sits at the motivational core of this project as it has been identified as a potential treatment of cachexia, through inhibition of the function of the eIF4F complex (Di Marco et al., 2012). PatA was isolated from Mycale sp. sponges found off west coast of New Zealand. In 1991, an initial report published a description of its 2-D structure and its cytotoxicity in human cell lines (Northcote et al., 1991). A simplified synthetic analogue des-methyl, des-amino Pateamine A (DMDA-PatA) (see Figure 1-2A) has been synthesized and shows near equal potency (Romo et al., 2004) and has been used as a tool to study the behaviour of PatA by proxy. As a demonstration of DMDA-PatA toxicity researchers compared the cytotoxicity of PatA with an existing cancer therapy, vinblastine (see Figure 1-2B). A notable feature is the striking uniformity of the IC\textsubscript{50} values in cell lines responding to PatA, almost all clustering at 10 nM, with few exceptions (Kuznetsov et al., 2009). DMDA-PatA, and by extension PatA has proven to be insensitive to P-glycoprotein mediated efflux. This resistance to efflux suggests that these molecules are likely to be more effective therapies against multi-drug resistant cancers (Kuznetsov et al., 2009), than therapeutics sensitive to removal by cancers employing a drug efflux strategy. Significantly, there are currently no therapeutics in the clinic that target translation initiation through the eIF4F complex, an exploitable target for cancer therapy (Pelletier, Graff, Ruggero, & Sonenberg, 2015). If PatA made it through clinical trials it would be the first compound to enter this new therapeutic territory.
In growing cells DMDA-PatA (and by extension PatA) shows nearly uniform IC$_{50}$ values across 32 cell lines demonstrating comparable cytotoxicity to vinblastine an existing anti-neoplastic agent (Kuznetsov et al., 2009). The simplified synthetic analogue DMDA-PatA had previously been deemed equipotent with its natural counterpart pateamine A in an in vitro reporter assay (Romo et al., 2004). Reprinted from Molecular Cancer Therapeutics, 2009, 8(5), 1250–60, Galina Kuznetsov, Potent in vitro and in vivo anticancer activities of des-methyl, des-amino pateamine A, a synthetic analogue of marine natural product pateamine A with permission from AACR.

1.3.1 Therapeutic window of Pateamine

Cancer therapies are infamous for having a raft of unpleasant, severe, and debilitating side-effects. This drives the biomedical field to search for new therapeutics with not only the ability to kill a range of cancers, but also considers the side-effect profiles and tolerability of a therapeutic. Early on in PatA’s discovery, researchers noted the contrast between toxic effects on cells under static growth conditions and those actively dividing (Northcote, Blunt, & Munro, 1991). P388, a murine cell line under active growth conditions exhibited an IC$_{50}$ of 0.15 ng/mL whereas at 90% confluence, under static growth conditions the BSC kidney epithelial cell line had a high tolerance to the cytotoxic effects of PatA evidenced by an IC$_{50}$ of 300 ng/mL. These outcomes were mirrored in more recent work with DMDA-PatA treated quiescent human fibroblasts, showing little to no toxic effects at the low nanomolar concentrations associated with its toxicity to cancer cells (Kuznetsov et al., 2009).

These effects suggest that PatA would be tolerated by somatic cells while being incredibly cytotoxic to rapidly growing cells - on the order of 1,000-2,000 times by these early studies. On paper, this indicates an acceptable therapeutic window, which refers to the range of useable concentrations between a drugs’ effectiveness as a therapy and unacceptable toxic side-effects. In vivo work in nude mice shows utility in the treatment of cancerous xenografts in MDA-MB-435 melanoma regression was achieved in 9 of 10 mice in comparison to 7 of 10 in paclitaxel at day 44 of the study (Kuznetsov et al., 2009). At study closure at day 65, half of the study’s cohort of mice were tumor free compared with a fifth of paclitaxel treated mice. It should be noted that the concentration of drug administered for success in these xenografts was extremely close to an empirically determined maximum tolerable dose. Despite this, not all xenograft models showed such promising results, some showed modest tumor regression. In LOX melanoma, less durable tumor regression was observed in comparison to paclitaxel. Modest regression was also reported in the remaining three tumor models, DLD-1 human colon cancer model, H522-T1 human non-small cell lung cancer model, and the NALM-6 leukemia model. Two other xenograft models, MiaPaca-2 pancreatic cancer and HT29 colon cancer, showed no significant response to therapy (Kuznetsov et al., 2009). This body of research indicates that pateamine A is in many ways a promising treatment for select cancers. The translation of this therapy to human trials is uncertain; the anti-cancer effects being close to a maximum tolerable dose is a concern for use as a therapy. Whether pateamine A finds a place in the
therapeutic space, either as a stand-alone therapy, in combination with existing therapies or in another therapeutic domain entirely (e.g. treatment of cachexia), it is undoubtedly a fascinating natural product with plenty of insights still to yield.

1.3.2 The problem: Pateamine availability

Total synthesis of Pateamine A was reported in a 1998 article by Daniel Romo (Romo et al., 1998), citing the compound’s unique structure, potent immunosuppressive effects, and high levels of cytotoxicity as key drivers of synthesis efforts. A few years later a near equipotent simplified analogue, DMDA-PatA was synthesised with 10 fewer steps in the chemical synthesis (Romo et al., 2004). Despite this, Pateamine A is not commercially available. Efforts to cultivate Mycale sp. Sponges for semi-commercial production of peloruside A, another Mycale sp. derived anti-neoplastic agent, were largely unsuccessful. This method of procuring marine natural products was never intended to be commercially feasible and was done on a small scale for research in what was described as an ‘interim supply’ of compounds (M. J. Page, Handley, Northcote, Cairney, & Willan, 2011). Unfortunately, predation of these farmed Mycale sponges by the nudibranch Hoplodrasis nodulosa meant that over the course of 6-7 years just over a 1.12 g peloruside A was obtained (M. J. Page et al., 2011). Had the endeavour to obtain peloruside A from sponges been successful, it would be reasonable to believe this means of production could have been mirrored for pateamine A. There are significant efforts being made at Victoria University of Wellington by the Harvey and Teesdale-Spittle group to produce peloruside A as well as pateamine A using synthetic chemistry approaches (Hemi Cumming et al., 2016). There are also retro-synthetic approaches using bacterial gene clusters expressed in a lab-culturable bacterial species (Owen et al., 2015) similar enough to the bacterial symbiont in Mycale to recombinantly express PatA by the Owen group.

1.3.3 What can Pateamine and eIF4A inhibition teach us about cachexia

The story of pateamine does not end at cancer and the applications do not stop at cancer therapy. Low dose (20 µg kg⁻¹) treatment with pateamine A has shown to rescue muscle wasting in vivo in mice with both C26 tumour induced muscle wasting, and the tumour-free TNF-α/IFN-γ model (Di Marco et al., 2012). More recent work has confirmed that eIF4A inhibition by several compounds with varied mechanisms proves that these effects are the result of eIF4A inhibition (Cramer et al., 2018). It was noticeable that low doses of PatA were still able to ameliorate the muscle wasting phenotype. These doses were well below concentrations where its anti-cancer effects tend to manifest, evidenced by no noticeable regression of the C26 tumours, suggesting it would also be well below the threshold for toxic effects. Although higher doses (50 µg kg⁻¹) both ameliorated cachexia and decreased the size of the tumours (Di Marco et al., 2012). The pleiotropic, dose-dependent effects alone hint at interesting biochemistry, with two effects mediated by treating the translation initiation complex at different dosage regimes. Perhaps more interesting from a clinical standpoint was the observation that at low doses there were no noticeable toxic manifestations of the compound on the treated animals. In the treatment of cachexia, a growing body of research has implicated factors including the cytokine IL-6 and the inducible nitric oxide synthase (iNOS) as well as the loss of myogenin and MyoD mRNA as the causative agents for muscle wasting (Di Marco et al., 2012). More recently, the signal transducer and activator of transcription 3 (STAT3) protein, a transcription factor and upstream effector of cytokines IL-6, TNF-α and IFN-γ, has been shown to be translationally repressed by eIF4A inhibition (Cramer et al., 2018). Cramer showed that that STAT3 protein levels are affected by eIF4A inhibitors without an effect on STAT3 mRNA levels suggesting that the effect is happening at the level of translation. In vitro INF-γ and TNF-α treatment of myotubes results in cytokine induced atrophy of the muscle fibres. The eIF4A inhibitors, pateamine A (PatA), hippuristanol, and silvestrol have all been shown to ameliorate these effects. New research has also added silvestrol and hippuristanol to the list of compounds affecting the iNOS/NO pathway.
cementing eIF4A inhibition as a potential central therapeutic target in the rescue of cachexia (Cramer et al., 2018).

1.3.4 How does eIF4A inhibition ameliorate cachexia at the molecular level?
Although PatA is known to interact with all three isoforms of eIF4A (Bordeleau et al., 2005), PatA’s anti-cancer and anti-cachetic effects are likely to be mediated primarily by its interaction with eIF4AI. There is a 4:1 abundance ratio in comparison to the eIF4AII isoform suggesting that at least based on abundance, eIF4AI is the main isoform (Yoder-Hill et al., 1993). Pateamine A encourages stabilisation of the favourable mRNA and ATP binding closed conformer, increasing helicase activity but preventing interaction with eIF4G (Iwasaki et al., 2019). Mechanistically, the anti-cancer properties of PatA at concentrations that lead to cell death are due to generalised protein synthesis inhibition. The sensitivity of tumor cells to its cytotoxic effects is likely a function of the protein demands of tumour cells going through unregulated division and the observation that many oncogenic transcripts are reliant on eIF4F for their efficient translation (Wolfe et al., 2015). Consequently, eIF4F components are often upregulated in cancers. The effects of eIF4A treatment can be direct, at the level of translation, whereby the mRNA level is unaltered, but the protein expression level is decreased. For example, as noted above STAT3 mRNA levels remain unaffected by eIF4A inhibition. However, eIF4A inhibition leads to a decrease in STAT3 and phospho-STAT3 protein in response to eIF4A. This was evidence that eIF4A inhibition was affecting STAT3 at the level of translation. The effects can also be indirect. STAT3 is a transcription factor, with many targets including IL-6. It is notable that IL-6 mRNA expression is lower after eIF4A inhibition. The decreased mRNA levels suggest that IL-6 protein levels have been indirectly affected, because inhibiting the production of protein should theoretically have no immediate effects on mRNA levels. STAT3 is the likely culprit for this disturbance.

A mechanism for the seemingly selective process by which inhibiting eIF4A lowers protein expression in a non-uniform manner has not been fully identified. Some relatively recent research proposes a model of stress granule-mediated translational repression as a mechanism by which eIF4A inhibitors could remove specific mRNAs. This repression of translation by removal to stress granules in this model ultimately leads to reversal of cachexia (Di Marco et al., 2012). It has been established that the degree of secondary structure present in the 5’ UTR is in direct proportion to the requirement for eIF4A for translation (Svitkin et al., 2001). Eukaryotic initiation factors are capable of binding and scanning unstructured 5’ UTRs however, even weak secondary structures enforce the requirement of the helicase activity of eIF4A for translation (Pestova & Kolupaeva, 2002). Di Marco implies that the therapeutic effects of eIF4A inhibition on models of cachexia are the result of the amount of 5’ UTR secondary structure present in eIF4A sensitive transcripts (Di Marco et al., 2012). In other words, that the amount of secondary structure in the 5’ UTR is the defining feature of an eIF4A-sensitive transcript. Our understanding of this system is evolving as new inhibitors of the system are developed and evaluated. Some of these are described in section 1.2.4, above. For example, recent research pertaining to the interactions of the rocaglates with eIF4A have shifted the understanding from translation dependent on 5’ UTR length and complexity, to one of sequence-specific binding (Iwasaki et al., 2016). By implication, other eIF4A inhibitors that strengthen the ability of eIF4A to bind mRNA may also be manifesting their effects on the proteome in a similar manner. Rocaglates have been shown to increase the affinity of the binding interaction of eIF4A to select for polyuridine sequences present in the 5’ UTR (Iwasaki et al., 2016). X-ray crystallography also showed that the formation of the mRNA-eIF4A dimer is required for rocaglates to ‘lock’ the complex together (Iwasaki et al., 2019). PatA’s interaction with mRNA and eIF4A has not been confirmed but is suspected to share this mechanism with rocaglates.
1.3.5 Is all mRNA born equal in the eyes of eIF4A?

It is clear that at high concentrations of eIF4A inhibitors, the reduction in availability of free eIF4A impacts on transcripts based on the 5’ UTR requirement for functional eIF4A (Wolfe et al., 2015). However, at lower concentrations, such as used in cachexia control, (Di Marco et al., 2012), it is possible that the selective removal of STAT3 and iNOS protein expression are a consequence of complex enzyme kinetics. The formation of the enzyme-substrates complex between eIF4A, ATP and mRNA are a requirement for rocaglate binding. In this sense rocaglates can be considered uncompetitive inhibitors of eIF4A as they require the assembled mRNA-eIF4A interface. The substrate preference of eIF4A for different sequences of mRNA is only beginning to be explored (Iwasaki et al., 2016). It is likely that eIF4A inhibitors bind more effectively to some mRNA sequences than they do to others. For example, recent publications suggest that rocaglates may favour binding to eIF4A-mRNA complexes at polypurine motifs. The field has not crystallised the characteristics of eIF4A dependent transcripts or whether interfering with other components of the eIF4F complex has the same effect. This preference for particular sequence motifs may arise through the strength of binding interaction of the inhibitor with the eIF4A-mRNA complex, with some sequences offering optimal binding sites. Alternatively, processing time may be an important factor. The rate of translation could be highly variable across the range of mRNAs the eIF4F system is responsible for translating. There is strong evidence that eIF4A responds differently to structurally differing mRNAs, including exclusively Poly-U containing, mRNAs containing a mixture of duplex and single stranded RNA, and mRNAs that are extensively duplexed. It was noted that the mRNA variety used had highly variable effects on the conformational dynamics of eIF4A (Harms, Andreou, Gubaev, & Klostermeier, 2014). Therefore, the time it takes for mRNA to be processed may be variable, and this could be a factor determining the seemingly selective effects of eIF4A inhibition, as uncompetitive inhibition is impacted by the lifetime of the enzyme-substrate complex.

PatA binding has been shown to be either a very strong or irreversible inhibitor of the function of eIF4A (James Henry Matthews, 2010). If PatA shares the other trait of rocaglates, namely the selectivity for polypurine motifs, this could also potentially explain the selectivity seen with eIF4A inhibition. However, recent work shows that hippocristanol still rescues the muscle wasting seen with in vitro models of cachexia (Cramer et al., 2018). This is notable, as hippocristanol is an allosteric regulator of eIF4A, and linked to a decreased affinity for mRNA (Cenic & Pelletier, 2016; Lindqvist et al., 2008), in contrast to PatA and the rocaglates. This is seeming evidence against the model of selective effects of eIF4A inhibition being a product of sequestering into SGs of mRNAs with specific 5’ UTR sequences or motifs such as polypurine motifs, hairpin loops or G-quadruplexes. Recent research suggests that the formation of classical secondary structures by (GGC)$_4$ motifs is favoured in comparison to G-quadruplexes and the role that G-quadruplexes play in 5’ UTR motif mediated translation initiation repression may have been overestimated (Waldron et al., 2018). Unpublished experiments done by Richard Little (R Little 2018, personal communication, 11 October) at Victoria University of Wellington laboratories suggest that polypurine motifs may be an enriched sequence in PatA sensitive transcripts.

Pateamine A and other eIF4A inhibitors show potential for the treatment of cachexia. There is ample evidence that the inhibition of eIF4A is responsible for the anti-cachectic outcomes researchers see in vivo. Current models implicate a loss of specific transcripts including iNOS and STAT3, while anabolic, muscle-generating transcripts such as myogenin and MyoD are rescued. The studies creating links between eIF4A inhibition and transcript loss or retention based on specific sequences and structural motifs are preliminary. Currently, nothing is known about the sequence selective effects of inhibiting other members of the eIF4F complex, and therefore whether any other eIF4F inhibitors should be considered for anti-cachectic drug development. The goal of this research is to
capture the effects both direct and indirect of translation inhibition by a selection of elf4E inhibitory compounds. These effects on translation should manifest in the proteome as a change in protein expression when compared to a population of untreated control cells.

2 Aims and objectives
Cachexia is a debilitating and sometimes fatal condition without current therapeutics. Targeting elf4A with inhibitory drugs like Pateamine A leads to a non-uniform drop in protein expression across the proteome. The goal of this study is to determine whether there is merit in targeting the components of elf4F individually. We aim to perturb the elf4F system in a targeted manner to study the global proteomic effects of treatment. We ask if we can achieve the same proteomic outcomes seen with Pateamine A using inhibitors for the other constituents of the elf4F system: ribavirin and 4E1RCat.

The objectives of this research were:

1) To determine the effective inhibitor concentrations (e.g. IC\textsubscript{50}, IC\textsubscript{10} and IC\textsubscript{1}) of two elf4E inhibitors, and a relevant control inhibitor, by pharmacologically challenging a model cell system.

2) To quantify proteomic effects, pharmacologically challenge cells with inhibitors of elf4E, and a control inhibitor, at concentrations determined in Objective 1, and extract, purify and analyse cellular protein abundance changes using LC-MS/MS.

3) To use gene ontologies and co-expression analysis to assess the cellular processes affected by elf4E inhibition, with the ultimate goal of generating insight pathways effected by low dose elf4E inhibitor treatment.

4) To identify any sequence motifs in the 5’ UTRs of proteins that change in response to elf4E inhibition, but that are not explained by the direct biological response to the treatment. Finally, to evaluate whether 5’UTR sequence motifs are equivalent to those that respond to low-dose elf4A treatment.

![Figure 2-1 A simple diagrammatic representation of the work done in this thesis, from MTT assay through to treatment, tryptic digestion, LC-MS\textsuperscript{2} analysis and to gene ontology (GO) and STRING analysis.](insert_image)
3 Materials and methods

3.1 Reagents

3.1.1 Cell Culture

96 well plates Corning, USA
Dulbecco’s Phosphate-Buffered Saline (DPBS) Life Technologies, NZ
Foetal Bovine Serum (FBS) Sigma-Aldrich, NZ
Roswell Park Memorial Institute-1640 media (RPMI-1640) GE Life Sciences, USA
HyClone™
Dulbecco’s Modified Eagle’s Media (DMEM) High modified GE Life Sciences, USA
+45000mg/L glucose +110mg/L sodium pyruvate
T25 flasks Corning, USA
T75 flasks Corning, USA
Trypan Blue, 0.4 % Life Technologies, NZ
Trypsin-EDTA, 0.05 % Life Technologies, NZ

3.1.2 Chemicals and Miscellaneous

α-Cyano-4-hydroxycinnamic acid (CHCA), 99 % Sigma-Aldrich, NZ
Acetone, >99 % Romil Ltd, UK
Acetic acid, glacial Merck, NZ
Acetonitrile, >99.9 % Carl Roth, NZ
Acetonitrile gradient grade for liquid chromatography Merck, NZ
Bovine serum albumin (BSA) ICP Biologicals, NZ
Chloroform BDH, UK
Complete protease inhibitor cocktail Sigma-Aldrich, NZ
Dithiothreitol (DTT) BioRad, NZ
Dimethyl sulfoxide, ≥99.9 % (DMSO) Sigma-Aldrich, NZ
Formic acid, 98-100 %
GlutaMAX™, 200 mM
Iodoacetamide, >99 %
Kimwipes®
LoBind tubes®
Methanol, 99.9 %
N, N-dimethylformamide (DMF), ≥99 %
Parafilm M®
Sinapinic acid
Sodium deoxycholate (SDC), ≥97 %
Sodium dodecyl sulphate (SDS)
Thiourea, 99 %
Trifluoroacetic acid (TFA), 0.1 % in H₂O
Triton-X-100
Trypsin, Mass Spectrometry Grade
Urea
dH₂O – water used was purified by reverse osmosis and distilled
ZipTip® pipette tips

3.1.3 Kits

DC™ Protein Assay

3.1.4 Buffers and Solutions

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution: 5mg/mL of MTT, in PBS thoroughly vortexed and stored in a foil sheath.
MTT solubiliser: 10% SDS: 50.0 g, 45% N, N-dimethylformamide: 225.0 mL, dilute to 500 mL with dH2O, pH adjusted to 4.5 with glacial acetic acid

Radioimmunoprecipitation assay (RIPA) lysis buffer: 25mM Tris, pH 7-8, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate (SDC), 1% Triton X-100, protease inhibitor - RIPA buffer was made to 0.99x final volume, protease inhibitor tablets were dissolved to 0.01x in dH2O to produce a 10x solution and combined to the produce the working solution.

Crude cell lysis buffer: 8M urea in dH2O.

Urea SDC solution: 8M urea, 5% (w/v) SDC in dH2O.

Urea-thiourea lysis solution: 6M urea, 2M thiourea in dH2O.

Alkylation buffer: 100 mM iodoacetamide in dH2O. Made fresh, tube covered in foil to minimise light exposure.

Reducing buffer: 10mM DTT in dH2O

Buffer A: 0.1% formic acid in H2O Optima® liquid chromatography/mass spectrometry.

Buffer B: 0.1% formic acid in acetonitrile.

CHCA matrix: 8 mg/mL α-Cyano-4-hydroxycinnamic acid (CHCA) in 50% acetonitrile, 0.1% TFA in H2O.

Sinapinic acid matrix: 10 mg/mL sinapinic acid in 50% acetonitrile, 0.1% TFA in dH2O.

Trypan blue solution: dH2O, 0.4% w/v trypan blue

Trypsin solutions: 20µg trypsin of trypsin was resolubilised in 20µL of dH2O and split into 1µg aliquots

3.2 Drug stocks (CHX, RBV and 4E1RCAT) – preparation and storage

Ribavirin (RBV) and 4E1RCat were purchased from Sigma-Aldrich and stored at -20 °C. Cycloheximide (CHX) was stored at 4 °C. Drugs were dissolved in DMSO and stored at -20 °C at a concentration of 50 mM for RBV and CHX. 4E1RCAT was dissolved at 4 mM. Drugs diluted in aqueous solutions were used within 24 hours to avoid degradation. Drug stock tubes were wrapped in parafilm to avoid water adsorption, a well-known property of DMSO.

3.3 Software

Xcalibur™ ThermoFisher Scientific, USA

Scaffold™ 4 Proteome Software, USA
3.4 Cell culture

3.4.1 Cell culture - strains and maintenance conditions HL60 and HT29
All cultures originated from existing liquid N\(_2\) freezer stocks at the Centre for Biodiscovery at Victoria University of Wellington. The HT29 cell line is derived from the colorectal adenocarcinoma of a 44-year-old caucasian female, originally isolated in 1964. The HT29 cell line has been used extensively in research, some examples of its use include its use as a measure of the effectiveness of anti-neoplastic compounds in drug discovery (Volpin et al., 2017) and to express genes due to its amenability to transfection (Morin, Vogelstein, & Kinzler, 1996). The HL60 cell line is an acute promyelocytic leukemia derived line originating from a 36-year-old caucasian female. The HL60 cell line are well-used to study myeloid differentiation (Birnie, 1988), it is commonly used as a first step assessing a compounds toxicity against human cells in MTT assays in drug discovery at the Victoria University of Wellington. Cell cultures were maintained at 37 °C in an incubator with a humidified atmosphere supplemented with 5% CO\(_2\). HL60 Cells were seeded by default at a density of 1x10\(^5\) cells/mL, HT29 cells were seeded at 2x10\(^5\) cells/mL, cells were maintained in the exponential phase of growth and were kept from reaching confluency by regular passaging. Generally, 3 days passed between passages for both cell lines this was a good general rule.

3.4.2 Cell culture media
HT29 and HL60 cells were cultured in RPMI media containing 10% (v/v) FBS and 2.05 mM L-Glutamine. DMEM was required when thawing cells stored in DMEM or when growing cells from the stocks of another lab in which the cells were previously cultured in DMEM. In these cases the media was supplemented with 10% (v/v) FBS and L-Glutamine, 2.05 mM. Cells grown in DMEM were transferred to RPMI and given at least one passage before they were used in any experiments.

3.4.3 Cell counting
Culture cell counts were performed using light microscopy and a 0.4% trypan blue solution to facilitate cell counting using a hemocytometer. The outer 4 and the middle squares in the grid were most commonly used. If cell counts were extremely consistent across 3 squares diagonally across the grid and were at enough numbers i.e. over 100 cells, then no more squares were counted. If cell numbers in a grid were below 50, depending on the consistency of the first 5 squares counted, it was sometimes deemed necessary to count all 9 squares in the grid, or re-count a new set of cells.

3.5 MTT cell proliferation assays
For all MTT proliferation assays, 96-well microtiter plates were used. The wells of the 96-well plate were seeded at 1.0 x 10\(^4\) cells/mL for both HL60 and HT29 cells at a final volume of 100 µL. The effects DMSO has above 0.5% were taken into consideration when selecting the top dose in the MTT assays, due to a minor calculation error early in the project the top dose of all drugs corresponded to a 1% DMSO concentration. As an example, the top dose of CHX and RBV was 500 µM, whereas the top dose of 4E1RCat was 40 µM. The two considerations when deriving the top dose for 4E1RCat were: the maximum achievable solubility in DMSO (4mM), and the maximum percentage of DMSO acceptable based on the literature. In HL60 cells a base proliferative decrease of ~3% is noticeable at 0.5% DMSO. All outer wells were filled with sterile distilled water to avoid evaporation affecting the...
volume in the outer wells. Assays were generally spaced apart in terms of passaging. For example, cells were harvested from 3 separate passages from the same flask or the same origin cells over the course of 3 passages, instead of 3 biological replicate flasks being grown side by side and assays performed simultaneously. A half-log (3.16-fold) serial dilution scheme was used for all drugs (see Figure 6-1). A relevant DMSO control was included at concentrations where DMSO is expected to influence cell growth. A media only blank and cell-only control are also included. Assay data was collected from triplicate wells using a plate reader set at 570 nm.

3.5.1 HL60 cell line
For the MTT assays with suspension cells the final well volume was made up to 100 µL. The media used to maintain cell lines was used to dilute the drug stocks in preparation for treatment. Drug preparation was done in the 96-well microtiter plate prior to addition of cells. The top dose of drug was prepared in a single well if feasible (i.e. total volume does not exceed well volume of 300µL). The well containing the top dose was split into three equal volumes among the triplicate wells, enough was prepared to leave 50µL in the wells of the top dose. The media-drug mix was moved between the remaining triplicate wells containing 50 µL of media and mixed thoroughly to prepare a half-log (3.16-fold) serial dilution. Due to difficulty encountered culturing the HL60 cell line, the HT29 cell line became the focus of the proteomic analyses. Towards the end of this thesis the problems with the HL60 cell line was resolved, and a mirror experiment was carried out in this cell line, however, there was insufficient time remaining to accommodate analysing the HL60 samples on the MS.

3.5.2 HT29 cell line
Adherent cell lines were allowed 14 hours to adhere to the bottom of the MTT wells before treatment. Drug doses were prepared in a re-useable plastic container with a 96-well format, this was necessary for the HT29 cell line as the cells are allowed 14 hours to settle down in the 96 well plate prior to treatment. HL60 cells can have their drug doses prepared in a 96 well plate and have cells injected into the drug doses, this is possible as they are a suspension cell line. The top dose was made in a single well of the re-useable plastic container, then split into 3 equal volumes, enough liquid was prepared to have an excess of 50 µL in the wells in the 96-well mimic container as it is difficult to fully recover all the liquid from the container. The top dose triplicate wells are used to construct the half-log dilutions down the length of the container, the media cells were cultured in was used to dilute the drug in each assay. After all the doses were constructed in the plastic container, they were transferred to the 96-well plate and left for 2 days.

3.5.3 Collecting dose response data
The wells of a 96-well plates were seeded at 1.0 x 10⁴ cells/mL of HL60 and HT29 cells at a final volume of 100 µL. A 20 µL aliquot of a 5 mg/mL MTT solution in PBS was pipetted into each well and the cells were given a 2-hour period to metabolise the MTT to formazan. After two hours the cells were lysed using the MTT solubiliser solution and left in an incubator overnight for the purple formazan crystals to dissolve. A (VERSAmax™) microplate reader set at 570 nm was used to read absorbance values. Excel was used to visualise general trends. Graphpad was used to generate graphs and get dose response values in the form of inhibitory concentration (IC) data.
3.6 Method of determining IC\textsubscript{10} and IC\textsubscript{1} from experimental data
Two concentrations of drug were initially going to be explored, the IC\textsubscript{10} and the IC\textsubscript{1}. For practical purposes and in the interest of time the IC\textsubscript{10} was selected. These were determined experimentally by MTT assay. In Prism, graphs were generated from the absorbances expressed as a percentage of untreated controls. A non-linear regression was fitted for each MTT assay replicate. To avoid determining IC\textsubscript{10} and IC\textsubscript{1} by eye, 90% of the top asymptote value and 99% of the top asymptote values for each regression line on the graph were calculated and the corresponding $\log_{10}$(drug concentration) determined for all replicates individually. These values were combined and averaged to give the expected drug concentration to achieve 10% inhibition and 1% inhibition respectively.

3.7 Preparing cells for pharmacological challenge
Biological replicates were grown to sufficient concentration to seed enough flasks at $2 \times 10^6$ cells. For example, HL60 cells were cultured to between $5-8.5 \times 10^6$ cells prior to treatment. T75 flasks were seeded at $2 \times 10^6$ and in the case of HL60 cells were treated immediately, whereas the HT29 cells are given 14 hours to adhere to the bottom of the flask. A 48-hour drug challenge was then applied to cells at IC\textsubscript{10} concentrations. HL60 and HT29 cells have a doubling time of about 24 hours under ideal conditions. The 48-hour treatment period was chosen to allow at least two mitotic divisions to occur. This treatment scheme was used to ensure the protein synthesis inhibitory effects of the drugs were given sufficient time to manifest. Inhibitory concentrations were determined by MTT proliferation assay described previously.

3.8 Sample preparation for use in LC-MS/MS

3.8.1 Cell lysis
Post-treatment cells were pelleted in a centrifuge at 300 xg for four minutes. The media was decanted, and cells were subsequently kept on ice. For the remainder of the lysis steps the cells were also kept on ice. The cell pellet was resuspended in 5-10 mL of ice-cold PBS and re-pelleted at 300 xg for a further four minutes, the PBS and all supernatant from this spin was discarded. Finally, 200 µL of the 8 M urea lysis solution was added to the cell pellet followed by vortexing. This combination of 8 M urea and the cell pellet was flash frozen at -80 °C three times, between freezes the pellet was allowed to defrost, followed by vortexing. The resulting lysate was transferred to 1.5 mL Eppendorf tubes.

3.8.2 Protein quantification
Protein quantification was necessary to assess how much trypsin would be used for tryptic digestion. Trypsin was added at a ratio of 1:50 of trypsin to protein. Two methods of protein quantification were explored.

3.8.2.1 NanoDrop (ThermoScientific)
The protein extracts were resuspended in a 50 µL solution of 8 M urea and quantified using a NanoDrop spectrophotometer. An 8 M urea blank was used to account for extraneous absorbance. 1 µL of sample proteins was placed on the contact point, the other contact was lowered into place with the sample between the two. The protein quantification $A_{280}$ setting was used. Although the ratio of trypsin used to treat a given sample is 1:50 this can vary widely and still achieve successful digestion of proteins in a sample, however, the NanoDrop was exceptionally inaccurate with huge variation, even when analysing the same sample multiple times. The NanoDrop was considered too inaccurate for protein quantification and was dropped in favour of the DC protein assay.
3.8.2.2 DC™ Protein Assay (Bio-Rad)
Proteins were resuspended in 100 µL of 8 M urea or however much was necessary to fully re-dissolve the proteins. Proteins were quantified using the DC protein Assay (BioRad). Assays were performed in a 96-well microplate. A BSA standard was made at 40 mg/mL in dH2O (40 µg/µL). Constructing a standard curve was achieved by performing 2-fold dilutions; 12 dilutions was sufficient to reach the lower end of the detection capacity of the assay. For each well containing standard or sample liquids were added in the order: 25 µL of reagent A → 5 µL sample/standard → 200 µL reagent B (yellow). The colour developed within 15 minutes and was stable for an hour. The standard generated a linear correlation between absorbance at 750 nm and protein concentration between 200 and 1500 µg/mL. Prism software from Graphpad was used to analyse the standard curve and sample data. For linear standard curves a simple linear regression function was fitted. A standard curve was generated with values outside the linear range, due to the small linear range of the assay this was extremely common. Due to the nature of tryptic digestion, often the amount of trypsin added is somewhat arbitrary and a 1:50 to 1:100 ratio of trypsin is more than sufficient to digest a protein sample. A sufficiently accurate result could be obtained by fitting a 2-phase decay non-linear regression to a standard curve starting at 40 µg/µL as the ratio of trypsin to protein will almost certainly be at a sufficient molar excess for digestion. Protein concentrations were interpolated based on the non-linear relationship between absorbance and protein concentration.

3.8.3 Protein precipitation

Chloroform-methanol precipitation

For every 100 µL of protein containing sample supernatant, 400 µL of methanol was added and agitated by vortexing. An aliquot of 100 µL chloroform was subsequently added, followed by agitation. 300 µL dH2O was added, agitated and the lysate-solvent mixture centrifuged at 13,000 G for two minutes. The aqueous phase containing chloroform was carefully removed, and a further 400 µL of methanol added and vortexed. A second centrifugation pelleted the precipitated proteins. The remaining liquid was removed, and the protein precipitate was taken to near-dryness in the centrivap concentrator. Excessive drying caused issues with re-dissolving the protein pellet. The pellet was resuspended in an 8 M urea solution.

Acetone precipitation

To the protein sample, four-times the sample volume of acetone cooled to a temperature of -20 °C was added. The protein-acetone solution was vortexed and incubated at -20 °C for one hour. The solution was subsequently centrifuged at 13,000 xg for 10 minutes. The acetone was removed with a pipette with care being taken not to disturb the pellet, which was not always visible. The pellet was left to air-dry, then resuspended in 50 µL of an 8 M urea solution.

3.8.4 Protein pre-treatment and tryptic digestion

A 100 mM dithiothreitol (DTT) solution was used to reduce cysteine residues, removing disulfide bridges, and give the protease used in subsequent steps free access to the lysine/arginine residues of the fully unfolded protein. DTT solution was applied to each Eppendorf-contained protein extract at a working concentration of 5 mM and heated at 70 °C for 20 minutes. An iodoacetamide solution was added at 5 mM to prevent disulfide bond formation by alkylating the reduced cysteines with carbamidomethyl groups. Sample urea concentration was lowered to 2 M prior with dH2O prior to trypsin addition. Trypsin was added to sample proteins at a 1:50 ratio and left to digest overnight at 37 °C.
3.8.5 Concentration, purification and desalting of peptides

Purification and desalting of the protein sample was achieved using a C-18 ZipTip®. A selection of solutions was made that filled the roles of conditioning the ZipTip column, rinsing the ZipTip column, binding the peptides to the C-18 resins, washing and desalting the peptides, and elution by a dilute solution of acetonitrile and neat acetonitrile.

- Wetting solution – 100% acetonitrile
- Cleaning solution – dH₂O
- Sample solution – peptides contained in a 2M urea solution
- Wash solution – 0.5% formic acid
- 1st Extraction solution – 0.5% formic acid in 1:1 (v/v) water: acetonitrile
- 2nd Extraction solution – 100% acetonitrile

Where possible, 100µL amounts of all solutions relating to the ZipTip procedure were used. During aspirating and dispensing of solutions the ZipTip was kept wet, and air was not allowed to enter the pipette tip. The wetting solution was aspirated and dispensed into waste 7-8 times. The cleaning solution was aspirated and dispensed into waste a minimum of 10 times to remove the acetonitrile wetting solution. The sample solution was aspirated and dispensed within a given sample tube a minimum of 10 times. The sample loaded on the pipette tip’s C-18 resins was washed by aspirating and dispensing the wash solution into the waste 10 times. A fresh LoBind tube was used in the following steps, the first extraction solution was aspirated and left within the pipette for 20 seconds then dispensed into the fresh tube. This action was repeated with the second extraction solution. The extraction solutions were evaporated to near dryness, using the centrivap concentrator. Peptides were resuspended in 0.1% formic acid, transferred to a liquid chromatography sample tube and submitted to the LC-MS/MS for analysis.

3.9 Matrix assisted laser desorption ionisation MS – time of flight (MALDI-TOF/MS)

Early in the optimisation phase of preparing proteins for MS analysis the presence of large amounts of polyethylene glycol (PEG) was noticed. Polyethylene glycol has a suppressive effect on the ionisation of molecules in electrospray MS. With this in mind MALDI-TOF/MS was used on samples to assay for PEG prior to transfer to the mass spectrometer. The main reason that MALDI-TOF was used is because PEG can be detected immediately after protein precipitation from the raw cell lysate. Tryptic digestion and the ZipTip processing are time consuming and being able to assay for PEG was a time-saving approach. Additionally, since proteins were re-dissolved in 8 M urea post precipitation and MALDI-TOF has a high salt tolerance it was a quick and easy way to detect PEG in samples and by a process of elimination identify the source of the PEG contamination. The Triton X-100 used in the RIPA lysis buffer used at the outset of this project is the suspected source of PEG contamination.

Matrix and MALDI

For mass spectrometry analysis, 1 µL of peptide-containing eluate was mixed with CHCA matrix in a 1:1 ratio and in a 1:10 ratio. Then 1 µL of each elution-matrix mixture was spotted onto a 384 well AB SCIEX Opti-ToF™ Cal Mix 5 plate and allowed to dry. The AB SCIEX TOF/TOF™ 5800 matrix-assisted laser desorption/ionisation time-of-flight tandem mass spectrometer (MALDI TOF/TOF) was calibrated using a 700-4000 m/z calibration mix. Many samples were in an 8 M urea solution when placed on a spot on the 384 well plate. These samples had high concentrations of urea salts present on the spot and often required the maximal laser setting of 4000 units.
3.10 LC-MS/MS settings and data analysis parameters

Peptides were separated using a flow rate of 0.2 µL/min and fractionated with a C18 column (Dionex, LC Packings, Netherlands). A 372 min buffer gradient was constructed from 0.1% formic acid (Buffer A) and 0.1% formic acid in 80% acetonitrile (Buffer B). The gradient was optimized to maximise the number of identified proteins using the gradient optimisation and analysis tool (GOAT) optimisation tool (Trudgian, Fischer, Guo, Kessler, & Mirzaei, 2014). The peptides in solution were ionised by electron spray ionisation with a silica tip emitter, with the voltage potential set at 2.2. Positive ion mode was used with the heated capillary temperature set at 200 °C and tube lens 160 V to permit entry of ions preferentially at 524.30 m/z. Ions with m/z range between 200 - 1850 m/z were analysed during the 372 min acquisition time using Fourier transform mass spectrometry (FTMS) in the Orbitrap, with data-dependent MS/MS on the top 6 intensity ions dynamically selected for collision-induced dissociation (CID) fragmentation and detection in the linear trap quadrupole (LTQ). The dynamic exclusion settings used were as follows: repeat count 1, repeat duration 30 s; exclusion list size 500; exclusion duration 90 s. A full-scan (500 ms maximum injection time) in the FTMS at a resolution of 30,000 identified the 6 highest abundance ions and selected them for CID (1.0 isolation width, normalised collision energy 35%, activation Q 0.25, activation time 30 ms) in the LTQ after accumulation of 500,000 ions with 10 ms maximum injection time. Between sample runs, the column was washed twice with a gradient from 2% Buffer B to 98% Buffer B across 30 min, the combination of washes used depended on how the chromatograms looked, if there was a lot of lipid at the end of the run a prolonged high %B wash was used. A minimum of two technical replicates were carried out on each of 3 or more biological replicate samples.

3.10.1 Data analysis 1: Protein ID

Mass spectra were analysed with both Sequest HT and Mascot search methods using Proteome Discoverer 2.1 (Thermofisher) against the entire Uniprot human proteome database (reviewed 07/09/2017). To aid in comparability between the data generated in this thesis and previous data collected by our lab group, an older Uniprot review was used. Parameters were set to a maximum of 2 missed cleavages with peptide lengths ranging from 5 to 144 amino acids selected. A precursor mass tolerance of 10 ppm and fragment mass tolerance of 0.8 Da were allowed. Modifications were as follows – Static: carbamidomethylation and dynamic: oxidation (methionine) and carbamylation (both N-terminal and at lysine).

Figure 3-1 A selection of gradients used for LC-MS² analysis and column cleaning. The 100% max value on the y-axis corresponds to an 80% ACN solution. a) The GOAT gradient is an optimisation to spread the data evenly across a run. b) a high %B wash employed in the final sample processing to ensure lipids were removed from the column prior to starting a new run. c) a standard linear wash gradient. d) A stronger wash gradient.
3.10.2 Data analysis 2: Protein quantification

Proteome Discoverer output files were then loaded into Scaffold Q+ (4.4.8) proteomics software for quantification analysis. Technical replicates were combined using MuDPIT processing (Kislinger, Gramolini, MacLennan, & Emili, 2005) and searches were again carried out against the Uniprot human proteome database (downloaded 07/08/2017). Protein abundance was quantified using the total TIC analysis method. Protein FDR thresholds were set to 1.0% and a peptide threshold of 0.1% FDR with proteins requiring at least 2 unique peptides to be identified. A protein abundance change, with a significance value less than 0.05 was considered significant.

The software converts the raw sequence files into potential peptide sequences. The peptide validator confirms whether the spectral data corresponds to a valid peptide. From this point, peptide sequences are matched against the Uniprot human proteome database for matches to defined protein sequences. The protein scorer assigns a quality score of the peptide sequence aligned with a known protein. Statistical due diligence from the FDR validator deals with false positives.
3.10.3 Analysis of proteome responses

Lists of proteins that changed in abundance to each treatment determined in Scaffold were compiled. The list of increasing, decreasing or combined change proteins were separately analysed for gene ontological (molecular function, cellular component, and biological process), pathway (KEGG and Reactome), and protein complexes (CORUM) enrichments through G:profiler (Reimand et al., 2016). Further analysis of clusters of proteins which had previously been found to be co-expressed was undertaken in STRING (Szklarczyk et al., 2019).

Figure 3-3 The processing workflow used to convert raw spectral data into peptide matches and ultimately to align sequences to known proteins to identify proteins from the spectral data. There are multiple nodes/modules used to perform statistics and validate findings.
3.11 Gprofiler and STRING analysis pipeline

- **Raw MS Files**
  - Spectral data from LC-MS

- **Proteome Discoverer**
  - Peptide spectrum matches (PSMs)
  - Protein identification

- **Scaffold**
  - Total ion current (TIC) quantification

- **FDR calculator**
  - Princeton Edu web-server
  - 10% FDR

- **List of accessions from Scaffold**

- **Uniprot + BLAST verification of gene names**

- **Gprofiler**
  - Biological process enrichment
  - Cellular component enrichment

- **STRING**
  - Co-expression analysis
  - Visual cluster representation

- **KEGG**

- **CORUM**
4 Results

4.1 IC_{10} and IC_{1} treatment data

One main objective of this project is to mimic the low-doses (IC_{10} and IC_{1}) of drug used in a previous proteomics experiments using patamine A. Obtaining growth inhibitory concentration (IC) data was achieved via MTT assay and non-linear regression analyses on data displayed in Figure 4-1, Figure 4-2, Figure 4-3, Figure 4-4, Figure 4-5, Figure 4-6, Figure 4-7. The MTT assays for ribavirin and cycloheximide generate excellent dose response curves in both cell lines, a key difference is the HT29 cell line not completely ceasing metabolism in response to treatment with the curves reaching their asymptotes well before zero. The 4E1RCat assay data displayed for the HT29 cell line demonstrate the impracticality of using a compound with low solubility and antiproliferative capacity. Below is a table containing the IC_{50}, IC_{10} and IC_{1} data in HT29 and HL60 cells for the control protein synthesis inhibitor cycloheximide (CHX), the eIF4E inhibitors ribavirin (RBV) and 4E1RCat as well as for the vehicle, dimethyl sulfoxide (DMSO) displayed in % (v/v). A minimum of three biological replicates were used to obtain IC data for all drugs. The vehicle dose response data was done to ensure that the inhibitory effects of the vehicle were well established. For CHX and RBV treatment concentrations, the IC_{10} and IC_{1} values did not include the effects of DMSO as the vehicle is diluted to beneath biological significance. For 4E1RCat this is not the case; the IC_{10} concentration was hard to establish in both cell lines, in this case the top dose of 40 µM was used as the IC_{10} concentration in both cell lines, indicated by a * in the table below.

Table 1 Growth inhibitory concentration (IC) data at 3 levels, the IC_{10} values were the values used for further treatments. *4E1RCat: this compound was not sufficiently active to generate a full growth inhibition curve, and so IC values could not be modelled. The highest concentration used (40 µM) gave a growth reduction equivalent to the 10% growth reduction seen with other treatments. Displayed in brackets beneath the IC values are the 95% confidence intervals. Note that curve fitting was not possible with all of the data and in some cases it was not possible to generate IC values in Prism despite having curves that superficially appeared model-able.

| Drug                    | IC_{1}  |   | IC_{10} |   | IC_{50} |   |
|-------------------------|---------|---|---------|---|---------|---|
|                         | HT29    | HL60 | HT29    | HL60 | HT29    | HL60 |
| Cycloheximide (µM)      | 0.004   | 0.005 | 0.038   | 0.048 | 0.305   | 0.349 |
|                         | (0.001 to 0.019) | (0.002 to 0.0178) | (0.020 to 0.071) | (0.025 to 0.088) | (0.226 to 0.412) | (0.259 to 0.471) |
| Ribavirin (µM)          | Not converged | Not converged | 11.9   | (5.03 to 28.3) | 10.9   | (5.15 to 22.9) | 81.4   | (35.7 to 185) | 68.3   | (39.1 to 119) |
| 4E1RCat* (µM)           | n/a     | n/a  | *40     | *40  | n/a     | n/a  |
| DMSO (%)                | Not converged | 0.621 | 0.390   | 1.52 | 1.46    | 3.45 |
|                         | (0.421 to 0.917) | (0.293 to 0.518) | (1.31 to 1.76) | (1.11 to 1.93) | (2.87 to 4.15) |
Ribavirin effects on HT-29 proliferation

Figure 4-1 Absorbance changes reflect metabolic activity revealed by MTT treatment of $10^5$ cells treated with increasing concentrations of ribavirin, with maximum inhibitor concentration limited to comfortably within the limit of solubility, with no more than 1% DMSO used in any dilution. Shown are the effects of ribavirin treatment on HT29 cell proliferation, graph generated in Prism. Note the failure to drop to total growth inhibition is not unusual in the HT29 cell line.

Cycloheximide effects on HT-29 proliferation

Figure 4-2 Absorbance changes reflect metabolic activity revealed by MTT treatment of $10^5$ cells treated with increasing concentrations of cycloheximide, no more than 1% DMSO used in any dilution. Shown are the effects of cycloheximide treatment on HT29 cell, graph generated in Prism.
**Figure 4-3** Absorbance changes reflect metabolic activity revealed by MTT treatment of 10^5 cells treated with increasing concentrations of 4E1RCat, with maximum inhibitor concentration limited by 4E1RCat solubility, with no more than 1% DMSO used in any dilution. Shown are the effects of 4E1RCat treatment on HT29 cell proliferation, graph generated in Prism. The DMSO vehicle at 1% v/v is likely to be partially contributing to the effects seen at the top dose of 40 µM.

**Figure 4-4** Absorbance changes reflect metabolic activity revealed by MTT treatment of 10^5 cells treated with increasing concentrations of ribavirin, with maximum inhibitor concentration limited to comfortably within the limit of solubility, with no more than 1% DMSO used in any dilution. Shown are the effects of ribavirin treatment on HL60 cell proliferation, graph generated in Prism. Note the drop to total growth inhibition is more noticeable in HL60 cells, presumably further increasing the ribavirin concentration would lead to total growth inhibition.
4.2 Effects of DMSO

To establish the biologically relevant anti-proliferative effects of DMSO in the cell lines used in this project, MTT assays were used to generate a dose response between DMSO % in solution and proliferation. These graphs are shown below. The minimum of 3 biological replicates rule was waived as this was exploratory and was done partly out of curiosity to ensure that the effects of the vehicle on cell lines was well established. This became useful when it became apparent that a calculation error had occurred that established that the top dose of each drug in the MTT assays corresponded to 1% DMSO which is slightly above the well-accepted 0.5% DMSO cut-off.

Figure 4.5 Absorbance changes reflect metabolic activity revealed by MTT treatment of $10^5$ cells treated with increasing concentrations of cycloheximide, no more than 1% DMSO was used in any dilution. Shown are the effects of cycloheximide treatment on HL60 cell proliferation, graph generated in Prism. Note the drop to total growth inhibition.

Figure 4.6 Effects of DMSO on HT29 cell proliferation, graph generated in Prism.
Figure 4-7 Effects of DMSO on HL60 cell proliferation, graph generated in Prism.
4.3 PEG contaminant detection by MALDI-TOF

Polyethylene glycol (PEG) is a polyether compound with an array of uses from medicine to plastics to molecular biology. It is commonly used as a plasticiser to change the thermal and mechanical properties of plastics to increase their pliability. Among its other uses, PEG is used in non-ionic detergents, often chemically added to the detergent molecule to increase its capacity to interact with water. In the mass spectrometry setting, PEG has the capacity to suppress ionisation of an analyte by competing for charge and lowering the capacity to generate peptide fragments, thus lowering the sensitivity of the analysis. Early in the course of this thesis it was discovered that PEG was present in test samples run on the LC-MS. A set of experiments were conducted to determine the source of PEG contamination. There were two expected sources of PEG contamination, the plastics used over the course of the experiment, and the non-ionic detergent Triton X-100 present in the RIPA buffer. Matrix assisted laser desorption/ionization – time of flight (MALDI-TOF) was used briefly in this thesis to detect PEG contamination. Due to most of the protein samples being dissolved in urea, and containing high salt concentrations the use of the maximum laser ablation setting was often necessary. The goal of this analysis was to achieve spectra containing peptide peaks without the characteristic series of peaks seen with PEG. These peaks differ by 44 mass units, and strongly resemble a statistical representation of a normal distribution. Figure 4-8, Figure 4-9 demonstrate spectra with only matrix peaks from the CHCA and peptide peaks respectively, these are placed here to give the reader an idea of what to expect from a MALDI-TOF spectra. (Figure 4-10, Figure 4-11, Figure 4-15, Figure 4-16) were all from samples processed using RIPA buffer and were evidence that RIPA buffer was the likely source of PEG. This was confirmed by samples processed using urea demonstrated in several of the figures below (Figure 4-10, Figure 4-11, Figure 4-15, Figure 4-16), these samples are free of PEG contamination and have clean peptide peaks free from the intrusion of the visually distinct PEG spectral motif.

Figure 4-8 Spectra using maximum laser intensity where nothing of interest is visible, a few matrix peaks are visible near the Y-axis.
Pre-digestion, peptides in 2 M urea were mixed with CHCA at a 1:10 ratio. Laser ablation at the maximum of 7000 units was used. There was a noticeable decrease in PEG between the two methods of precipitation (Figure 4-10, Figure 4-11), however still abundant at concentrations too high to run on the LC-MS.

Figure 4-9 A spectrum showing matrix peaks near the Y-axis and demonstrating visible peptide peaks.

Figure 4-10 MALDI-MS/MS spectrum of a RIPA buffer precipitated PEG-contaminated sample following chloroform-methanol precipitation.
The figures below are post-digestion, ZipTipped peptides mixed with CHCA at a 1:10 ratio, Polyethylene glycol (PEG) is absent. They are a good demonstration of the spectrum expected from a complex protein sample without the presence of PEG.

Figure 4-12 MALDI-MS/MS spectrum of a typical peptide sample (sample A22).
The figures below show urea lysed, post-digestion, ZipTipped peptides mixed with CHCA at a 1:10 ratio. This is included as an example of polyethylene glycol that may come from another source, such as autoclaved pipette tips. Note the low abundance of PEG in comparison to peaks associated with the MS signal of the CHCA matrix on the left side of Figure 4-15.
4.4 A side-by-side comparison of protein precipitation methods

Side-by-side comparisons were made using the chloroform-methanol and acetone methods using known-protein standards (Bovine serum albumin) and HT29 raw lysates to compare the efficiency and reproducibility of the two methods. The chloroform-methanol method produces an almost ‘fluffy’ pellet, which is useful for redissolving precipitated proteins. However, if sample proteins are not visible by eye, dislodging the pellet when removing the organic phase is a quick way to lose sample without realising it. As a result, although the chloroform-methanol method gave higher recovery in most cases, it occasionally lead to very low protein recovery. In contrast, the acetone precipitation method proved effective and robust and was adopted throughout this thesis.
4.5 Assessing the effectiveness of lysis solutions without non-ionic detergents

A core component of this thesis was the DC Protein Assay, it was used extensively to quantify protein concentrations (Figure 4-17). Although the assay recommends working in the linear range of the assay, it was noted that the working range of the assay was inconveniently small, in response to this a larger range of concentrations were used and a non-linear curve fitted.

![DC Protein Assay - Standard Curve using Bovine Serum Albumin](image)

Figure 4-17 A standard curve of bovine serum albumin (BSA) was constructed using the DC protein assay, a portion of the graph between 200-1500µg/mL has a direct linear relationship between absorbance and BSA concentration. A non-linear regression is fitted to the curve.

To eliminate PEG contamination alternative buffers without non-ionic detergent (e.g. Triton-X100, NP40) were explored for protein extraction efficiency. 1x10⁶ cells were lysed using each buffer. Analysis was done using Prism, a non-linear 2-phase decay function was used to model the above standard curve to allow calculation of the protein concentrations in each sample.

| Lysis condition (1x10⁶ cells) | Protein concentration (µg/µL) | Efficiency relative to RIPA buffer (%) | Total extracted protein (µg) |
|-----------------------------|-------------------------------|--------------------------------------|-----------------------------|
| RIPA lysis buffer           | 2.10                          | 100                                  | 420                         |
| 8M urea                     | 1.39                          | 66.2                                 | 278                         |
| 8M urea (replicate)         | 1.36                          | 64.8                                 | 272                         |
| 8M urea + 5% SDC            | 0.87                          | 41.4                                 | 174                         |
| dH₂O + protease inhibitor   | 0.45                          | 21.4                                 | 90                          |

Table 2 Lysis buffer versus protein concentration for several lysis methods. All buffers besides RIPA buffer were flash frozen at -80 °C three times to aid in the lysis process.

Although RIPA buffer was ~1/3 more effective than 8 M urea, it was significantly better than 8 M urea containing SDC and distilled water with protease inhibitor.
4.6 Mass spectrometry optimisation – chromatograms

Early attempts to generate spectra were largely unsuccessful, it was noted that samples from the earliest attempts were very low in protein content, comparable to the spectra for unrelated samples shown in figures Figure 4-18 and Figure 4-19. These have very few peaks in the middle of the gradient where most peptides would cluster and slightly more peaks corresponding to hydrophobic molecules, more than likely lipids. The change in the chromatograms between the exosome derived proteins and the proteins derived from a whole cell lysate with a comparatively high protein abundance is almost directly related to the protein concentration of the sample loaded on the MS. The exosome data show low protein abundances, toward the lower end of detection in the DC Protein Assay. It is used to demonstrate the relationship between loaded protein and the resulting chromatograms. Although visually assessing a chromatogram is not a reliable way of predicting the number of protein IDs generated in Proteome Discoverer, it is generally possible to determine the characteristic chromatograms of a low protein abundance sample (Figure 4-18, Figure 4-19). Note that the lysis method used in some samples of this section is a variation of the 8 M urea lysis solution containing 6 M urea and 2 M thiourea. This method was discontinued as the thiourea would crystallize at low temperatures which was inconvenient for later centrifugation steps.

Figure 4-18 LC-MS/MS total ion count chromatogram of exosomes lysed using 6M urea + 2M thiourea. Number of recognised proteins: <50. The sample was obtained using 8 M urea lysis to replace the RIPA lysis buffer, exosomes prepared by Deanna Dupre included to show the resulting spectra when peptide concentration is insufficient.

Figure 4-19 LC-MS/MS total ion count chromatogram of exosomes lysed using 6M urea + 2M thiourea. Number of recognised proteins: <10. The protocol I used to replace the RIPA lysis buffer, exosomes prepared by Deanna Dupre included to show the resulting spectra when peptide concentration is insufficient.
Figure 4-21: LC-MS/MS total ion count chromatogram of HT29 cells lysed using 6M urea + 2M thiourea. Number of proteins identified with a high level of confidence in proteome discoverer: 967.

Figure 4-20: LC-MS/MS total ion count chromatogram of HT29 cells lysed using 6M urea + 2M thiourea. Number of proteins identified with a high level of confidence in proteome discoverer: 1234. Demonstrating the effectiveness of my digestion and processing protocol.

Figure 4-22: LC-MS/MS total ion count chromatogram of HT29 cells lysed using 6M urea + 2M thiourea. Number of proteins identified with a high level of confidence in proteome discoverer: 1286.
Mass spectrometry results

It will become apparent in the MS results that data was only collected in the HT29 cell line. This was due in part to the difficulties encountered with culturing the HL60 cells and in part to availability of the mass spectrometer as it is a shared instrument with high usage and was unavailable for large periods of time whilst under repair. A mirror experiment was set up in the HL60 cell line and the cells processed to the point where they could be analysed on the mass spectrometer, however, in the interest of time and availability of the instrument, the HT29 cell data was the focus of the MS analyses. First Set of MS results: HT29 cells - 4E1Rcat (3.2 µM) and DMSO at (0.4% v/v)

This set of data was not used to evaluate the effects of 4E1Rcat. It is provided as example of the effects of the protein loading concentration (total protein) on the number of protein hits in Proteome Discoverer. There is a correlation between total protein loaded and the number of proteins identified. In this preliminary experiment, a calculation error meant that the appropriate amount of 4E1Rcat was not used, the target concentration of 40 µM was missed and cells were instead treated at 3.2 µM. The matching DMSO control data was obtained at the IC10 of DMSO, rather than at the DMSO concentration that corresponded to the 4E1Rcat treatment. 8M Urea (instead of 6M urea + 2M thiourea) was used as the lysis buffer. Membranes were not pelleted after cell lysis, protein precipitation occurred in acetone with membranes still present, protein concentrations post-precipitation were highly variable.

Table 3: More proteins are identified in Proteome Discoverer in response to higher total protein run on the MS. Displayed are: Samples 1-3: DMSO (0.4% v/v), 4-6: 4E1Rcat (3.2 µM)

| Sample #     | A750 (averaged triplicate) | Protein conc. (µg/µL) | Total protein (µg) | Protein IDs (Replicate 1; 2) |
|--------------|-----------------------------|-----------------------|--------------------|------------------------------|
| Control - 1  | 0.224                       | 0.8                   | 26.5               | 834; 783                     |
| Control - 2  | 0.267                       | 1.2                   | 41.4               | 781; 786                     |
| Control - 3  | 0.231                       | 0.8                   | 28.5               | 926; 849                     |
| 4E1Rcat - 4  | 0.354                       | 2.5                   | 87.3               | 1211; 1182                   |
| 4E1Rcat - 5  | 0.293                       | 1.5                   | 52.8               | 1040; 1039                   |
| 4E1Rcat - 6  | 0.271                       | 1.2                   | 43.1               | 985; 959                     |

Samples were reconstituted in a final volume of 110 µL of 0.1% formic acid. The protein yield was sampled post acetone precipitation, and large amounts of variability between samples was observed. The correlation between protein yield and the number of proteins identified in Proteome Discover influenced the methods used for later experiments. Samples for subsequent experiments that had low initial concentrations were normalised by reconstituting the sample in a lower volume, thus increasing the concentration of protein. The mass spectrometer uses 25 µL for each replicate, and two technical replicates was considered enough. Therefore between 60 and 110 µL, there is room to adjust sample volume to increase the consistency of protein identification.

Second set of MS results: HT29 cells - Untreated, CHX (0.043 µM) and RBV (11.1 µM)
Normalling protein abundance to 100 µg of total protein run on the MS leads to a large improvement in the number of proteins identified in Proteome Discoverer. Displayed: 1-3: Untreated cells, 3-6: Cycloheximide (0.043 µM), 7-9: Ribavirin (11.1 µM)

### Table 4

| HT29 Sample # | AP50 (averaged triplicate) | Protein Conc. (µg/µL) | Total protein (µg) | Dilution factor | Normalised protein abundance (µg) | Protein IDs (Replicate 1; 2) |
|---------------|-----------------------------|-----------------------|-------------------|----------------|-----------------------------------|-----------------------------|
| Control - 1   | 0.484                       | 3.9                   | 290.6             | 2.9            | 100                               | 1209; 1137                 |
| Control - 2   | 0.457                       | 3.3                   | 250.7             | 2.5            | 100                               | 1171; 1169                 |
| Control - 3   | 0.392                       | 2.2                   | 168.1             | 1.7            | 100                               | 1362; 1246                 |
| CHX - 4       | 0.460                       | 3.4                   | 255.0             | 2.6            | 100                               | 1315; 1209                 |
| CHX - 5       | 0.476                       | 3.7                   | 278.5             | 2.8            | 100                               | 1010; 1100                 |
| CHX - 6       | 0.311                       | 1.2                   | 93.5              | n/a            | 93.5                              | 1291; 1226                 |
| RBV - 7       | 0.402                       | 2.4                   | 179.5             | 1.8            | 100                               | 1106;1096                  |
| RBV - 8       | 0.376                       | 2.0                   | 151.0             | 1.5            | 100                               | 1264;1121                  |
| RBV - 9       | 0.296                       | 1.1                   | 82.7              | n/a            | 82.7                              | 1291;1149                  |

4.7.2 Third set of results: HT29 cells – DMSO control (1%) and 4E1RCat (40 µM)

This data was used for subsequent analysis, and replaced data obtained from the first set of MS results.

### Table 5

Normalising protein abundance leads to a consistent improvement in the number of identified proteins. Displayed are: Samples 1-3: DMSO (1% v/v), 3-6: 4E1RCat (40 µM)

| HT29 Sample # | AP50 (averaged triplicate) | Protein Conc. (µg/µL) | Total protein (µg) | Dilution factor | Normalised protein abundance (µg) | Protein IDs (Replicate 1; 2) |
|---------------|-----------------------------|-----------------------|-------------------|----------------|-----------------------------------|-----------------------------|
| Control - 1   | 0.309                       | 1.6                   | 123.0             | 1.2            | 100                               | 1120; 1172                 |
| Control - 2   | 0.354                       | 2.2                   | 167.3             | 1.7            | 100                               | 1389; 1242                 |
| Control - 3   | 0.333                       | 1.9                   | 145.6             | 1.5            | 100                               | 1218; n/a                  |
| 4E1RCat - 4   | 0.357                       | 2.3                   | 170.6             | 1.7            | 100                               | 1200; n/a                  |
| 4E1RCat - 5   | 0.344                       | 2.1                   | 156.8             | 1.6            | 100                               | 1040; 1395                 |
| 4E1RCat - 6   | 0.301                       | 1.5                   | 116.0             | 1.2            | 100                               | 1235; 1122                 |

4.8 Further analysis of MS data post-protein identification

After processing in Proteome Discoverer, data was moved into Scaffold and four treatment conditions were constructed and analysed. Comparisons were as follows: untreated controls against the cycloheximide (CHX) treatment, untreated controls against ribavirin (RBV), a 1% DMSO control against 4E1RCat and lastly untreated controls versus the 1% DMSO control, essentially a DMSO treatment condition.
4.8.1 Gene ontological enrichment analysis using G profiler

Global proteome changes were computed in Scaffold prior to GO analysis in the G profiler online software. Inclusion as a significant change was managed internally by the Scaffold software, which uses a decoy generation strategy to manage the FDR. No formal cut-off was applied with respect to protein abundance in terms of fold-changes. Despite this, on closer inspection the Scaffold software appears to have imposed cut-off boundaries, the lower threshold for an upregulated protein based on the four analyses run in Scaffold is a fold-change of $> 1.1$ and the lower threshold for a downregulated protein appeared to be capped at a fold change of $> 0.0002$.

Table 6 gene ontology enrichment by biological process (BP) and cellular component (CC). The molecular function (MF) GO domain was included in one analysis. The top 5 BP and CC are displayed, the intention of the selection process was to pick the top 5 unique processes, components and MF if applicable. If 5 unique processes did not exist, the nested ontology terms were included, despite the smaller ontology contributing to the enrichment seen in the larger ontology. Ontological enrichments are reported separately for proteins that increase in abundance (UPs) in response to the treatment, and those that go down (DOWNs). Ontology term name, adjusted p-values from G:profiler and the number of proteins observed to change in abundance that are associated with the ontology (intersection size) are reported.

### Cycloheximide UPs

| source | term name                                                                 | adjusted p value | intersection size |
|--------|---------------------------------------------------------------------------|------------------|-------------------|
| GO:BP  | RNA splicing, via transesterification reactions with bulged adenosine as nucleophile | 4.57 x 10^{-2}   | 6                 |
| GO:BP  | mRNA splicing, via spliceosome                                             | 4.57 x 10^{-2}   | 6                 |
| GO:BP  | RNA splicing, via transesterification reactions                           | 4.80 x 10^{-2}   | 6                 |
| GO:CC  | ribonucleoprotein complex                                                  | 1.53 x 10^{-5}   | 11                |
| GO:CC  | catalytic step 2 spliceosome                                               | 6.10 x 10^{-5}   | 5                 |
| GO:CC  | spliceosomal complex                                                       | 8.82 x 10^{-5}   | 6                 |

### Cycloheximide DOWNs

| source | term name                                                                 | adjusted p value | intersection size |
|--------|---------------------------------------------------------------------------|------------------|-------------------|
| GO:BP  | regulation of protein catabolic process                                    | 1.07 x 10^{-4}   | 9                 |
| GO:BP  | positive regulation of protein modification by small protein conjugation or removal | 4.63 x 10^{-4}   | 6                 |
| GO:BP  | regulation of protein modification by small protein conjugation or removal | 6.19 x 10^{-4}   | 7                 |
| GO:BP  | proteolysis involved in cellular protein catabolic process                | 2.20 x 10^{-3}   | 10                |
| GO:BP  | proteasome-mediated ubiquitin-dependent protein catabolic process         | 2.80 x 10^{-2}   | 7                 |
| GO:CC | proteasome complex | 2.10 x 10^{-3} | 4 |
| GO:CC | endopeptidase complex | 2.23 x 10^{-4} | 4 |
| GO:CC | peptidase complex | 6.80 x 10^{-3} | 4 |
| GO:CC | ubiquitin conjugating enzyme complex | 3.16 x 10^{-2} | 2 |
| GO:CC | MCM complex | 4.81 x 10^{-2} | 2 |

**Ribavirin UPs**

| source | term name | adjusted p value | intersection size |
|--------|-----------|------------------|------------------|
| GO:BP | nuclear-transcribed mRNA catabolic process, nonsense-mediated decay | 1.41 x 10^{-4} | 7 |
| GO:BP | SRP-dependent cotranslational protein targeting to membrane | 7.72 x 10^{-4} | 6 |
| GO:BP | RNA catabolic process | 1.46 x 10^{-3} | 9 |
| GO:BP | ribosome biogenesis | 3.79 x 10^{-2} | 7 |
| GO:BP | maturation of LSU-rRNA | 3.14 x 10^{-2} | 3 |
| GO:CC | ribonucleoprotein complex | 2.81 x 10^{-8} | 17 |
| GO:CC | cytosolic ribosome | 2.73 x 10^{-4} | 6 |
| GO:CC | nucleolus | 3.71 x 10^{-4} | 13 |
| GO:CC | polysome | 5.82 x 10^{-4} | 5 |
| GO:CC | cytosolic part | 1.42 x 10^{-3} | 7 |

**Ribavirin DOWNs**

| source | term name | adjusted p value | intersection size |
|--------|-----------|------------------|------------------|
| GO:MF | cadherin binding involved in cell-cell adhesion | 1.34 x 10^{-3} | 3 |
| GO:MF | cadherin binding | 4.30 x 10^{-3} | 6 |
| GO:MF | cell-cell adhesion mediator activity | 2.62 x 10^{-2} | 3 |
| GO:MF | cell adhesion mediator activity | 4.30 x 10^{-2} | 3 |
| GO:MF | cell adhesion molecule binding | 4.35 x 10^{-2} | 6 |
| GO:CC | cell-cell adherens junction | 5.65 x 10^{-3} | 4 |
| GO:CC               | term name                      | adjusted p value | intersection size |
|--------------------|--------------------------------|------------------|-------------------|
| adherens junction  |                                | 3.22 x 10^{-2}   | 6                 |
| proteasome complex |                                | 3.30 x 10^{-2}   | 3                 |
| endopeptidase complex |                            | 3.45 x 10^{-2}   | 3                 |
| anchoring junction  |                                | 3.74 x 10^{-2}   | 6                 |

4E1RCat UPs

| source | term name                                                                 | adjusted p value | intersection size |
|--------|---------------------------------------------------------------------------|------------------|-------------------|
| GO:BP  | SRP-dependent cotranslational protein targeting to membrane              | 2.46 x 10^{-7}   | 7                 |
| GO:BP  | nuclear-transcribed mRNA catabolic process, nonsense-mediated decay       | 1.34 x 10^{-6}   | 7                 |
| GO:BP  | translational initiation                                                  | 1.13 x 10^{-6}   | 8                 |
| GO:BP  | heterocycle catabolic process                                             | 3.03 x 10^{-5}   | 10                |
| GO:BP  | ribosomal large subunit biogenesis                                        | 9.21 x 10^{-3}   | 4                 |
| GO:CC  | cytosolic large ribosomal subunit                                         | 1.26 x 10^{-9}   | 7                 |
| GO:CC  | large ribosomal subunit                                                   | 2.16 x 10^{-9}   | 8                 |
| GO:CC  | ribonucleoprotein complex                                                 | 4.62 x 10^{-7}   | 12                |
| GO:CC  | cytosolic part                                                            | 1.57 x 10^{-5}   | 7                 |
| GO:CC  | polysomal ribosome                                                        | 2.72 x 10^{-5}   | 4                 |

4E1RCat DOWNs

| source | term name                  | adjusted p value | intersection size |
|--------|----------------------------|------------------|-------------------|
| GO:CC  | nucleoid                   | 1.48 x 10^{-3}   | 3                 |
| GO:CC  | mitochondrial nucleoid      | 1.48 x 10^{-3}   | 3                 |
| GO:CC  | nuclear replisome           | 4.27 x 10^{-2}   | 2                 |
The full tables generated from the GO analysis are provided in Appendix Table 11, Table 12, Table 13, Table 14. They are large and contain nested ontological terms, in which an ontological hierarchy is enriched by the ontologies nested below it. This table was constructed as a way to highlight the processes and cellular compartments in which our treatments seem to be generating the most significant enrichments in the HT29 cell line, revealing the major cellular response to the treatments.
5 Discussion

5.1.1 The research questions

The research performed in this thesis aims to gather more information surrounding the unknown consequences of inhibiting the elf4E component of the elf4F complex. Pateamine A has demonstrated the ability to selectively disturb protein expression at low doses, such that there is not a global lowering of translation in proportion to the disturbance, but rather has selective effects with interesting outcomes. Other elf4A inhibitors, hippuristanol and silvestrol have mirrored these effects \textit{in vitro} and the outcome of this selective lowering of protein expression has demonstrable therapeutics effects \textit{in vivo}, namely the rescue of cachexia. Research has demonstrated that rocaclates can change the sequence selectivity of the 4A helicase with its mRNA target (Iwasaki, Floor, & Ingolia, 2016) – this selectivity is facilitated by motifs in the mRNA 5’ UTR, and is one potential explanation for the non-uniform effects of Pateamine A on the proteome.

Previous research by our laboratory has demonstrated that at low dose treatment, equivalent to an IC$_{10}$ or below in the cell lines treated, Pateamine A disturbs protein expression in a pattern across the global proteome. This in contrast to the selected control drug, cycloheximide which uniformly lowers global protein expression. This research was an extension of the proteomic work with Pateamine A. Treatment involved two main drugs, 4E1RCat and ribavirin, 4E1RCat acts at the elf4E-elf4G interface preventing interaction and so interfering with cap-recognition. Ribavirin acts as an m7G cap mimic and competes with mRNA vying for translation. This distinction may have functional outcomes as elf4E interaction with elf4G is likely to have a regulatory or stimulatory effect.

In this research, the elf4F complex was perturbed by 4E1RCat and ribavirin treatment and the proteomic outcome investigated to see if a consistent pattern emerged. This research attempted to generate insight into inhibition of elf4F in the context of cachexia. It is still uncertain how the cachexia rescuing effects of elf4A inhibitors are mediated. The work was therefore designed to address a hypothesis that the selective anti-cachectic effects of inhibition of elf4A are mediated by the elf4F complex.

The structure of the experiments I undertook was chosen to attempt to answer a core set of questions:

1. Is there a pattern in the upregulation or downregulation of proteins in response to low dose elf4E inhibition? Does this pattern mirror the effects seen with elf4A inhibition?

2. Are there elf4E dependent transcripts and what characteristics (e.g. motifs in the 5’ UTR), if any, do they possess? Do these characteristics mirror those seen with elf4A inhibition?

In answering these questions, I hoped to identify whether the anti-cachectic effects of inhibition of elf4A is solely mediated by elf4A itself, or whether there is a potential effector role for the elf4F complex as a whole. Furthermore, the work should reveal whether there is a possibility of therapeutic intervention at elf4E to ameliorate cachexia, much like elf4A inhibition.
5.2 Practical work and technique focused discussion

5.2.1 Cell culture

5.2.1.1 Obtaining reproducible treatment data
Getting reproducible drug response data from multiple MTT assays was a challenge. The low solubility of 4E1Rcat in comparison to ribavirin and cycloheximide caused significant trouble when collecting treatment data. Low solubility combined with 4E1Rcat’s low toxicity and the cytotoxic effects of DMSO was a major challenge for data collection and made the task of fitting a non-linear regression open to interpretation. The variability of the data could be caused by several factors, one of them I addressed by the running an assay at two cell concentrations. Fig 6.6 shows the effects of cell number on the outcome of an MTT assay. I noticed early on that using the standard number of cells (10,000) appropriate for an HL-60 assay made the wells look exceptionally crowded when using the significantly larger HT-29 cells. In response to this, I ran an assay to check how cell number would affect the outcome of an assay. Decreasing the number of cells to 2,500 has a subtle, but noticeable effect at the lower drug concentrations, with a steeper drop in cell activity in comparison to the other three curves. Although the other three curves show significant variability at the high drug concentrations, they show a marked consistency at the lower doses.

In the MTT assay results of 4E1Rcat in the HT29 cell line, there appeared to be limited absorbance changes, even at high concentration. This may be due low efficacy of the compound in this cell line. However, I wished to establish that it did not arise from the optical properties of the 4E1Rcat treatment, as the compound has both a strong optical absorbance and low aqueous solubility, potentially leading to scattering effects. However, formazan crystals were formed during the MTT metabolism step of the assay in the treated HT29 cells, confirming that they remain metabolically active. Furthermore, the equivalent assay with HL60 cells does show a complete loss of metabolic activity showing that the 4E1Rcat solution is unlikely to interfere with the absorbance readings from the MTT assay. This trend of cell quiescence was seen consistently across all the HT29 MTT assays.

5.2.1.2 Practicalities of cell maintenance

5.2.1.2.1 Growth characteristics relate to freezing methodology
The original stocks from which cells in this thesis were derived grew exceptionally well, despite being nearly 20 years old (frozen in 1998). HT29 cells grown over the course of this thesis were aggressively detached using a combination of trypsin and EDTA. When detaching cells from culture flasks, initially the goal was to separate the cells into individual ‘colony forming units’, however, cells thawed using this method tended to grow at a significantly slower rate than observed in the original stocks. Original liquid nitrogen stocks recovered from the thawing process with notable ease and rapidly adhered and begun to grow within 1-2 days. Closer inspection of the original stocks late in this thesis yielded some insights: cells from the original stocks were cryopreserved in clusters, which meant that cells must have been less aggressively detached from their cell culture flasks. These cells could have been frozen using a different method such as the ‘Mr. Frosty’ cryogenic isopropanol containers, the concentration of cryoprotectants or freezing media used may also have been optimised. Freezing cells in clusters is likely to be the main reason for the original stocks’ excellent post-thaw growth.

5.2.1.2.2 HL60 cells
The HL60 suspension cells used in this thesis generally behaved well. However, growing cultures growing would frequently collapse, seemingly without explanation. It is unlikely that this is due to cells becoming over-confluent, as suggested by empirical evidence in the form experiments with
taking cells left in the incubator with the same media for >2 weeks and coaxing the cells back to a healthy state with fresh media. This was done out of an interest in understanding the cell line better. It seems to indicate that HL60 cells are robust to the effects of metabolic acids and high confluency. Another separate issue with HL60 cells that added to trouble in the tissue cell culture suite seemed to arise from cells that were frozen poorly or thawed poorly, leading to large numbers of dead cells. Unlike adherent cells, removing debris is a challenge when dealing with suspension cell lines. Attempts to slow-centrifuge cells in the hope that the cells which should have more mass than the debris surrounding them would be enriched showed little benefit. Even at the lowest speed settings, the centrifuged pellet would contain still contain significant quantities of debris.

5.2.1.2.3 **HT29 cells**

HT29 cells have peculiar growth patterns, distinct from most other cell lines observed over the course of this thesis. They tend to form pseudo-glandular structures and grow in an almost bacterial manner, with the colony expanding and even growing in a 3-D orientation rather than the traditional adherent cells which tend to form a monolayer, see Figure 6-2. The nature of HT29 cell growth made visual assessments of confluence challenging, a combination of observing the coverage of the flask’s surface and the colour of phenol red in culture media was required to assess proximity to the confluence threshold. HT29 cells, much like the HL60 cells, suffered from the same issues seen where the culture seemed to collapse for no apparent reason. Similar experiments were done with HT29 cells regarding response to metabolic acid and high confluency, and the conclusion was again that HT29 cell culture collapse is unlikely to be due to these factors, refer to Figure 6-3, Figure 6-4, Figure 6-5.

5.2.1.2.4 **Rationale for removing PenStrep from media**

Cell culture was initially performed using a pre-made mix of penicillin and streptomycin. Use of antibiotics in cell culture media was discontinued prior to data collection using MTT assays and drug treatment regimens for several reasons. PenStrep was avoided primarily due to research demonstrating genome wide gene regulation and expression changes caused by anti-biotic use in cell culture implicating a molecular interaction within cells (Ryu, Eckalbar, Kreimer, Yosef, & Ahituv, 2017). Secondarily, bacteria can grow in media laden with antibiotics, with a sufficiently large bacterial or fungal load in a contamination event. Cell culture could be accurately described as a waiting game. Cultures where PenStrep is present will take longer for a contamination to become evident; removing PenStrep allowed mistakes to be rectified and new cultures to be started, generally within 24 hours of a contamination event.

5.2.1.2.5 **Problems with thawing, absence of glutamine and contamination**

Over the course of this thesis extensive trouble was encountered when culturing cells. The cause being three-fold. When attempting to start up cultures from freezer stocks, the recovery of cells from thaw, an unnoticed change to L-glutamine-free media, and periodic contamination compounded to make identifying the sources of problems difficult. Effectively caring for cells that have undergone sub-optimal freezing or thawing was a skill learned late in this thesis. Rinsing off dead cells was especially useful for adherent cells, as dead cells and debris can be selectively removed from the culture. Dead cells can acidify media inducing metabolic stress, and debris and secreted molecules from dying cells were also observed to inhibit the sustained, healthy growth of cells in culture. Initial orders of RPMI-1640 contained glutamine, the indicator of the presence or lack of glutamine is a small piece of text on the bottle. At various points during the year cells were dying for seemingly no reason, it is suspected that various orders of media with or without glutamine may explain this. The most useful approach was getting an experienced cell culture user to observe and criticise my technique in the cell culture environment, it became apparent that
assumptions about the sterility of pieces of equipment that had not been autoclaved had been made, this is likely to be a significant source of contamination across the course of this thesis. Trouble-shooting in the cell culture environment was an important component of this thesis, identifying the problem is the main issue and several simple tests were employed to quickly determine what was causing the problem. For contamination issues, the first step was to test potentially contaminated reagents by transferring an appropriate amount into uncontaminated media and incubating it for 1-2 days, to see if any bacteria or fungi grew. For cells that were not growing well, a different stock of the same cell line could be thawed to see if the problem was isolated at the individual level to the liquid nitrogen stock. It could also be checked if media was causing growth issues by seeing if an available HeLa cell line would grow in the media causing trouble. Alternatively, cells could be given more time to recuperate, and be given new media every few days to see if they could make a recovery.

It was noted that HT29 cells grow slowly in media without glutamine, but HL60 cells start to die and refuse to grow, this may have been a fourth confounding factor that made identifying the problem more difficult, this was realised late in the thesis.

5.2.1.2.6 A criticism of cell counting

Cell counting was an important component of this project. However, it was also something that seemed to be a ballpark measure at times. The variability is likely to be a result of three phenomena, firstly, although the samples were thoroughly mixed by pipette prior to sampling, after a sample had been removed and placed on the glass adjacent to the hemocytometer squares, cells would have a chance to settle to the bottom within the globule of media. The second source of variability was the inconsistent pattern of capillary-like uptake of media between the glass coverslip and the glass of the hemocytometer. This inconsistency of can lead to some regions of the hemocytometer having significantly more or less cells. Lastly, small volumes were used, typically 2 µL was diluted in 18 µL, a 10-fold dilution, if the settling of cells in the globule of media is occurring then sampling using small volumes could encounter a problem when sampling of regions of lower or higher cell density. Prior to treatment with compounds, roughly 2 million HT29 or HL60 cells were plated. Whether 2 million cells were plated or 1.5 million cells or 2.5 million matters, see fig 6.6. There are also carry-over effects when it comes to sample processing on the mass spectrometer. Ideally there should be a comparable number of cells between treatment conditions and minimal normalisation towards the later stages, where the volume that peptides are reconstituted in can be varied to ensure consistent concentrations are run on the mass spectrometer. In this sense errors in cell counting could carry through all the way to mass spectrometry stage. There are two points in this project where an accurate cell count will have the most effect on experimental data, the cell counting required to plate 10,000 cells in an MTT well and the cell counting prior to plating up for drug treatment. Although we control for variability between MTT assays by taking absorbance as a percentage of a given control, accurately plating 10,000 cells per well every time improves the comparability of assays. The same principle applies to plating for drug treatment. Cell counting is time consuming and extends the hours spent in the tissue culture suite with every sample that requires counting. Increased consumable used in the form of plastic falcon tubes for the centrifugation process, and pipette tips for the counting are generally necessary, the extra handling can also occasionally result in contamination events, and the mental burden of doing mundane basic mathematics where mental energy could best be applied elsewhere. Cell counting using a hemocytometer is still an important skill in the wet-lab scientists’ arsenal, however, it is 2019 and automated tools have existed for several years now, perhaps it is time to put away the archaic technology and move into the future.
5.2.1.3 Defining a biological replicate in the context of this study

Biological replicates were defined as a culture that had spent at least one passage in its own flask before being seeded and used for either an MTT assay or a treatment at a selected concentration of drug. Technical replicates are defined as either two cultures seeded from the same initial cell culture and used as part of an MTT assay or drug treatment or, in the case of MS data collection, peptide solutions were sampled twice, and thus two technical replicates were combined for later analysis.

5.2.2 Cell lysis and protein handling optimisation

The preparatory steps prior to mass spectrometry involved cell lysis, protein precipitation, disulfide bridge removal, subsequent capping of cysteines with carbamidomethyl moieties, tryptic digestion, desalting and concentration of peptides. This process required extensive optimisation to improve the spectral data abundance and quality. The methodologies for cell lysis and protein precipitation were evaluated, and steps taken to increase peptide abundances as described below.

5.2.2.1 Protein extraction/precipitation methodology choice

Plastics used for protein-related work were not autoclaved to lower the risk of plasticiser leaching into samples. LoBind Tubes were also used as a precaution, reducing the risk of peptides bonding with the plastic when drying in the centrivap concentrator.

5.2.2.1.1 Cell lysis/protein extraction optimisation

Following recognition of PEG contamination, the lysis solution needed to be changed to one not containing non-ionic detergents as these are a source of PEG. The efficiency of several lysis solutions was assessed - RIPA buffer, 8M urea, 8M urea + SDC, and dH2O with added protease inhibitor. The lysis solutions were also combined with 3 quick freeze/thaw cycles in a -80°C freezer, with vortexing of lysate in between. This was an added step to increase the extraction efficiency. RIPA buffer is a purpose-designed lysis buffer and the most aggressive solution, liberating a total of 420 µg of protein this is likely to be the maximum amount that can be extracted. Due to losses during the acetone precipitation a solution as close to the RIPA buffer efficiency as possible was desirable. Human cell lines are generally easy to lyse and don’t require an aggressive lysis procedure for protein liberation; 8M urea is used as a solubilising agent in later steps in the protein extraction protocol, thus it was logical to test it as a lysis solution. The 8M urea lysis was simple and deemed efficient enough to be used extensively.

5.2.2.1.2 Protein precipitation

Two methods were investigated for protein precipitation – acetone, and chloroform-methanol (C-M). I found the acetone precipitation to be simpler, more consistent, and predictable in its ability to precipitate proteins from cell lysates. Previous research performed by our laboratory using C-M suggested that it was the more effective of the two precipitation methods. The slightly more complex C-M precipitation outperformed the acetone precipitation in raw protein yield, but results were more variable overall. Ultimately, the reliability and utility of acetone precipitation were the deciding factors and this method was employed for the data acquisition phase of this project.

5.2.2.1.3 Protein loading optimisation for LC-MS/MS

A need for optimising protein loading concentration by normalising became apparent after the first round of mass spectrometry data was collected. This need came to light after the first round of data of 4E1RCat and DMSO treated HT29 cells was captured. A large discrepancy between protein abundance between samples 1-6 was noted, with some as low as ~25 µg and some as high as ~90 µg. This could have been a consequence of seeding density due to poor cell counting, loss of cells when aspirating media, or an error in the estimation of protein abundance using the DC assay. The
analysis in graphpad was done using a non-linear regression to extract protein concentration using a standard curve of known protein abundances, this strategy may also have contributed to some of the variability. However, the incredibly strong correlation between post-precipitation protein concentration and the number of identified proteins in Proteome Discoverer suggest that the DC assay and non-linear regression are not at fault. The above observations are evidence that protein concentration in a sample and the number of protein IDs are strongly linked, indicating that using around 80-100 µg is likely to give a high number of protein IDs.

5.2.2.2 The polyethylene glycol saga

5.2.2.2.1 Rationale for exhaustive removal of PEG from samples

The reason PEG contamination became an issue is two-fold. Another student doing affinity pull-down of a low abundance protein initially used RIPA buffer for cell lysis, the RIPA recipe includes Triton X-100 a non-ionic PEG containing detergent. This became an issue as PEG tends to lead to ion suppression of sample peptides. This becomes a significant issue when working with low abundance proteins where the sensitivity decrease caused by PEG ion suppression manifests as an inability to detect peptides from a sample. The second issue is that the mass spectrometer is a shared instrument and the PEG takes significant washing to remove from the LC column, another user interested in low-abundance proteins was concerned that the presence of PEG was lowering the sensitivity of the mass spectrometer, although later recognised as a contamination within their own samples. Previous research done by our lab group did not run into the problem of PEG contamination despite the use of RIPA buffer, this is likely because these experiments were observing a whole proteome and the protein abundances were well in excess of the PEG present. The work performed in this thesis is likewise a whole proteome approach with high protein abundances, and so should not theoretically have run into any issues, however, it was noted that a good run from the previous research was expected to yield ~800 protein IDs, and a bad run about ~600. In contrast, an excellent run over the course of this thesis would yield up to 1400 proteins and a bad run was expected to yield 800 or below protein IDs. This discrepancy of protein yields could come as direct consequence of ion suppression by PEG that may never have been addressed had such stringency in identifying and removing the source of PEG been implemented.

5.2.2.2.2 MALDI-TOF use for PEG detection

The MALDI-TOF was used as a tool for analysing samples because it is quick, simple and samples require less processing (desalting, tryptic digestion) before a sample can be analysed as MALDI is more tolerant to high salt concentrations. This allows PEG contamination to be detected more rapidly in comparison to the LC-MS/MS. For a given MALDI experiment the matrix, generally CHCA is combined with a few microlitres of sample at ratios of 1:10 and 1:100, a single microlitre is spotted and dried, this drying period can be very brief or, in the case of high urea concentrations can take up to an hour. Polyethylene glycol has a characteristic fragmentation pattern, with a difference of 44 mass units which closely resembles the shape of a normal distribution (Figure 4-11). In summary, MALDI-TOF is quick, simple, salt-tolerant and has low preparatory time, the 44-repeating pattern of PEG is easy to spot. Using this technique, samples were verified to be free of PEG to identify the ideal methodology for sample preparation for LC-MS/MS analysis.
5.2.3 Mass spectrometry

5.2.3.1 Mass spectrometry interpretation software
Proteome Discoverer (Mascot, Sequest databases), Scaffold and the Princeton Edu FDR calculator were used to identify peptides and protein matches.

5.2.3.1.1 Rationale of static and dynamic modification settings
There are settings within Proteome Discoverer to address peptide modifications in a given proteomics experiment. A static modification refers to those modifications which have been intentionally imposed on the sample peptides, in this case cysteine residues were capped with carbamidomethyl groups from the iodoacetamide treatment to prevent disulfide bridges from reforming. Dynamic modifications refer to modifications that may arise non-uniformly to the sample imposed intentionally or unintentionally because of sample handling. Urea was used extensively as a lysis buffer and a means to re-dissolve proteins. At times the urea/sample solutions would reach as high as 56 °C, increasing the likelihood of carbamylation at the N-terminus and at lysine residues. Oxidation at lysine residues, and deamidation of amide sidechains were also considered as dynamic modifications. Tryptic digestion occurs at lysine and arginine residues, and extensive carbamylation and oxidation can lead to poorly digested polypeptides, this did not become a problem during this thesis. Below is an example of protein ID numbers improving in response to addition of modifications. In addition, a readout has been added from the Proteome Discoverer interface showing a selection of heavily modified peptides some with multiple modifications per peptide chain. It is important to note that most peptides identified have no modifications, with a small proportion having 1 modification, usually carbamidomethylation at a cysteine residue, and an even smaller minority having 1 or more dynamic modification. Adjusting the dynamic modification settings resulted in a slight improvement to the number of proteins identified. Overall the processing methodologies used to prepare samples for LC-MS/MS analysis worked well, samples were not degrading and the effect of using urea was relatively minor.

1. Only fixed carbamidomethylation (no dynamic)
   Total high-quality proteins: 1225
   Total PSMs (peptide sequence matches): 22271

2. +oxidation (only)
   Total high-quality proteins: 1234
   Total PSMs (peptide sequence matches): 22655

3. +carbamoylation (only)
   Total high-quality proteins: 1246
   Total PSMs (peptide sequence matches): 22421

4. +oxidation +carbamoylation
   Total high-quality proteins: 1249
   Total PSMs (peptide sequence matches): 22811

5. +carbamoylation +deamidation
   Total high-quality proteins: 1249
   Total PSMs (peptide sequence matches): 21711
5.2.3.2 Label-free protein quantification from MS/MS spectra

The desire to use label-free tools for protein quantification offers benefits that include limited manipulation of the original biological sample over the course of an experiment, saving time and reagents. The techniques for quantification based on MS spectra have expanded and many of these are available with their own strengths and weaknesses depending on the nature of the experiment.

MS Spectra were analysed using licensed software (Scaffold 4) using in-built label-free methods of quantification. Total ion current (TIC) is a no-nonsense method that is very robust, however, iBAQ which has been developed with an ability to correct for proteolytic fragments from a given protein also looks to be an effective method. Spectral counting employs a similar alignment method as used for calculating mRNA abundance in an RNA-seq experiment, where the ‘reads’, in this case validated peptides are aligned with a known protein sequence. This method was ignored as there are well known biases, as larger proteins will generate more peptides and it is affected by how the machine is running on a given day. This research follows on from previous research performed by our group in which TIC and iBAQ were both used, and thus both techniques were used for protein quantification from the MS spectra. If a significant difference became apparent between the two quantification methods, it was noted and assessed.

5.2.3.2.1 TIC - Total ion current quantification

TIC is a label-free quantification tool and an extension of spectral counting. It is reliable and more robust to some of the problems that plague spectral counting as a quantification technique. For a TIC quantification the average of the TIC for all of the MS/MS spectra that identify a protein is used as a quantitative measure. With the TIC method each spectral count is assigned a unique abundance value (Asara, Christofk, Freimark, & Cantley, 2008).

5.2.3.2.2 iBAQ - Intensity based absolute quantification

iBAQ is a relatively new quantification tool that works very well for protein quantification with various methods of correcting for bias. The central concept of iBAQ is taking the sum of peak intensities of all peptides matching to a specific protein this value is taken and divided by the number of theoretically observable peptides. This process is corrective and aims to control for the capacity of larger proteins to have their peptides overrepresented in a sample. The process of
figuring out which peptide fragments are theoretically possible is aided by knowing the digestion enzyme, in this case trypsin cutting at lysine/arginine (unfortunately followed by proline). The values from this iBAQ method are an accurate proxy for protein levels (Schwanhäuser et al., 2011).

5.2.3.3 Statistical techniques for dealing with false positives
Multiple testing correction with Bonferroni usually makes all results insignificant. False discovery rate algorithms are necessary to figure out what is worth exploring further, FDR as a concept is interesting, it invites the possibility of encountering false positives (e.g. discovering a protein not present in a sample) while attempting to minimise false negatives (e.g. not recognising a correctly identified protein as significant). As an example, below in the first treatment data run through the mass spectrometer. Aiming to identify a change in protein expression between the 4E1Rcat treated cells and the DMSO treated cells a list of 1052 proteins was identified. Of these, at 5% FDR no significant change was noted, at 10% FDR, 2 protein changes became significant, at 15% 20 protein changes became significant (18 proteins increasing in expression and 2 decreasing) and at 20% FDR 22 changes were identified as significant. At an FDR of 15%, it would be expected that 15% of the changes identified as significant are erroneous, corresponding to 3 insignificant changes being falsely identified.

Figure 5-2 A table of 1052 proteins identified between the 4E1Rcat and DMSO treatment conditions (only showing the top 22). Four FDR stringencies, 5, 10, 15, 20% were applied. Cells highlighted in blue were scored as significant by the FDR calculation. FDR calculations were run through a web service retrieved from http://qvalue.princeton.edu/.

5.2.3.3.1 Multiple testing correction
With the rise of computational biology and the mountains of data modern biologists are capable of generating, the use of multiple testing correction is essential. High-throughput experiments, including RNA-seq studies and proteomics aimed at identifying global protein expression changes between treatments, are examples of molecular biology experiments where multiple testing correction is used. The problem being solved by multiple hypothesis testing is that when tests are done enough, chance plays a part in determining whether significant findings will be deemed insignificant and vice versa. As an example, if 100 proteins from experimental data are known to be unaffected by a treatment, i.e. levels don’t change between a treatment and a control, at a defined statistical confidence cut-off, of α = 0.05, five of these 100 by pure chance will have p-values that would encourage rejection of the null hypothesis (i.e. give a response that would be assumed to indicate a change, or a failure to change, between treatments). The risk of falsely discovering a protein is something accepted as part of a proteomics experiment, as the goal is to generate leads which can be validated by molecular biology techniques. The problem with this is that t-tests
individually are frequently undertaken at an α = 0.05, generally speaking. However, as the number of tested outcomes gets higher the absolute number of false positives increases. The t-tests performed for a given protein are a comparison between the untreated control and the treatment condition, if the distributions of peptide ‘counts’ or another quantitative measure between control and treatment are the same, the p-values would be uniformly distributed (Figure 5-3A). Conversely, if there are changes in a quantitative measure between treatment and control, the distributions would overlap less and thus in terms of p-values the distribution would be skewed towards lower values (figure 5-3B). As an example, in a case where most protein levels are unaffected by drug treatment, and a small proportion are affected, statistically you have a case where the affected proteins which will have p-values skewed and closer to 0 and the p-values from the unaffected proteins which will have uniformly distributed p-values between 0 and 1 comingling in the same analysis. Multiple testing corrections like the Benjamini-Hochberg attempt to address this by essentially overlaying the histograms by summation of the p-value distributions of treated and untreated conditions. Then drawing a line across the uniform portion of the distribution crossing into the affected and unaffected proteins the p-values above the line are true-positives. Based on the general FDR of 0.05, less than 5% of the significant results will be false positives (figure 5-3C). The False Discovery Rate (FDR) approach attempts to control the proportion of false discoveries in the results. A decision is made beforehand to accept a proportion ‘α’ of false discoveries, then a threshold is calculated in a way that ensures that the global FDR is expected to be at worst equal to α.

Figure 5-3 A) An example of an even statistical distribution, if peptide ‘counts’ or another quantitative measure between control and treatment are the same, the p-values would be spread uniformly. B) If there are changes in a quantitative measure between treatment and control, the distributions would overlap less, and p-value distribution would be skewed towards lower values. C) An example of a distribution in which both A) and B) are present in the same analysis, a Benjamini-Hochberg-type correction is applied by the red-line across the uniform portion of the graph.
5.2.3.3.2 False discovery rates (FDR)
False discovery is a statistical concept that involves accepting an erroneous outcome or ‘false positive’ as a true positive outcome. In statistical analyses, there is an inherent risk that a type 1 error, a false positive, or a type 2 error in which a statistically significant result is discounted as insignificant, a false negative. The goal of FDR is to minimise false negatives, at the cost of introducing false positives into an analysis at an unspecified rate, in this study the FDR is set at 10%. While the analysis now captures most if not all of the potentially significant findings, they are now interspersed with erroneous results which cannot readily be separated from correctly identified results.

http://qvalue.princeton.edu/ - is a web-based server that performs a Benjamini-Hochberg-like multiple testing correction in which the FDR rate can be specified.

5.2.3.3.3 Decoy protein database strategies for FDR estimation
Decoy protein databases are an empirical strategy for estimating FDR, the basic concept involves generating sets of ‘decoys’ which are a set of distinct generated peptide/protein sequences that don’t correspond to actual peptides/proteins. These decoys are seeded among experimentally identified peptides and the final number of decoys that make it through in the list of identified peptide spectrum matches (PSMs) is an accurate way of empirically determining FDR. Generated decoys are generally beholden to criteria that try to ensure:

1. Similar amino acid distributions as target protein sequences.
2. Similar protein length distribution as target protein sequence list.
3. Similar numbers of proteins as target protein list.
4. Similar numbers of predicted peptides as target protein list.
5. No predicted peptides in common between target and decoy sequence lists.

(Speicher, 2010)
There are two core strategies for generating decoys that can satisfy the above criteria, the first and simplest is a ‘reversal’ strategy that involves reversing the sequence of a peptide/protein to obtain a new entity with the exact constituent amino acids but a non-existent (typically) counterpart in the realm of protein/peptide sequences. The second, a ‘stochastic’ strategy employed by search engines like Mascot, one of the primary protein validation search engines used for protein identification in this thesis. This involves the generation of proteins in a pseudorandom manner, proteins are random in some aspects while following amino acid bias patterns or even using a Markov chain model to better imitate real proteins by mimicking micro-patterns such as single or double amino acid repeats or highly basic/acidic regions (Speicher, 2010).

Multiple testing is a fundamental component of modern biology, and statistical-mathematical and empirical-computational methods in the proteomics field are used to ensure that the number of false-positives and false-negatives are minimised within a given statistical analysis. Corrections like the Bonferroni and Benjamini-Hochberg adjustments are common in this space, modern proteomics also uses an empirical-computational approach to false discovery that involves seeding an analysis with known decoys to model the FDR rate. These two approaches are fundamental to this thesis and have been employed extensively on the path from raw LC-MS² through to PSM identification, to lists of identified and quantified proteins and finally to lists of proteins up or downregulated in response to a treatment.
5.3 Insights from gene ontology and STRING analysis

The FDR adjustment used alongside Scaffold’s existing multiple testing corrections was deemed too stringent as it removed all significant hits from the three main treatment conditions, cycloheximide, ribavirin and 4E1RCat. The DMSO condition however, gained an additional 163 hits at a 10% FDR adjustment through the Princeton Edu web server (Migliaccio, Rotondi, & Auricchio, 2006). In response to this, it was decided that on the whole, the 10% FDR adjustment was too stringent for the main treatments, where the focus on a concentration that produced a small growth defect resulted in few hits. In contrast, it was too generous for use in the DMSO control analysis, which had many hits and accepting a 10% FDR would mean including a large number of false positives. Therefore, the analyses discussed here use the up and downregulated hit list from the Scaffold software, depending only on its multiple testing corrections to limit false positives. Gene ontologies (GOs) are categories of processes, functions, and locations that use standard terms to describe the characteristics of biological systems. An ontology analysis moves the analytics away from what the individual protein or gene specifically does in a molecular sense and attempts to generate new insights by contextualising the specific molecular mechanisms of each individual protein responding to a treatment into more general processes. For example, a DNA helicase specifically unwinds DNA, but is in a more general sense involved in replication. Although at the molecular level the DNA helicase melts and unzips complementary base pairing, its function as a helicase is in the context of providing access to polymerases and other DNA replication proteins to the now single stranded DNA which is important for the replicative process. The use of standard GO terminology ensures it is possible to group proteins together under common ontological terms, and therefore also to analyse protein changes under the three GO domains – Biological Process, Molecular Function, and Cellular Component. Ontological analyses are useful for understanding what processes, functions and cellular locations are associated with a proteome response to a treatment. There are additional ways of analysing proteome responses, beyond the GO terms. These include investigation of changes in sets of proteins that relate to specific pathways, which can be obtained through pathway databases, such as KEGG (Tanabe & Kanehisa, 2012). There are also databases, such as the CORUM database, which can be interrogated to identify whether responding proteins are found in the same complex. Another analysis tool used is STRING (Szklarczyk et al., 2017), this is a visualisation tool that constructs a network of protein nodes based on the confidence with which the nodes interact. Interaction networks can be based on a range of features, including appearance in the same publications, correlation in databases (such as GO and KEGG) and co-expression. This latter analysis reveals clusters of proteins in a set that have been shown to be co-transcriptionally regulated. Proteins in a co-expression network are generally involved in the same biological responses, and these networks can be a useful tool, in addition to GO term analysis, to understand the proteins that appear in the proteome response data. In our analyses, due the small number of hits, in the form of upregulated and downregulated proteins, analyses were sometimes done with a combination of up and downregulated hits to improve the confidence relating to affected processes. The STRING analyses were constructed in this manner, with the exception of the up and downregulated proteins in the DMSO condition, where no benefit was achieved by doing analyses on the combined hits. It is important to note that the appearance of a protein in the GO analysis does not indicate that it will form part of a cluster in the STRING diagrams. All the hits from the Scaffold analysis were included in the GO and STRING analyses. However, in the STRING analyses if the protein did not have partners from the same analysis the node was not included in the STRING output.
5.3.1 Cycloheximide (69 hits)

In the work presented here, cycloheximide is used as a control to evaluate which changes are occurring due to general translational stress, and which are specific to the compound under study. Cycloheximide is expected to decrease protein synthesis uniformly across the protein expression profile of the cells treated at IC_{10}. We may also see a stress response signal due to stalled translation at the ribosome.

GO term enrichment analysis reveals that spliceosomal process and ribonucleoproteins are upregulated in the cycloheximide treatment (Table 7). This assessment is supported by the STRING analysis of the combined up and downregulated proteins (Figure 5-4). This shows a spliceosome-related protein cluster as well as a ribosomal protein cluster. The upregulation of the ribonucleoproteins by cycloheximide is expected as it directly affects protein synthesis at the level of the ribosome. It is likely that cycloheximide is removing a portion of the protein output capacity of the cells and the cellular response is to produce more ribosomes to counteract the loss. The spliceosomal process could also be explained by this observation as new ribosomes will need to be built by the remaining functional ribosomes in the cell and mRNA is the precursor to both the rRNA and ribonucleoproteins.

Proteins involved in ubiquitination and protein degradation via the proteasomal process are downregulated.

Interestingly, associated with the proteosomal components in the cluster diagram are proteins involved in ubiquitination and energy metabolism in the form of enzymes involved in the production of NADPH and ATP. The proteosome is energy dependent and requires a ubiquitination signal for degradation of proteins.

The CORUM and KEGG analyses showed no changes in the upregulated hits condition. In the downregulated hits condition, three complexes showed up in the CORUM analysis: PA28-20S proteasome, the OTUB1-UBC13-MMS2 complex and the MCM4-MCM6-MCM7 complex. These are involved in proteasomal degradation, ubiquitination and DNA replication respectively. These three processes being downregulated in response to protein synthesis inhibition is logical. The balance of protein synthesis is disturbed, and the cells are responding by downregulating the degradation of proteins via ubiquitination and the proteasome. Under normal circumstances proteins will be replaced by protein synthesis. Since cycloheximide has perturbed protein synthesis, downregulating the turnover of proteins is a necessary adaptation to preserve remaining functional proteins. The decrease in DNA replication may be a generalised stress response to the toxicity of cycloheximide, an instruction to cease or lower replicative efforts. In the combined analysis of up and downregulated proteins the CORUM analysis pulled out an additional complex that is not involved in proteasomal degradation that didn’t show up in either of the separate analyses, the SF3b complex which recognises the branch point adenosine of pre-MRNA as part of the splicing process (Rakesh, Joseph, Bhaskara, & Srinivasan, 2016). In the combined condition, the KEGG analysis showed two pathways termed proteasome (as expected from the GO and STRING analyses) but also arginine biosynthesis. Response of the arginine biosynthetic pathway to translational stress has been observed previously (Venturi et al., 2018).
5.3.2 Ribavirin (83 hits)

The two main processes seen in the upregulated hits are cell adhesion/cadherin and proteasomal proteins involved in protein degradation.

Interestingly far more proteins seem to be downregulated in response to ribavirin treatment, and subsequently more processes are affected. Three main processes seem to be ribosome biogenesis, protein targeting to the endoplasmic reticulum (ER), and RNA metabolism. The CORUM analysis of the downregulated hits showed two complexes, the Nop56p-associated prerRNA complex presumably a complex involved in ribosomal biogenesis (Hayano et al., 2003), and cytoplasmic ribosome. The KEGG analysis also notes that the ribosome is downregulated. Taken together these results suggest that ribavirin is influencing the ribosome and its biogenesis.

No new clusters or processes appeared in the combined analysis. The enrichment for ontologies relating to cell-cell adhesion and cadherin binding is unique to the results found with ribavirin and points to a function of ribavirin that is not related to translational inhibition. This is not unexpected, as ribavirin has been noted to have multiple cellular targets. The observation of a downregulation in cadherin binding proteins is consistent with a recent publication which suggests this may be part of the mechanism through which ribavirin can protect against viral infections, at least in combination with an interferon treatment (Rendón-Huerta et al., 2013).
5.3.3 4E1RCat (47 hits)

Upregulated proteins and processes in response to 4E1RCat involve ribosome biogenesis, protein targeting to the ER, and RNA metabolism.

The downregulation change in response to 4E1RCat seems to be minimal, the affected processes involve the mitochondrial proteins HADHB (involved in beta-oxidation) (Naiki et al., 2014), ATAD3A (a heavily expressed mitochondrial membrane protein involved in nucleoid organisation, and various aspects of growth and metabolism) (Li & Rousseau, 2012), and TFAM (a mitochondrial transcription factor) (Litonin et al., 2010). Also affected is the eukaryotic replisome, a complex required for rapid and accurate chromosome replication (Yeeles, Janska, Early, & Diffley, 2017).

The CORUM analysis pulls out three clusters of which the cytoplasmic ribosome and 60S ribosomal subunit clusters are nested ontological terms. This means that the 60S ribosomal cluster term contributes to the significance of the cytoplasmic ribosome term. Also, in this analysis is the Nop56p-associated pre-rRNA complex, as noted for ribavarin. The CORUM analysis indicates effects on ribosome biogenesis. The KEGG analysis confirms this with ribosome being the only process identified. The effects on mitochondrial proteins and processes and effects on eukaryotic replication were unexpected. No additional insights were obtained through the combined analysis.
Comparisons between treatments

The number of hits aligns with the expectations from the drugs in terms of molecular specificity within the cell. Ribavirin, the drug with the most expected interactions within the cell (1.2.4.2), has the largest number of hits, whereas 4E1RCat, with only two molecular interactions (1.2.4.1), has the least hits. Cycloheximide is middling. This may be superficial, but it is interesting to note, nonetheless. Unique to the 4E1RCat treatment is the downregulation of mitochondrial nucleoid proteins and the eukaryotic replisome. For the ribavirin treatment, upregulation of cell adhesion molecules and proteins associated with the cell adhesion process is also unique. It is noticeable that responses to ribavirin are in the opposite direction to those observed in other treatments. For example, the decrease in ribosome and ribosome biogenesis is in contrast to the increase in proteins associated with these ontologies found for cycloheximide and 4E1Rcat. A trend of converse regulation between ribavirin and 4E1RCat, protein targeting to the ER is a downregulated process in the RBV treatment and an upregulated process in the 4E1RCat treatment. In the same vein proteosomal proteins and processes are seen in the cycloheximide and RBV, however in cycloheximide they are downregulated and upregulated in ribavirin this process is upregulated. Finally, RNA metabolism is another process that is downregulated in the RBV treatment but upregulated in the 4E1RCat treatment.
5.3.5 DMSO (364 hits)
The intention of the DMSO treatment was as a control, and so it is not a treatment aligned with the aims and objectives of this thesis. It was required, due to the low solubility and low activity of 4E1Rcat, which combined to require a high DMSO concentration in its treatment. However, the sheer strength of the response to 1% DMSO in the HT29 cells is intriguing. The 1% DMSO treatment condition roughly corresponds to a growth inhibition of between 30-40%. In the context of this study this would be considered a medium-high dose treatment, which explains the magnitude of hits seen in the DMSO condition. I strongly think that 4E1Rcat is having a molecular effect on the cells. Observation of cell pellet sizes post-drug treatment indicates that 4E1Rcat is contributing to lowering proliferation, as cells in the DMSO control run alongside 4E1Rcat had noticeably smaller pellets, which were stained red interestingly (4E1Rcat is red in solution). Although appropriate DMSO controls were run in tandem with the 4E1Rcat treatment it may not be possible to deconvolute the effects that DMSO is exerting on the proteome, it may be possible that effects we see in the 4E1Rcat condition have been tweaked by the action of DMSO. Although it is possible to overlay the proteomic effects of the treatment and control and subtract the DMSO controls’ proteomic effects from those exerted by 4E1Rcat. However, this might not be convincingly possible, it may be that some of the effects seen in the 4E1Rcat treatment are a result of the combined efforts of the two compounds. The decision to analyse the DMSO condition proved worthwhile as some of the processes affected by such a general toxic agent appeared in the treatment conditions by compounds that were expected to be far more selective comparatively.

DMSO has wide-ranging effects on cells, upregulated processes ranked by significance include targeting to the ER (localisation and targeting), translation (initiation, peptide biosynthesis and ribosome biogenesis), RNA catabolism (cellular nitrogen catabolism, aromatic compound catabolism, nuclear base-containing compound catabolism and heterocycle catabolism). Some minor upregulated processes include DNA conformation change and energy metabolism.

The processes that respond to DMSO through protein downregulation include RNA metabolism, translation, splicing, protein folding and exocytosis.

DMSO is likely to have general toxic effects on the cell, perhaps interestingly in the context of the other treatments, a high proportion of the processes seen in the other treatments are also seen in the DMSO condition. Localisation to the ER, effects on translation, RNA metabolism and catabolism, splicing and minor effects on energy metabolism are seen on aggregate in the three treatment conditions. In this sense, DMSO appears to be delivering responses typical of a translation inhibitor. This was an unexpected finding, and not anticipated at the outset of the study. However, investigation of the literature revealed a 2019 paper which investigates the effects of DMSO on cells (Verheijen et al., 2018). The study included a proteomic analysis, for which data (presented in the supplementary information Tables 3 and 4) is consistent with the results presented here. Although analysed through a different process, focusing on pathways from the ConsensusPathDB with the Reactome database, the proteomic data shows exceptional q-values (down to $10^{-49}$) associated with translation and the ribosome, making these the strongest pathway hits in their proteomic study.
Figure 5-7 STRING diagram of upregulated proteins expression change in response to treatment with DMSO at 1%, equivalent the concentration in the 4E1RCat top.

Figure 5-8 STRING diagram of downregulated proteins expression change in response to treatment with DMSO at 1%, equivalent the concentration in the 4E1RCat top dose.
5.3.6 Summary of insights from GO and STRING analysis of treatments

In summary, the drug treatment effects on the HT29 cells largely result in ontological enrichments related to processes that they are the directly targets of the treatments. In terms of named processes, the treatments caused many of the same effects, which include ribosomal processes, splicing, protein degradation, targeting to the ER and energy metabolism. It seems highly likely that proteomic outcomes in response to each compound are a direct result of the perturbation of the molecular process in combination with general toxic responses to treatment and the subsequent effects on the cell. Some unique responses do appear in the ribavirin treatment in the form of effects on cell adhesion and expression of cadherins in ribavirin. Likewise, in the 4E1RCat treatment condition mitochondrial nucleoid and the appearance of the eukaryotic replisome are unique. The revelation that DMSO causes cellular responses that are consistent with protein synthesis was unexpected. Furthermore, it brings into doubt the proteome responses observed in response to 4E1Rcat. This compound required high concentrations of DMSO to solubilise it at a concentration of 1% (v/v) DMSO sufficient to cause a growth defect in the HT29 cells at. Even though the up-and down-regulated proteins associated with the 4E1Rcat treatment have been determined in relation to the DMSO control, it is difficult to confidently rule out that noted enrichments might arise from experimental artifacts. Conversely, the low number of varying proteins and enriched proteins found with the 4E1Rcat treatment may arise simply because 4E1Rcat-induced changes are being masked by the cellular response to DMSO.

5.4 General discussion

5.4.1 The purpose and power of a whole-proteome experiment

Mass spectrometry is an excellent tool for investigating protein expression at a global level and prospecting for interesting biological outcomes and generation of hypotheses to further explore and validate using molecular biology techniques. Generally, biases and enrichment from sample handling and data analysis are minimised to ensure that any enrichment noticed in a given proteomics experiment are a consequence of biological activities and processes, rather than a consequence of a given method of protein isolation and analysis. Proteomics often begins without a prior hypothesis in mind and is a prospective analysis tool. Therefore, the software we use to analyse our spectral data and the methods of cell lysis and protein precipitation are likely to bias the analysis. However, these biases can be mitigated by a thorough understanding of the analytical tools, and the enrichment outcomes of a given wet-lab methodology. An example of a false enrichment discovery by members of our lab group involved noticing that treatments from HT29 and HL60 cells treated at IC10 values with PatA were heavily enriched for extracellular exosomes. Although initially interesting, retrospectively the extraction methods removed many membrane-bound proteins and enriched for cytosolic proteins; extracellular exosomes are derived from the membrane and cytosolic components, which could explain this enrichment.

5.4.2 The significance of ‘low dose’

Previous research suggests that PatA has its anti-cachectic effects at low doses, well below anti-tumour dosing. Since the nature of PatA’s anti-cachectic effects are still poorly understood and may involve the eIF4F complex, it is appropriate when targeting another component of the same complex to treat it at the same dosing scheme as this will help clarify whether it is low dose eIF4A inhibition mediating the effects, or whether the complex as a whole plays a role in cachectic reversal.

5.4.3 4E1RCat and separating the DMSO effects from drug treatment effects

The effects of compounds on proliferative or metabolic activity of human cancer cell lines is a way to establish the potency of a drug. Cell lines have variable responses to the anti-proliferative effects of
a given treatment. The MTT assay is a mainstay in drug discovery, the principle of this assay is the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (yellow) to its corresponding formazan (purple) by actively metabolising cells. This assay was used extensively in this thesis as a tool to assay the anti-proliferative effects of compounds selected for this study. Dimethyl sulfoxide (DMSO), the solvent used to reconstitute the drugs used in this thesis has demonstrable anti-proliferative effects. Data was collected to establish the effects of the vehicle on the cell lines used. DMSO alone was shown to have an IC₅₀ of 0.151% (v/v in buffer) in HT29 cells and 0.621% (v/v in buffer) in HL60 cells. DMSO controls were included in the analysis to address the effects DMSO has on cells. A concentration of 0.1% (v/v in buffer) has been established as having negligible cytotoxic effects, evidenced by the literature (Timm, Saaby, Moesby, & Hansen, 2013) and backed by MTT assays performed over the course of this thesis. DMSO controls were only relevant to the 4E1RCat treatment conditions, where the 100-fold drop in concentration from the stock concentration in 100% DMSO to 1% DMSO in the top dose, which corresponds to a ~IC₅₀ treatment, is not enough of a dilution to avoid the cytotoxic effects of DMSO. For CYCLOHEXIMIDE and ribavirin, the ability to dissolve these drugs at 50 mM and their cytotoxicity being significantly higher than 4E1RCat mean that in the process of diluting the drugs to their appropriate IC₅₀ values in treatment conditions, the DMSO concentration is low enough to not warrant controlling for the effects of DMSO. As an example, the IC₅₀ of ribavirin in HT29 cells of 11.1 µM is more than 1000-fold lower than the 50 mM starting stock concentration. Cycloheximide with an IC₅₀ of 0.0427 µM (42.7 nM) is also below 1000-fold more dilute than the starting stock. The IC₅₀ of DMSO generated from an assay of HT29 cells treated with DMSO was 0.4% by volume. This contrasts with the top dose of 4E1RCat treatment, which was selected as the ~IC₅₀ treatment value which has a DMSO concentration of 1%. These findings conflict, as by my treatment data an IC₅₀ for DMSO treatment is at a lower percentage than used in the top dose in the 4E1RCat treatment condition used as an IC₅₀. One could interpret this as 4E1RCat being protective against the effects of DMSO, however, this is unlikely. Another interpretation is that since the 4E1RCat data was assayed on six separate occasions, that it is the more reliable collection of data. Whereas the dose response to DMSO, was initially completed as an exploratory experiment and subsequently an IC₅₀ was generated. The assay was only replicated two times, if replicated a few more times the IC₅₀ may shift. Over the course of this project, it has been repeatedly noted that 4E1RCat is not particularly cytotoxic to cells (see Figure 4-3). When dissolve 4E1RCat, significant solubility issues were encountered, consistent with some online resources which showed conflicting solubility values for dissolution in DMSO. Efforts to dissolve the drug by sonication were unsuccessful, the drug eventually became soluble at a concentration of 4 mM, about 10-fold lower than the 50 mM stocks of RBV and CYCLOHEXIMIDE. Early attempts to solubilise at higher concentrations were motivated by the low cytotoxicity expected from 4E1RCat. These concerns manifested in the form of a struggle to get 4E1RCat to kill cells even at the top dose of 40 µM, at this dose the DMSO concentration is 1% making it hard to determine whether 4E1RCat is having any effects or whether the effects of DMSO are being observed. RBV by contrast was soluble up to 180 mM and demonstrated by dose response curves to be significantly more cytotoxic by comparison. As mentioned in the introduction 4E1RCat only has 2 molecular interactions, with 4EBP and eIF4E, in this sense it is pharmacologically ‘clean’ (1.2.4.1). However, due to its low cytotoxicity (see Table 1) and low solubility (see 4.2), the approximate IC₅₀ value is influenced by the DMSO vehicle which is at 1%, this may confound results. The appropriate DMSO controls have been generated in response to the conditions necessary for 4E1RCat to be useable as a treatment. Despite this, we may expect that 4E1RCat will have a small uniform lowering effect on protein synthesis in line with its molecular function as an eIF4E inhibitor.

5.4.4 Ribavirin, a ‘dirty’ drug with multiple interactions
It has been noted in the literature that Ribavirin, a commercially available clinical drug, has a suite of molecular interactions at a range of concentrations. Ribavirin mainly targets enzymes involved in metabolism, but also polymerases, consistent with its status as a guanosine mimic. This is further
evidenced by its ability to be misincorporated into viral mRNA at millimiolar concentrations by viral RNA-dependent RNA polymerases, Crotty et al suggest that the effect of this on viral replication is an increase in mutation rates in response to misincorporation of ribavirin into the viral genome to a catastrophic level, however a caveat to this is that the researchers used ribavirin at 20 times higher a concentration than clinically relevant doses, at 100 µM (Crotty, Cameron, & Andino, 2001). This interaction with RNA-dependent RNA polymerase is not the central mediator of its broad anti-viral activity. This has been attributed to inhibitory effects on inosine monophosphate dehydrogenase (IMPDH) by ribavirin 5'-monophosphate, leading to depletion of intracellular pools of GTP (Leyssen, Clercq, & Neyts, 2006). Strong evidence for this model of interfering with viral replication, is that treatment with ribavirin results in a 4-log reduction in viral RNAs which can be partially reversed by the addition of guanosine. Researchers noted, interestingly, that despite suspected involvement of IMPDH in ribavirin’s anti-viral activity, mycophenolic acid, another IMPDH inhibitor had no anti-viral effect (Lanford et al., 2002). As mentioned previously (1.2.4.2), despite conflicting findings (Westman et al., 2005; Yan et al., 2005), ribavirin has been confirmed to interact with eIF4E at micromolar concentrations (Kentsis et al., 2005, 2004). The conflicting results were suggested to be a result of free-eIF4E (often referred to as apo-eIF4E) being sensitive to structural changes at pH changes between 7.5-8. Some backing for this is the observation by Westman et al (Westman et al., 2005) that ribavirin bound to eIF4E at 2-4 orders of magnitude lower than originally reported by Kentsis et al (Westman et al., 2005), suggesting that buffer conditions in the conflicting papers could be lowering the capacity of eIF4E to bind ribavirin. Ribavirin is unlikely to exert its molecular effects in its unmetabolized form, ribavirin is metabolised in the liver and intracellularly. Since the intracellular metabolism is the form relevant to a cell culture, this will be the focus. Ribavirin is the substrate of adenosine kinase which converts ribavirin into ribavirin monophosphate (RMP), which in turn is the substrate of monophosphate and diphosphate kinases which respectively dephosphorylate, and triphosphorylate RMP into ribavirin triphosphate (RTP). In most cell types RTP dominates at concentrations 20-100 times that of RMP (T. Page & Connor, 1990). Viruses function within the cell and the cellular machinery is co-opted for their replicative processes, thus the relevance of such an in-depth assessment of ribavirin’s mode of action in an anti-viral setting is relevant to the effects it may have on cellular processes. With such a wide variety of molecular targets at a variety of concentrations, it is important to acknowledge this when treating cells, as the effects on the proteome may be confounded by the other interactions within the cell. The effects of ribavirin are slightly less certain in comparison to cycloheximide or 4E1RCat due to the variety of molecular targets it is known to interact with, refer to section 1.2.4.2. We expected that there would be a general lowering effect on protein synthesis as some proportion of eIF4E will be competitively inhibited by ribavirin. There may be other effects as ribavirin lowers the GTP pool within the cell which may lead to a cellular response for guanosine producing enzymes or enzymes involved in generating or using GTP. Although other processes, such as those associated with cell-cell adhesion, were found, the relatively clean ontological enrichment profile of ribavirin for processes associated with translation validate its potential for studies of this kind.

5.4.5 Cycloheximide – control protein synthesis inhibitor

Cycloheximide is a eukaryotic protein synthesis inhibitor used extensively in molecular biology to stop the cellular production of protein. Cycloheximide was selected as a control compound due to its known protein synthesis inhibition by blocking translation at the ribosome (Schneider-Poetsch et al., 2010). More importantly cycloheximide inhibits all protein synthesis in a non-selective manner, thus it was an appropriate tool to use as a positive control in these experiments as the goal is to tease out any specific effects of eIF4E inhibitors from the change in the proteome that could be a result of a biological response to translation stress, giving a skewed or selective effect on protein expression.
5.4.6 Regulatory network influence on translational output

It is not apparent that the magnitude of change any individual protein can make to a cell is in proportion to its abundance. In fact, the opposite can be true. If you take a set of 100 proteins randomly selected from a proteome, it is likely that some of these proteins will have structural, enzymatic, or regulatory roles within the cell. However, the majority of the proteins in which change is detected are likely to be high abundance structural and enzymatic proteins. Transcription factors are the most extreme example of a proteins with extremely low abundance but correspondingly extreme effects on gene expression and protein synthesis in the cell in response to growth factors and the cellular stresses within an environment. Transcription factors, despite being encoded by 6% of the genome (Barabási, Gulbahce, & Loscalzo, 2011) and so the second largest group of genes, are generally the lowest in abundance by far. Evidence of this being that only 5% of all TFs have been purified and characterised (Ngagore et al., 2013).

Whilst transcription factors provide one example of how an abundance change in a protein can cause broad change within a cell, they are not alone in having this property. Barabási et al describe non-transcription factor related abnormalities in an insightful way - “The impact of a specific genetic abnormality is not restricted to the activity of the gene product that carries it, but can spread along the links of the network and alter the activity of gene products that otherwise carry no defects.” (Barabási, Gulbahce, & Loscalzo, 2011). This insight can be extended to the outcome of translational inhibition. For example, pateamine A is suspected to give selective translation inhibition of transcripts with specific 5' UTR structures in a manner similar to rocaglates (Iwasaki et al., 2016). Due to the nature of cellular regulatory networks, if the translationally repressed gene product is part of a network – and especially if it is a key regulator – its down-regulation can have flow on effects on protein expression lower down in the pathway. This observation can be used to potentially trace the effects of a treatment and its effects on proteins that are identified through a proteomics experiment and potentially finding a regulator, such as a transcription factor, or set of processes responsible for the changes seen in protein expression between treated and untreated cells.

5.4.7 The search for 5’ UTR motifs

It has been observed that motifs in the 5’ UTR sequences are often involved in mRNA regulation. This can happen in two ways. In the first, the sequence itself is a recognisable element that can be bound by a regulatory protein that can either increase or decrease some aspect of expression of these mRNAs. An example of this is the 5’-Terminal Oligopyrimidine tracts (TOP) motif present in 30% of transcripts in actively growing mammalian cells (Pichon et al., 2012), the TCT motif is also a requirement for transcription for the majority of the TOP containing mRNAs (Pichon et al., 2012).

The second way is by the motif generating a higher order secondary structure that indirectly interferes with translation by enforcing a requirement for helicase activity prior to expression, as mentioned in the introduction (see 1.2.1). Even for a transcript with a 5’ UTR with minimal secondary structure, translation can be severely inhibited if eIF4A is not present. Although the intention initially was to explore 5’ UTR motifs to see if there were transcripts that respond to eIF4E treatment in the manner observed with pateamine A (previous work by our lab group) and presumably part of the puzzle that explains why pateamine A can rescue cachexia at low doses. We have not ruled out that there are eIF4E responsive transcripts. At a glance our eIF4E treatment data does not present enough proteins that cannot be explained by a direct biological response effects of ribavirin and 4E1RCat treatment. In terms of running motif enrichment analyses, statistically speaking small numbers of proteins, and their transcripts are likely to generate false leads by way of randomness. We have not been exhaustive in our separation of biological responder transcripts and eIF4E inhibitor sensitive transcripts on the current analyses approach in the interest of time.
However, it is in many ways justified as a replacement drug for 4E1RCat would need to be found and a higher dose of ribavirin will need to be used to elicit a greater proteome response to justify running a series of motif enrichment analyses. As it currently stands, we do not have enough data to deny or confirm the existence of eIF4E inhibition sensitive transcripts.

5.5 Conclusion

The findings in this thesis are unable to meaningfully test the notion that low dose eIF4E inhibition has selective effects that mirror outcomes from pateamine A treatment. This arises because the eIF4E inhibitors used in this study each have problems associated with their use. Ribavirin has a suite of molecular interactions within the cell which can cloud the interpretation of the proteomic outcome in response to treatment. The major problem was the low efficacy of 4E1RCat and its overlapping proteome response with the DMSO vehicle. The 4E1RCat was chosen due to its reported selectivity for eIF4E inhibition, and it was intended to use this to aid abstraction of eIF4E-mediated effects from the proteome response of the polypharmacological ribavirin. Together, these create uncertainty in interpreting the proteome responses observed as being specific to eIF4E inhibition. However, the ontological enrichments observed with ribavirin do support its use in ongoing studies. Furthermore, many of the proteins observed to decrease in abundance in response to eIF4E inhibitors appear to be associated with the direct biological response to the known effects of the inhibitors, such as their effect on translation, as revealed by GO term and co-expression analysis. Relatively few protein changes are left to be explained in terms of translational repression based solely on a 5’UTR sequence motif. This small number coupled with the uncertainty of the origin of their translational repression led to the conclusion that sequence motif analysis would not be meaningful. The major outcomes of this work are, cycloheximide, ribavirin and 4E1RCat exert effects on the proteome consistent with their classification as translation inhibitors. An interesting finding comes in the form of DMSO our solvent of choice’s effects on translation, seemingly consistent with a translation inhibitor at a growth inhibition of 30-40%. Although there are a few examples of proteins and protein and protein clusters that may not be easily explained by a direct biological response of the cell line to perturbation by the treatments applied in this thesis. There is not enough information to ruled out the existence of eIF4E sensitive transcripts. This study has paved the way for future experimentation with higher doses of ribavirin and a replacement inhibitor for 4E1RCat or another strategy to disentangle the effects of 4E1RCat from the DMSO vehicle it is dissolved in.

5.6 Future directions

Due to time restraints treatment data was obtained only in the HT29 cell line. The continuation of this project would involve confirming whether the observations seen in HT29 cells in response to treatment are mirrored in other cell lines. A mirror experiment in the HL60 cell line has been performed and these cells will be processed and run on the mass spectrometer to see if the data supports the findings seen in the HT29 cells, an experiment that unfortunately could not be accommodated in the timeline of this thesis due to instrumental down-time. Although initially the intention was to quantify proteins using both TIC and iBAQ, in the interest of time TIC was used exclusively for protein quantification. Further research could make use of the robust iBAQ method and see if any new proteins appear in the analyses or other proteins disappear. The use of more potent or selective eIF4E inhibitors would be an ideal way to confirm these results, especially if this allows avoidance of a solvent vehicle control such as DMSO. This may not be possible however, as 4E1RCat and another commonly used inhibitor, 4EGI-1, have comparable solubility. In fact, 4E1RCat seems to have the edge in this department. Based on the literature, 4E1RCat is the more frequently used of the two, and unfortunately alternative direct eIF4E inhibitors may not yet exist. Another possibility, if it is impossible to find a stronger eIF4E inhibitor would be to repeat the IC_{10} treatments with DMSO added to the cycloheximide and ribavirin treatments and perhaps pateamine A as well to
see if the proteomic effects of DMSO can be convincingly subtracted from a treatment. It would be worthwhile investigating the effects of ribavirin at a higher concentration to see if sufficient proteins can be downregulated, which fall outside the direct biological response to the translational stress, to investigate 5’ UTR motifs associated with reduction in protein expression. Future research could also test the effects of hippuristanol or other available eIF4A inhibitors on the proteome response to see if it matches the proteomic effects of pateamine A in HT29 and HL60 cells. Another possible avenue to explore could involve replicating the effects on muscle fibers, the muscle fibers responding to pateamine A could be subjected to a proteomic analysis to gather more information about the specific effects of pateamine A on the proteome of muscle cells, ideally from in vivo studies, and compared to the effect of ribavirin or another eIF4E inhibitor.
6 Appendices

Top dose = 500µM (CHX and RBV), 40µM (4E1RCat)

![Plate plan example](image)

Figure 6-1 Plate plan example done in triplicate following a half-log (3.16) serial dilution scheme. DMSO controls were included at concentrations where DMSO is expected to influence cell growth. A media only blank and cell-only control are also included.

![Figure 6-2](image)

Figure 6-2 Shown at 10x magnification. Healthy cells from the original stock from 1999, after 1 passage. Note the propensity of the cell line to form colonies, also note the healthy look and regularity of the glandular structures.
Figure 6-3 Shown at 10x magnification, HT29 cells grown in depleted acidic media at high confluence for ~1 week. Note, that the glandular structures are becoming jagged looking and the dead cells in suspension.

Figure 6-4 40x magnification - a closer look at cells from the same flask as figure 6-3. Note the debris and apoptotic blebs in the environment surrounding the cells.
Figure 6-5 100x magnification - a closer look at cells from the same flask as figure 6-3. Note the number of apoptotic blebs in the environment surrounding the cells.
Figure 6-7 The effects of cell seeding density on proliferative signal from the MTT assay.

Figure 6-6 A comparison of the proliferative effects of cycloheximide on HT29 and HL60 cells. Note how in HT29 cells the graph does not level out at 0, whereas the HL60 cells do.
Figure 6-8 Relating to section 4.7.2, three biological replicates are displayed for the untreated controls, cycloheximide and ribavirin. Displayed in the left panels is the unfiltered LC-MS² chromatogram, in the right panels the base peaks are isolated from the unfiltered data.
Figure 6-9 Relating to section 4.7.3, three biological replicates are displayed for 4E1RCat and the DMSO control. Displayed in the left panels is the unfiltered LC-MS² chromatogram, in the right panels the base peaks are isolated from the unfiltered data.
### Table 7: Proteins responding to treatment with cycloheximide.

| Identified Proteins                                                                 | T-Test (p-Value) | Up or Down regulated | Identified Proteins                                                                 | T-Test (p-Value) | Up or Down regulated |
|----------------------------------------------------------------------------------|------------------|----------------------|-------------------------------------------------------------------------------------|------------------|----------------------|
| PSME2 protein OS=Homo sapiens GN=PSME2 PE=2 SV=1                                | 0.048            | Down                 | Cluster of Sialic acid synthase OS=Homo sapiens GN=NANS PE=1 SV=2 (SIAS_HUMAN)     | 0.045            | Up                   |
| Cluster of cDNA FLJ54047, highly similar to Alpha-1 catenin (Cadherin-associated protein) OS=Homo sapiens PE=2 SV=1 (B4E2G8_HUMAN) | 0.048            | Down                 | Testicular tissue protein Li 27 OS=Homo sapiens PE=2 SV=1                           | 0.039            | Up                   |
| SUMO-conjugating enzyme OS=Homo sapiens PE=2 SV=1                               | 0.047            | Down                 | cDNA FLJ76092, highly similar to Homo sapiens 5'-nucleotidase, cytosolic II-like 1 (NT5C2L1), mRNA OS=Homo sapiens PE=2 SV=1 | 0.034            | Up                   |
| Endoribonuclease LACTB2 OS=Homo sapiens GN=LACTB2 PE=1 SV=2                      | 0.046            | Down                 | Cluster of 26S protease regulatory subunit 6A OS=Homo sapiens GN=PSMC3 PE=1 SV=1 (E9PM69_HUMAN) | 0.033            | Up                   |
| Ubiquitin-conjugating enzyme E2 K OS=Homo sapiens GN=UBE2K PE=1 SV=3             | 0.046            | Down                 | Cluster of Epididymis secretory protein Li 21 OS=Homo sapiens GN=HEL-S-21 PE=2 SV=1 (V9HWG9_HUMAN) | 0.032            | Up                   |
| Cluster of Ubiquitin thioesterase OS=Homo sapiens PE=2 SV=1 (B4DPDS5_HUMAN)      | 0.044            | Down                 | Vacuolar protein sorting-associated protein 29 OS=Homo sapiens GN=VPS29 PE=1 SV=1 | 0.03             | Up                   |
| MARCKS-related protein OS=Homo sapiens GN=MARCKSL1 PE=1 SV=2                    | 0.036            | Down                 | 39S ribosomal protein L39, mitochondrial OS=Homo sapiens GN=MRPL39 PE=1 SV=3         | 0.028            | Up                   |
| Cluster of cDNA PSEC0016 fis, clone NT2RM1001076, highly similar to Procollagen-lysine,2-oxoglutarate 5-dioxygenase 3 (EC 1.14.11.4) OS=Homo | 0.036            | Down                 | Cluster of Serine/threonine-protein phosphatase PP1-alpha catalytic subunit OS=Homo sapiens GN=PPP1CA PE=1 SV=1 (PP1A_HUMAN) | 0.028            | Up                   |
| Gene Name | Description | Expression | Fold Change | p-value |
|-----------|-------------|------------|-------------|----------|
| Cluster of cDNA FLJ55694, highly similar to Dipeptidyl-peptidase 1 (EC 3.4.14.1) OS=Homo sapiens PE=2 SV=1 (B4DJQ8_HUMAN) | RNA binding protein (Autoantigenic, hnRNP-associated with lethal yellow) long isoform variant (Fragment) OS=Homo sapiens GN=RALY PE=1 SV=1 | Down | 0.034 | 0.027 | Up |
| Splicing factor 3B subunit 3 OS=Homo sapiens GN=SF3B3 PE=1 SV=4 | Cluster of Small nuclear ribonucleoprotein-associated protein N OS=Homo sapiens GN=SNRPN PE=1 SV=1 (RSMN_HUMAN) | Down | 0.031 | 0.026 | Up |
| Synaptic vesicle membrane protein VAT-1 homolog OS=Homo sapiens GN=VAT1 PE=1 SV=2 | Cluster of Protein canopy homolog 2 OS=Homo sapiens GN=CNPY2 PE=1 SV=1 (CNPY2_HUMAN) | Down | 0.031 | 0.025 | Up |
| Cluster of Spermine synthase OS=Homo sapiens GN=SMS PE=1 SV=2 (SPSY_HUMAN) | Serpin B6 OS=Homo sapiens GN=SERPINB6 PE=1 SV=1 | Down | 0.03 | 0.017 | Up |
| L-amino adipate-semialdehyde dehydrogenase-phosphopantetheinyl transferase OS=Homo sapiens GN=AASDHPPT PE=1 SV=2 | 40S ribosomal protein S12 OS=Homo sapiens GN=RPS12 PE=1 SV=3 | Down | 0.026 | 0.017 | Up |
| Cluster of Proteasome subunit alpha type OS=Homo sapiens GN=PSMA6 PE=1 SV=1 (G3V295_HUMAN) | ATP synthase subunit gamma OS=Homo sapiens PE=2 SV=1 | Down | 0.026 | 0.015 | Up |
| Cluster of DNA replication licensing factor MCM4 OS=Homo sapiens GN=MCM4 PE=1 SV=5 (MCM4_HUMAN) | cDNA FLJ39996 fis, clone STOMA2002166, highly similar to Splicing factor 3B subunit 4 OS=Homo sapiens PE=2 SV=1 | Down | 0.024 | 0.015 | Up |
| Cluster of Epididymis secretory protein Li 71 OS=Homo sapiens GN=HEL-S-71 PE=2 SV=1 (V9HW41_HUMAN) | Stathmin OS=Homo sapiens GN=STMN1 PE=2 SV=1 | Down | 0.022 | 0.014 | Up |
| Cluster of cDNA FLJ76962, highly similar | Eukaryotic translation initiation factor 3 subunit | Down | 0.021 | 0.013 | Up |
| Gene Name                                                                 | OS          | GN          | PE | SV | Fold Change | Status |
|--------------------------------------------------------------------------|-------------|-------------|----|----|-------------|--------|
| to Homo sapiens nucleolar protein 5A (56kDa with KKE/D repeat) (NOL5A), mRNA | Homo sapiens | E8K9K6_HUMAN | 2  | 1  | 0.02        | Down   |
| Similar to NADH dehydrogenase (Ubiquinone) 1 alpha subcomplex, 9 (39kD) | Homo sapiens | EIF3M       | 1  | 1  | 0.02        | Up     |
| Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1                   | Homo sapiens | PIN1        | 1  | 1  | 0.016       | Down   |
| Hsp70-binding protein 1                                                  | Homo sapiens | HSPBP1      | 1  | 1  | 0.015       | Down   |
| Cluster of DDAH2                                                        | Homo sapiens | HEL-S277    | 1  | 1  | 0.015       | Down   |
| Cluster of Elongation factor 1-alpha 2                                   | Homo sapiens | EEF1A2      | 1  | 1  | 0.015       | Down   |
| E3 ubiquitin-protein ligase CHIP                                          | Homo sapiens | STUB1       | 1  | 1  | 0.015       | Down   |
| Synaptogyrin-2 (Fragment)                                                | Homo sapiens | SYNGR2      | 1  | 1  | 0.014       | Down   |
| DNA replication licensing factor MCM6                                    | Homo sapiens | MCM6        | 1  | 1  | 0.013       | Down   |
| Gene Name and Description | Expression | Change | Description |
|---------------------------|------------|--------|-------------|
| Cluster of Epididymis luminal protein 220 OS=Homo sapiens GN=HEL-S-70 PE=2 SV=1 (V9HW80_HUMAN) | 0.013 | Down | Peroxiredoxin-5, mitochondrial OS=Homo sapiens GN=PRDX5 PE=1 SV=4 |
| Proteasome subunit beta type OS=Homo sapiens PE=2 SV=1 | 0.013 | Down | Cluster of RNA-binding motif protein, X chromosome OS=Homo sapiens GN=RBMX PE=1 SV=3 (RBMX_HUMAN) |
| Splicing factor 3B subunit 5 OS=Homo sapiens GN=SF3B5 PE=1 SV=1 | 0.01 | Down | cDNA FLJ90381 fis, clone NT2RP2005035, highly similar to Calumenin OS=Homo sapiens PE=2 SV=1 |
| Cluster of Epiplakin OS=Homo sapiens GN=EPPK1 PE=1 SV=1 (A0A087X1U6_HUMAN) | 0.01 | Down | Asparagine--tRNA ligase, cytoplasmic OS=Homo sapiens GN=NARS PE=1 SV=1 |
| UPF1 regulator of nonsense transcripts homolog (Yeast), isoform CRA_b OS=Homo sapiens GN=UPF1 PE=4 SV=1 | 0.0087 | Down | Aspartate aminotransferase OS=Homo sapiens PE=2 SV=1 |
| Cluster of Glutamate dehydrogenase OS=Homo sapiens PE=2 SV=1 (B4DMFS_HUMAN) | 0.0083 | Down | Glyoxylate reductase/hydroxyacyl and reductase OS=Homo sapiens GN=GRHPR PE=1 SV=1 |
| 40S ribosomal protein S10 OS=Homo sapiens GN=RPS10 PE=1 SV=1 | 0.0075 | Down | Dynein heavy chain 14, axonemal OS=Homo sapiens GN=DNAH14 PE=2 SV=3 |
| S- (hydroxymethyl)glutathione dehydrogenase OS=Homo sapiens GN=ADH5 PE=2 SV=1 | 0.0073 | Down | |
| Aminocyclase-1 OS=Homo sapiens GN=ACY1 PE=4 SV=1 | 0.0065 | Down | |
| Phosphomannomutase OS=Homo sapiens GN=PMM2 PE=1 SV=1 | 0.0059 | Down | |
| Protein Description                                                                 | p-value | Status |
|------------------------------------------------------------------------------------|---------|--------|
| Fatty acid synthase OS=Homo sapiens GN=FASN PE=1 SV=3                              | 0.0012  | Down   |
| Cluster of BUB3-interacting and GLEBS motif-containing protein ZNF207 OS=Homo sapiens GN=ZNF207 PE=1 SV=1 (J3QRS9_HUMAN) | 0.0003  | Down   |
Table 8 Proteins responding to treatment with ribavirin.

| Identified Proteins                                                                 | T-Test (p-Value) | Up or Down regulated | Identified Proteins                                                                 | T-Test (p-Value) | Up or Down regulated |
|-------------------------------------------------------------------------------------|------------------|----------------------|-------------------------------------------------------------------------------------|------------------|----------------------|
| Cluster of Histone H2B type 1-J OS=Homo sapiens GN=HIST1H2BJ PE=1 SV=3 (H2B1J_HUMAN) | 0.0067           | Down                 | Ribosomal L1 domain-containing protein 1 OS=Homo sapiens GN=RSL1D1 PE=1 SV=3        | 0.0087           | Down                 |
| Cluster of Epididymis luminal protein 220 OS=Homo sapiens GN=HEL-S-70 PE=2 SV=1 (V9HW80_HUMAN) | 0.037            | Down                 | cDNA FLJ56566, highly similar to Small glutamine-rich tetrapeptide-containing protein A OS=Homo sapiens PE=2 SV=1 | 0.046            | Down                 |
| Cluster of Probable ATP-dependent RNA helicase DDX17 OS=Homo sapiens GN=DDX17 PE=1 SV=1 (A0A1W2PQ51_HUMAN) | 0.014            | Down                 | Cluster of cDNA, FLJ92825, highly similar to Homo sapiens SAR1a gene homolog 1 (S. cerevisiae) (SARA1), mRNA OS=Homo sapiens PE=2 SV=1 (B2R679_HUMAN) | 0.021            | Down                 |
| Cluster of RPS4X protein (Fragment) OS=Homo sapiens GN=RPS4X PE=2 SV=2 (Q96IR1_HUMAN)    | 0.019            | Down                 | Cluster of NOP56 protein (Fragment) OS=Homo sapiens GN=NOP56 PE=2 SV=1 (A0PJ92_HUMAN) | 0.038            | Down                 |
| Cluster of Serine hydroxymethyltransferase, mitochondrial (Fragment) OS=Homo sapiens GN=SHMT2 PE=1 SV=1 (G3V4W5_HUMAN) | 0.00086          | Down                 | Protein dpy-30 homolog OS=Homo sapiens GN=DPY30 PE=1 SV=1                              | 0.017            | Down                 |
| Cluster of Ribosomal protein L7, isoform CRA_a OS=Homo sapiens GN=RPL7 PE=4 SV=1 (A0A024R814_HUMAN) | 0.029            | Down                 | Hypoxanthine-guanine phosphoribosyltransferase OS=Homo sapiens GN=HPRT1 PE=1 SV=2    | 0.027            | Down                 |
| Ribosomal protein L23, isoform CRA_b OS=Homo sapiens GN=RPL23 PE=3 SV=1                | 0.0075           | Down                 | DNA helicase OS=Homo sapiens GN=MCM3 PE=2 SV=1                                      | 0.0016           | Down                 |
| 40S ribosomal protein S3 OS=Homo sapiens GN=RPS3 PE=1 SV=2                             | 0.033            | Down                 | ATP synthase subunit delta, mitochondrial OS=Homo sapiens GN=ATP5D PE=1 SV=2         | 0.002            | Down                 |
| Gene Name | Description | Fold Change | Expression | Genes | Description | Fold Change | Expression |
|-----------|-------------|-------------|------------|-------|-------------|-------------|------------|
| Cluster of Epididymis secretory protein Li 71 | OS=Homo sapiens GN=HEL-S-71 PE=2 SV=1 (V9HW41_HUMAN) | 0.038 | Down | BUB3-interacting and GLEBS motif-containing protein ZNF207 | OS=Homo sapiens GN=ZNF207 PE=1 SV=1 | 1.00E-04 | Down |
| Cluster of Staphylococcal nuclease domain-containing protein 1 | OS=Homo sapiens GN=SND1 PE=1 SV=1 (SND1_HUMAN) | 0.009 | Down | cDNA FLJ57877, highly similar to Cleavage and polyadenylation specificity factor 7 | OS=Homo sapiens PE=2 SV=1 | 0.046 | Down |
| 40S ribosomal protein S3a | OS=Homo sapiens GN=RPS3A PE=2 SV=1 | 0.045 | Down | Endothelial differentiation-related factor 1 | OS=Homo sapiens GN=EDF1 PE=1 SV=1 | 0.029 | Down |
| Cluster of cDNA FLJ59240, highly similar to Far upstream element-binding protein 1 | OS=Homo sapiens PE=2 SV=1 (B4DWL1_HUMAN) | 0.014 | Down | RNA-binding protein 42 | OS=Homo sapiens GN=RBM42 PE=1 SV=1 | 0.00061 | Down |
| Cluster of Hydroxysteroid (17-beta) dehydrogenase 4, isoform CRA_b (Fragment) | OS=Homo sapiens GN=HSD17B4 PE=2 SV=1 (A0A0S2Z4J1_HUMAN) | 0.048 | Down | | | |
| 40S ribosomal protein S13 | OS=Homo sapiens GN=RPS13 PE=1 SV=2 | 0.043 | Down | Cluster of Keratin, type I cytoskeletal 18 | OS=Homo sapiens GN=KRT18 PE=1 SV=2 (K1C18_HUMAN) | 0.043 | Up |
| ATP synthase subunit O, mitochondrial | OS=Homo sapiens GN=ATP5O PE=1 SV=1 | 0.03 | Down | Cluster of Actin, alpha skeletal muscle | OS=Homo sapiens GN=ACTA1 PE=1 SV=3 (A6NL76_HUMAN) | 0.036 | Up |
| 60S ribosomal protein L10a | OS=Homo sapiens GN=RPL10A PE=1 SV=2 | 0.0051 | Down | Cluster of Annexin | OS=Homo sapiens GN=ANXA2 PE=3 SV=1 (A0A024R5Z7_HUMAN) | 0.04 | Up |
| 40S ribosomal protein S30 | OS=Homo sapiens GN=FAU PE=1 SV=1 | 0.015 | Down | Heterogeneous nuclear ribonucleoprotein K | OS=Homo sapiens GN=HNRNPK PE=1 SV=1 | 0.021 | Up |
| Gene Name | Description | OS | GN | PE | SV | Log2FoldChange | Direction | Significance |
|-----------|-------------|----|----|----|----|---------------|-----------|-------------|
| Cluster of Cyclin-dependent kinase 16 | OS=Homo sapiens GN=CDK16 PE=1 SV=1 (CDK16_HUMAN) | Homo sapiens | CDK16 | 1 | 1 | 0.014 | Down | 5 |
| 2'-5'-oligoadenylate synthetase 3, 100kDa, isoform CRA_a | OS=Homo sapiens GN=OAS3 PE=4 SV=1 | Homo sapiens | OAS3 | 4 | 1 | 0.024 | Down | 1 |
| Heterogeneous nuclear ribonucleoprotein R | OS=Homo sapiens GN=HNRNPR PE=1 SV=1 | Homo sapiens | HNRNPR | 1 | 1 | 0.043 | Down | 1 |
| Protein POF1B | OS=Homo sapiens GN=POF1B PE=1 SV=3 | Homo sapiens | POF1B | 1 | 3 | 0.012 | Down | 3 |
| Testicular tissue protein Li 75 | OS=Homo sapiens PE=2 SV=1 | Homo sapiens | L75 | 1 | 1 | 0.033 | Down | 1 |
| cDNA FLJ76387, highly similar to Homo sapiens splicing factor, arginine/serine-rich 9 (SFRS9), mRNA | OS=Homo sapiens PE=2 SV=1 | Homo sapiens | SFRS9 | 1 | 1 | 0.041 | Down | 1 |
| Cluster of DNA helicase | OS=Homo sapiens PE=2 SV=1 (B3KMX0_HUMAN) | Homo sapiens | B3KMX0 | 1 | 1 | 0.046 | Down | 1 |
| Epidermal growth factor receptor kinase substrate 8-like protein 1 | OS=Homo sapiens GN=EPS8L1 PE=1 SV=1 | Homo sapiens | EPS8L1 | 1 | 1 | 0.046 | Down | 1 |
| Small nuclear ribonucleoprotein Sm D2 | OS=Homo sapiens GN=SNRPD2 PE=1 SV=1 | Homo sapiens | SNRPD2 | 1 | 1 | 0.003 | Down | 1 |
| Prefoldin subunit 2 | OS=Homo sapiens GN=PFDN2 PE=1 SV=1 | Homo sapiens | PFDN2 | 1 | 1 | 0.047 | Down | 1 |
| Ubiquitin carboxyl-terminal hydrolase 14 | OS=Homo sapiens GN=USP14 PE=1 SV=2 | Homo sapiens | USP14 | 1 | 2 | 0.022 | Up | 1 |
| Protein S100-A6 | OS=Homo sapiens GN=S100A6 PE=1 SV=1 | Homo sapiens | S100A6 | 1 | 1 | 0.049 | Up | 1 |
| Cluster of Annexin A1 | OS=Homo sapiens GN=ANXA1 PE=1 SV=2 (ANXA1_HUMAN) | Homo sapiens | ANXA1 | 1 | 2 | 0.0009 | Up | 5 |
| Cluster of Aspartate aminotransferase | OS=Homo sapiens GN=GOT2 PE=4 SV=1 (A0A024R6W0_HUMAN) | Homo sapiens | GOT2 | 4 | 1 | 0.0081 | Up | 1 |
| Cluster of MHC class I antigen (Fragment) | OS=Homo sapiens GN=HLA-A PE=3 SV=1 (E5BB16_HUMAN) | Homo sapiens | HLA-A | 1 | 3 | 0.042 | Up | 1 |
| Cluster of Proteasome subunit alpha type | OS=Homo sapiens GN=PSMA6 PE=1 SV=1 (G3V295_HUMAN) | Homo sapiens | PSMA6 | 1 | 1 | 0.02 | Up | 1 |
| Cluster of ARP3 actin-related protein 3 homolog (Yeast), isoform CRA_a | OS=Homo sapiens GN=ACTR3 PE=3 SV=1 (A0A024RAI1_HUMAN) | Homo sapiens | ACTR3 | 1 | 3 | 0.011 | Up | 1 |
| Cluster of DNA helicase | OS=Homo sapiens PE=2 SV=1 (B3KMX0_HUMAN) | Homo sapiens | B3KMX0 | 1 | 1 | 0.046 | Down | 1 |
| Prefoldin subunit 2 | OS=Homo sapiens GN=PFDN2 PE=1 SV=1 | Homo sapiens | PFDN2 | 1 | 1 | 0.047 | Down | 1 |
| Ubiquitin carboxyl-terminal hydrolase 14 | OS=Homo sapiens GN=USP14 PE=1 SV=2 | Homo sapiens | USP14 | 1 | 2 | 0.022 | Up | 1 |
| Protein S100-A6 | OS=Homo sapiens GN=S100A6 PE=1 SV=1 | Homo sapiens | S100A6 | 1 | 1 | 0.049 | Up | 1 |
| Cluster of Annexin A1 | OS=Homo sapiens GN=ANXA1 PE=1 SV=2 (ANXA1_HUMAN) | Homo sapiens | ANXA1 | 1 | 2 | 0.0009 | Up | 5 |

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| Gene Name | Description | OS | GN | PE | SV | Regulation | Description |
|-----------|-------------|----|----|----|----|------------|-------------|
| U1 small nuclear ribonucleoprotein 70 kDa | OS=Homo sapiens GN=SNRNP70 PE=1 SV=2 | 0.022 | Down | 14-3-3 protein sigma | OS=Homo sapiens GN=SFN PE=1 SV=1 | 0.033 | Up |
| Cluster of Hydroxysteroid dehydrogenase-like protein 2 | OS=Homo sapiens GN=HSDL2 PE=1 SV=1 (HSDL2_HUMAN) | 0.0084 | Down | Cluster of Kallikrein 1 | OS=Homo sapiens GN=KLNI PE=3 SV=1 (A0A1R3UCE8_HUMAN) | 0.0086 | Up |
| ADP-sugar pyrophosphatase | OS=Homo sapiens GN=NUDT5 PE=1 SV=1 | 0.032 | Down | Ubiquitin thioesterase | OS=Homo sapiens PE=2 SV=1 | 0.01 | Up |
| Cluster of 40S ribosomal protein S6 | OS=Homo sapiens GN=RPS6 PE=2 SV=1 (Q96DV6_HUMAN) | 0.032 | Down | Cluster of Tight junction protein ZO-1 (Fragment) | OS=Homo sapiens GN=TJP1 PE=2 SV=1 (A9CQZ8_HUMAN) | 0.02 | Up |
| Septin-9 | OS=Homo sapiens GN=SEPT9 PE=1 SV=2 | 0.0077 | Down | SUMO-activating enzyme subunit 2 | OS=Homo sapiens GN=UBA2 PE=1 SV=2 | 0.043 | Up |
| Cluster of FGFR2-BICC1 fusion kinase protein | OS=Homo sapiens GN=FGFR2-BICC1 PE=2 SV=1 (V5YQU3_HUMAN) | 0.027 | Down | ATP synthase subunit gamma | OS=Homo sapiens PE=2 SV=1 | 0.0007 2 | Up |
| Cluster of UPF1 regulator of nonsense transcripts homolog (Yeast), isoform CRA_b | OS=Homo sapiens GN=UPF1 PE=4 SV=1 (A0A024R7L5_HUMAN) | 0.04 | Down | ATP-binding cassette sub-family E member 1 | OS=Homo sapiens GN=ABCE1 PE=1 SV=1 | 0.045 | Up |
| Cluster of 40S ribosomal protein S10 | OS=Homo sapiens GN=RPS10 PE=1 SV=1 (RS10_HUMAN) | 0.038 | Down | Nuclear transport factor 2 | OS=Homo sapiens GN=NUTF2 PE=1 SV=1 | 0.05 | Up |
| Ras-GTPase activating protein SH3 domain-binding protein 2, isoform CRA_a | OS=Homo sapiens GN=G3BP2 PE=4 SV=1 | 0.0082 | Down | Coatomer subunit delta | OS=Homo sapiens GN=ARCN1 PE=2 SV=1 | 0.015 | Up |
| Phosphoserine aminotransferase | OS=Homo sapiens PE=2 SV=1 | 0.0067 | Down | Tubulin-folding cofactor B (Fragment) | OS=Homo sapiens GN=TBCB PE=1 SV=8 | 0.015 | Up |
| Cluster of Pyrroline-5-carboxylate reductase | OS=Homo sapiens PE=2 SV=1 | 0.04 | Down | SEC13-like 1 isoform b variant (Fragment) | OS=Homo sapiens PE=2 SV=1 | 0.0058 | Up |
| Gene Name                                                                 | ENSEMBL ID | Fold Change | Regulation | Description                                                                                                                                                                                                 |
|--------------------------------------------------------------------------|------------|-------------|------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Endoribonuclease LACTB2                                                  | E7D7X9_HUMAN | 0.018       | Down       | Apoptosis-associated speck-like protein containing a CARD                                                                                                                                                |
| Cluster of Acetyl-CoA acetyltransferase, cytosolic variant (Fragment)    | Q59GW6_HUMAN | 0.0057      | Down       | Cluster of cDNA, FLJ93976, highly similar to Homo sapiens COP9 homolog (COP9), mRNA                                                                                                                      |
| Cluster of cDNA FLJ75871, highly similar to Homo sapiens staufen, RNA    | A8K622_HUMAN | 0.024       | Down       | Cluster of cDNA FLJ55936, highly similar to Polypyrimidine tract-binding protein 2                                                                                                                        |
| Nucleolar RNA helicase 2                                                 | DDX21      | 0.018       | Down       | 26S proteasome non-ATPase regulatory subunit 14                                                                                                                                                    |
| Cluster of Rho guanine nucleotide exchange factor 1                      | ARHG1      | 0.0012      | Down       | Putative uncharacterized protein DKFZp686B04128                                                                                                                                                    |
| Identified Proteins                                                                 | T-Test (p-Value) | Up or Down regulated | Identified Proteins                                                                 | T-Test (p-Value) | Up or Down regulated |
|-----------------------------------------------------------------------------------|------------------|----------------------|------------------------------------------------------------------------------------|------------------|----------------------|
| Cluster of Keratin, type I cytoskeletal 18 OS=Homo sapiens GN=KRT18 PE=1 SV=2 (K1C18_HUMAN) | 0.034            | Down                 | 60S ribosomal protein L8 OS=Homo sapiens GN=RPL8 PE=1 SV=2                         | 0.034            | Up                   |
| Cluster of Epididymis luminal protein 33 OS=Homo sapiens GN=HEL-S-72p PE=2 SV=1 (V9HW22_HUMAN) | 0.047            | Down                 | Cluster of Hypoxia up-regulated protein 1 OS=Homo sapiens GN=HYOU1 PE=1 SV=1 (HYOU1_HUMAN) | 0.025            | Up                   |
| Cluster of Dehydrogenase/reductase SDR family member 2, mitochondrial OS=Homo sapiens GN=DHRS2 PE=1 SV=4 (DHRS2_HUMAN) | 0.024            | Down                 | Cluster of ATP-dependent RNA helicase A OS=Homo sapiens GN=DXH9 PE=1 SV=4 (DHX9_HUMAN) | 0.05             | Up                   |
| Cluster of Titin OS=Homo sapiens GN=TTN PE=1 SV=1 (A0A0A0MTS7_HUMAN)              | 0.0014           | Down                 | Peptidyl-prolyl cis-trans isomerase FKBP4 OS=Homo sapiens GN=FKBP4 PE=1 SV=3       | 0.012            | Up                   |
| Cluster of Adenylate kinase 2, mitochondrial OS=Homo sapiens GN=AK2 PE=1 SV=2 (KAD2_HUMAN) | 0.014            | Down                 | Cluster of Bifunctional glutamate/proline--tRNA ligase OS=Homo sapiens GN=EPRS PE=1 SV=5 (SYEP_HUMAN) | 0.027            | Up                   |
| Trifunctional enzyme subunit beta, mitochondrial OS=Homo sapiens GN=HADHB PE=1 SV=3 | 0.032            | Down                 | Cluster of Proteasome subunit alpha type-1 OS=Homo sapiens GN=PSMA1 PE=1 SV=1 (PSA1_HUMAN) | 0.002            | Up                   |
| Cluster of Adenylyl cyclase-associated protein OS=Homo sapiens PE=2 SV=1 (B2RDY9_HUMAN) | 0.022            | Down                 | Cluster of Sequestosome-1 OS=Homo sapiens GN=SQSTM1 PE=1                          | 0.015            | Up                   |
| Gene Name                                                                 | SV=1 (SQSTM_HUMAN) | Up/Down | Description                                                                                           | p-Value |
|---------------------------------------------------------------------------|---------------------|---------|-------------------------------------------------------------------------------------------------------|---------|
| Cluster of ATPase family AAA domain-containing protein 3A (Fragment)      | 0.043               | Down    | cDNA FLJ75881, highly similar to Homo sapiens transferrin receptor (p90, CD71) (TFRC), mRNA OS=Homo sapiens PE=2 SV=1 | 0.013   |
| Lysozyme C OS=Homo sapiens GN=LYZ PE=1 SV=1                               | 0.022               | Down    | cDNA, FLJ93269, highly similar to Homo sapiens mitochondrial ribosomal protein L15 (MRPL15), nuclear gene encoding mitochondrial protein, mRNA OS=Homo sapiens PE=2 SV=1 | 0.044   |
| Cluster of EH-domain containing 4, isoform CRA_a OS=Homo sapiens GN=EH4 PE=3 SV=1 | 0.02                | Down    | 60S ribosomal protein L34 OS=Homo sapiens GN=RPL34 PE=1 SV=3                                         | 0.0095  |
| Cytochrome c oxidase subunit 6B1 OS=Homo sapiens GN=COX6B1 PE=1 SV=2      | 0.048               | Down    | Isocitrate dehydrogenase [NADP] OS=Homo sapiens PE=2 SV=1                                             | 0.023   |
| Ubiquitin/ISG15-conjugating enzyme E2 L6 OS=Homo sapiens GN=UBE2L6 PE=1 SV=4 | 0.019               | Down    | 60S ribosomal protein L35 OS=Homo sapiens GN=RPL35 PE=1 SV=2                                         | 0.015   |
| Cysteine--tRNA ligase, cytoplasmic OS=Homo sapiens GN=CARS PE=1 SV=1      | 0.048               | Down    | Epididymis secretory protein Li 102 OS=Homo sapiens GN=HEL-S-102 PE=2 SV=1                            | 0.0016  |
| H/ACA ribonucleoprotein complex subunit 3 OS=Homo sapiens GN=NOP10 PE=1 SV=1 | 1.00E-04           | Down    | Aspartate aminotransferase OS=Homo sapiens PE=2 SV=1                                                 | 0.043   |
| Cluster of PURA protein (Fragment) OS=Homo sapiens GN=PURA PE=2 SV=1 (Q2NLC9_HUMAN) | 0.011               | Down    | Cluster of Heat shock 70 kDa protein 4L OS=Homo sapiens                                                | 0.014   |
| Description                                                                 | P-value | Direction | Description                                                                                     | P-value |
|-----------------------------------------------------------------------------|---------|-----------|-------------------------------------------------------------------------------------------------|---------|
| Mitochondrial transcription factor A OS=Homo sapiens PE=4 SV=1              | 0.021   | Down      | cDNA FLJ60607, highly similar to Acyl-protein thioesterase 1 (EC 3.1.2.-) OS=Homo sapiens PE=2 SV=1 | 0.037   |
| DNA helicase OS=Homo sapiens GN=MCM3 PE=2 SV=1                             | 0.005   | Down      | Glutamate--cysteine ligase regulatory subunit OS=Homo sapiens GN=GCLM PE=1 SV=1                  | 0.042   |
| Isochorismatase domain-containing protein 2 OS=Homo sapiens GN=ISOC2 PE=1 SV=1 | 0.024   | Down      | Acyl-protein thioesterase 2 OS=Homo sapiens GN=LYPLA2 PE=1 SV=1                                 | 0.00073 |
| Cluster of Heme oxygenase 2 (Fragment) OS=Homo sapiens GN=HMOX2 PE=1 SV=1  |         |           | Cluster of cDNA FLJ54671, highly similar to Calcium-binding mitochondrial carrier protein Aralar2 OS=Homo sapiens PE=2 SV=1 (B722E2_HUMAN) | 0.035   |
| Cluster of 60S ribosomal protein L3 OS=Homo sapiens GN=RPL3 PE=1 SV=2 (RL3_HUMAN) | 0.012   | Up        | Cluster of cDNA FLJ54671, highly similar to Calcium-binding mitochondrial carrier protein Aralar2 OS=Homo sapiens PE=2 SV=1 (B722E2_HUMAN) | 0.033   |
| Cluster of Ribosomal protein L10 isoform A (Fragment) OS=Homo sapiens GN=RPL10 PE=2 SV=1 (XSD2T3_HUMAN) | 0.0034  | Up        | Bifunctional coenzyme A synthase OS=Homo sapiens GN=COASY PE=1 SV=4                              | 0.0063  |
| Cluster of 60S ribosomal protein L7a OS=Homo sapiens GN=RPL7A PE=1 SV=2 (RL7A_HUMAN) | 0.046   | Up        | Sulfiredoxin-1 OS=Homo sapiens GN=SRXN1 PE=1 SV=2                                              | 0.018   |
| Cluster of Thioredoxin reductase 1, cytoplasmic OS=Homo sapiens             | 0.041   | Up        | Nuclear protein localization 4 homolog (S.                                                     | 0.044   |
| Identified Proteins | T-Test (p-Value) | Up or Down regulated | Identified Proteins | T-Test (p-Value) | Up or Down regulated |
|---------------------|------------------|----------------------|---------------------|------------------|----------------------|
| Cluster of Epididymis luminal protein 33 OS=Homo sapiens GN=HEL-S-72p PE=2 SV=1 (V9HW22_HUMAN) | 0.00068 | Down | Cluster of D-dopachrome tautomerase OS=Homo sapiens GN=DDT PE=2 SV=1 (Q53Y51_HUMAN) | 0.042 | Down |
| Cluster of Filamin-A OS=Homo sapiens GN=FLNA PE=1 SV=4 (FLNA_HUMAN) | 0.00013 | Down | Cluster of Alpha-enolase OS=Homo sapiens GN=ENO1 PE=1 SV=2 (ENOA_HUMAN) | 0.043 | Down |
| Cluster of Polyadenylate-binding protein OS=Homo sapiens PE=2 SV=1 (B4DQX0_HUMAN) | 2.00E-04 | Down | Cluster of Eukaryotic translation initiation factor 5A (Fragment) OS=Homo sapiens GN=EIF5A PE=1 SV=8 (I3L397_HUMAN) | 0.043 | Down |
| Cluster of Heterogeneous nuclear ribonucleoprotein K, isoform CRA_d OS=Homo sapiens GN=HNRPK PE=4 SV=1 (A0A024R228_HUMAN) | 0.00034 | Down | Cluster of Heterogeneous nuclear ribonucleoprotein H2 OS=Homo sapiens GN=HNRNPH2 PE=1 SV=1 (HNRH2_HUMAN) | 0.043 | Down |
| Cluster of 60S acidic ribosomal protein P2 OS=Homo sapiens GN=RPLP2 PE=1 SV=1 (RLA2_HUMAN) | 0.00066 | Down | Protein DJ-1 OS=Homo sapiens GN=PARK7 PE=1 SV=1 | 0.043 | Down |
| Cluster of T-complex protein 1 subunit zeta-2 | 0.0011 | Down | Splicing factor 3A subunit 1 OS=Homo | 0.043 | Down |
| Gene Name | Organism | GO ID | Description | p-value | Fold Change |
|-----------|----------|-------|-------------|---------|-------------|
| Thioredoxin | Homo sapiens | | Cluster of SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily e, member 1, isoform CRA_a OS=Homo sapiens GN=SMARCE1 PE=4 SV=1 (A0A024R1S7_HUMAN) | 0.043 | Down |
| Protein S100 | Homo sapiens | | cDNA FLJ37476 fis, clone BRAWH2012827, highly similar to Homo sapiens BH3 interacting domain death agonist (BID), transcript variant 1, mRNA OS=Homo sapiens PE=2 SV=1 | 0.044 | Down |
| Cluster of cDNA FLJ75422, highly similar to Homo sapiens capping protein (actin filament) muscle Z-line, alpha 1, mRNA OS=Homo sapiens PE=2 SV=1 (A8K0T9_HUMAN) | | | Methylosome subunit plCln OS=Homo sapiens GN=CLNS1A PE=1 SV=1 | 0.044 | Down |
| Cluster of Nucleosome assembly protein 1-like 1 (Fragment) OS=Homo sapiens GN=NAP1L1 PE=1 SV=1 (H0YHC3_HUMAN) | | | Pyridoxine 5'-phosphate oxidase variant (Fragment) OS=Homo sapiens PE=2 SV=1 | 0.044 | Down |
| Cluster of Cold shock domain containing E1, RNA-binding, isoform CRA_a OS=Homo sapiens GN=CSDE1 PE=4 SV=1 (A0A024R0E2_HUMAN) | | | Cluster of Ribonuclease T2 OS=Homo sapiens GN=RNASET2 PE=1 SV=1 (A0A087WZM2_HUMAN) | 0.045 | Down |
| Protein POF1B | Homo sapiens | | Cluster of UPF1 regulator of nonsense transcripts homolog (Yeast), isoform CRA_b OS=Homo sapiens GN=UPF1 PE=4 SV=1 (A0A024R7L5_HUMAN) | 0.045 | Down |
| Gene Name                                                                 | OS  | PE  | SV  | Log2 Fold Change | Expression Status | FDR   | P-Value | Log2 Fold Change | Expression Status | FDR   | P-Value |
|--------------------------------------------------------------------------|-----|-----|-----|------------------|-------------------|-------|---------|------------------|-------------------|-------|---------|
| Cluster of Chromobox protein homolog 3                                  | Homo sapiens | CBX3 | 1  | 0.0004 2         | Down              | 0.046 |        |                   |                   |       |         |
| Catechol O-methyltransferase                                             | Homo sapiens | COMT | 1  | 0.0008 4         | Down              | 0.046 |        |                   |                   |       |         |
| GRB2 protein (Fragment)                                                  | Homo sapiens | GRB2 | 2  | 0.011            | Down              | 0.046 |        |                   |                   |       |         |
| Cluster of PDZ and LIM domain protein 5                                  | Homo sapiens | PDLIM5 | 5  | 0.0008 1         | Down              | 0.047 |        |                   |                   |       |         |
| Cluster of Testicular secretory protein Li 8                            | Homo sapiens | S100P | 2  | 1.00E-04         | Down              | 0.048 |        |                   |                   |       |         |
| Cluster of cDNA FLJ56531, highly similar to UV excision repair protein   | Homo sapiens | PSMB6 | 1  | 0.0006 5         | Down              | 0.049 |        |                   |                   |       |         |
| Cluster of Band 4.1-like protein 1                                       | Homo sapiens | CLINT1 | 2  | 0.0011           | Down              | 0.049 |        |                   |                   |       |         |
| Clathrin interactor 1 isoform 2 (Fragment)                               | Homo sapiens | CLINT1 | 2  | 0.0006 7         | Down              | 0.049 |        |                   |                   |       |         |
| Gene Name | Fold Change | Gene Name | Fold Change |
|-----------|-------------|-----------|-------------|
| Aminoacylase-1 | 0.0003 | Cluster of Histone H4 | 0.0009 |
| OS=Homo sapiens | 1 | OS=Homo sapiens | 9 |
| GN=ACY1 PE=4 | SV=1 | GN=HIST1H4A PE=1 | SV=2 |
| ATP synthase subunit delta, mitochondrial | 1.00E-04 | Cluster of Thioredoxin reductase 1, cytoplasmic | 0.0005 |
| OS=Homo sapiens | Down | OS=Homo sapiens | Up |
| GN=ATP5D PE=1 | SV=1 | GN=TXNRD1 PE=1 | SV=3 |
| BJ-HCC-24 tumor antigen | 0.0003 | Cluster of 4F2 cell-surface antigen heavy chain | 0.001 |
| OS=Homo sapiens | Down | OS=Homo sapiens | Up |
| GN=SLC3A2 PE=1 | SV=3 | GN=SLC3A2 PE=1 | SV=3 |
| Cluster of Myosin light chain 6B | 0.0013 | Histone H1.5 | 6.00E-04 |
| OS=Homo sapiens | Down | OS=Homo sapiens | Up |
| GN=MYL6B PE=1 | SV=1 | GN=HIST1H1B PE=1 | SV=3 |
| Cluster of Ras-GTPase activating protein SH3 domain-binding protein 2 | 0.0013 | High mobility group protein B2 | 0.0006 |
| OS=Homo sapiens | Down | OS=Homo sapiens | Up |
| GN=G3BP2 PE=4 | SV=1 | GN=HMGB2 PE=1 | SV=2 |
| Cluster of Tropomyosin alpha-3 chain | 0.0014 | Cluster of 40S ribosomal protein S10 | 0.0001 |
| OS=Homo sapiens | Down | OS=Homo sapiens | Up |
| GN=TPM3 PE=1 | SV=2 | GN=RPS10 PE=1 | SV=1 |
| Inorganic pyrophosphatase | 0.0016 | Cluster of Voltage-dependent anion channel 2 | 0.0007 |
| OS=Homo sapiens | Down | OS=Homo sapiens | Up |
| GN=PPA1 PE=1 | SV=2 | GN=VDAC2 PE=4 | SV=1 |
| Acyl carrier protein, mitochondrial | 0.0016 | Histone H2A.V | 0.0009 |
| OS=Homo sapiens | Down | OS=Homo sapiens | Up |
| GN=NDUFAB1 PE=1 | SV=3 | GN=H2AFV PE=1 | SV=3 |
| Cluster of Epidermal growth factor receptor kinase substrate 8-like protein 1 | 0.0017 | ATP synthase subunit gamma | 0.0003 |
| OS=Homo sapiens | Down | OS=Homo sapiens | Up |
| GN=EPS8L1 PE=1 | SV=1 | GN=H2AFV PE=1 | SV=3 |
| Gene Description                                                                 | P-value | Direction | Gene Description                                                                                     | P-value | Direction |
|--------------------------------------------------------------------------------|---------|-----------|-----------------------------------------------------------------------------------------------------|---------|-----------|
| Cluster of S100A10 protein (Fragment) OS=Homo sapiens GN=S100A10 PE=2 SV=1 (Q6FG5ES_HUMAN) | 0.0023  | Down      | Cluster of Methylcrotonoyl-CoA carboxylase beta chain, mitochondrial OS=Homo sapiens GN=MCCC2 PE=1 SV=1 (MCCB_HUMAN) | 0.0009 6 | Up        |
| Lactoylglutathione lyase OS=Homo sapiens GN=HEL-S-74 PE=2 SV=1                  | 0.0022  | Down      | Cluster of Transcriptional activator protein Pur-alpha OS=Homo sapiens GN=PUA PE=1 SV=2 (PUA_HUMAN)       | 0.0009 6 | Up        |
| Cluster of Nuclear autoantigenic sperm protein OS=Homo sapiens GN=NASP PE=1 SV=2 (NASP_HUMAN) | 0.0021  | Down      | Dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 2 OS=Homo sapiens GN=RPN2 PE=1 SV=3 | 0.001   | Up        |
| Cluster of cDNA FLJ76823, highly similar to Homo sapiens splicing factor, arginine/serine-rich 6 (SFRS6), mRNA OS=Homo sapiens PE=2 SV=1 (A8KS88_HUMAN) | 0.0023  | Down      | Isocitrate dehydrogenase [NAD] subunit, mitochondrial OS=Homo sapiens GN=IDH3B PE=1 SV=1                | 0.0007 5 | Up        |
| Proteosome subunit beta type OS=Homo sapiens PE=2 SV=1                         | 0.002   | Down      | ATP-dependent 6-phosphofructokinase, liver type OS=Homo sapiens GN=PFKL PE=1 SV=6                      | 0.0004 7 | Up        |
| Spermine synthase OS=Homo sapiens GN=SMS PE=1 SV=2                            | 0.0022  | Down      | cDNA FLJ52100 OS=Homo sapiens PE=2 SV=1                                                              | 0.0003 6 | Up        |
| Succinate-CoA ligase [GDP-forming] subunit beta, mitochondrial OS=Homo sapiens GN=SUCLG2 PE=1 SV=2 | 0.0018  | Down      | SF3A2 protein (Fragment) OS=Homo sapiens GN=SF3A2 PE=2 SV=1                                        | 0.0012  | Up        |
| cDNA FLJ59206, highly similar to Eukaryotic translation initiation factor 4B OS=Homo sapiens PE=2 SV=1 | 0.0019  | Down      | Cytochrome c1, heme protein, mitochondrial OS=Homo sapiens GN=CYC1 PE=1 SV=3                         | 0.0013  | Up        |
| Cluster of DDAH2 OS=Homo sapiens GN=HEL-S-277 PE=1                            | 0.002   | Down      | Cluster of Epididymis tissue sperm binding protein Li 3a OS=Homo                                        | 0.0015  | Up        |
| SV=1 (V9HW53_HUMAN)                            | sapiens PE=1 SV=1 (E9KL35_HUMAN)                             | 0.0025 | Down | Cluster of Inorganic pyrophosphatase 2, mitochondrial OS=Homo sapiens GN=PPA2 PE=1 SV=2 (IPYR2_HUMAN) | Cluster of Transmembrane 9 superfamily member OS=Homo sapiens GN=SMBP PE=2 SV=1 (Q96I5S_HUMAN) | 0.0016 | Up |
|-----------------------------------------------|---------------------------------------------------------------|--------|------|-------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------|--------|----|
| Cluster of Keratin, type I cytoskeletal 18 OS=Homo sapiens GN=KRT18 PE=1 SV=2 (K1C18_HUMAN) | RNA binding protein (Autoantigenic, hnRNP-associated with lethal yellow) long isoform variant (Fragment) OS=Homo sapiens GN=RALY PE=1 SV=1 | 0.003  | Down | Cluster of Keratin, type I cytoskeletal 18 OS=Homo sapiens GN=KRT18 PE=1 SV=2 (K1C18_HUMAN) | Cluster of Keratin, type I cytoskeletal 18 OS=Homo sapiens GN=KRT18 PE=1 SV=2 (K1C18_HUMAN) | 0.0017 | Up |
| Cluster of Alpha-actinin-4 OS=Homo sapiens GN=ACTN4 PE=1 SV=2 (ACTN4_HUMAN) | Succinate--CoA ligase [ADP/GDP-forming] subunit alpha, mitochondrial OS=Homo sapiens GN=SUCLG1 PE=1 SV=4 | 0.0032 | Down | Cluster of Peptidyl-prolyl cis-trans isomerase A OS=Homo sapiens GN=PPIA PE=1 SV=2 (PPIA_HUMAN) | Cluster of Peptidyl-prolyl cis-trans isomerase A OS=Homo sapiens GN=PPIA PE=1 SV=2 (PPIA_HUMAN) | 0.0017 | Up |
| 40S ribosomal protein S12 OS=Homo sapiens GN=RPS12 PE=1 SV=3 | Cluster of MHC class I antigen (Fragment) OS=Homo sapiens GN=HLA-A PE=3 SV=1 (E5BBI6_HUMAN) | 0.0029 | Down | Cluster of Peptidyl-prolyl cis-trans isomerase A OS=Homo sapiens GN=PPIA PE=1 SV=2 (PPIA_HUMAN) | Cluster of Peptidyl-prolyl cis-trans isomerase A OS=Homo sapiens GN=PPIA PE=1 SV=2 (PPIA_HUMAN) | 0.0022 | Up |
| Cluster of Eukaryotic peptide chain release factor GTP-binding subunit ERF3A OS=Homo sapiens GN=GSPT1 PE=1 SV=1 (ERF3A_HUMAN) | Cluster of Histone 1, H1e OS=Homo sapiens GN=HIST1H1E PE=2 SV=1 (A3R0T8_HUMAN) | 0.0027 | Down | Cluster of Histone 1, H1e OS=Homo sapiens GN=HIST1H1E PE=2 SV=1 (A3R0T8_HUMAN) | Cluster of Histone 1, H1e OS=Homo sapiens GN=HIST1H1E PE=2 SV=1 (A3R0T8_HUMAN) | 0.002  | Up |
| Cluster of Dynein heavy chain 12, axonemal OS=Homo sapiens GN=DNAH12 PE=1 SV=2 (E9PG32_HUMAN-DECOY) | RPL21 protein OS=Homo sapiens GN=RPL21 PE=2 SV=1 | 0.0032 | Down | Cluster of Putative deoxyribonuclease TATDN1 OS=Homo sapiens GN=TATDN1 | Cluster of Putative deoxyribonuclease TATDN1 OS=Homo sapiens GN=TATDN1 | 0.0023 | Up |
| Gene Name | Description | OS | SV | PE | SV | Gene Name | Description | OS | SV | PE | SV | p-value | Fold Change |
|-----------|-------------|----|----|----|----|-----------|-------------|----|----|----|----|---------|-------------|
| Alcohol dehydrogenase [NADP(+)] | Homo sapiens | 3 | 1 | 2 | 1 | Homo sapiens | Cluster of TOB3 | Homo sapiens | 2 | 1 | 2 | 1 | 0.0032 | Down |
| 0.0023 | Up |
| Cluster of Triosephosphate isomerase | Homo sapiens | 3 | 1 | 1 | 2 | Homo sapiens | H/ACA ribonucleoprotein complex subunit 3 | Homo sapiens | 1 | 1 | 1 | 2 | 0.0035 | Down |
| 0.0019 | Up |
| Cluster of 26S proteasome non-ATPase regulatory subunit 4 | Homo sapiens | 1 | 2 | 1 | 2 | Homo sapiens | Cluster of Mitochondrial transcription factor A | Homo sapiens | 4 | 1 | 1 | 1 | 0.0035 | Down |
| 0.0018 | Up |
| Cluster of ADP-ribosylation factor 1 | Homo sapiens | 2 | 1 | 1 | 1 | Homo sapiens | Cysteine-tRNA ligase, cytoplasmic | Homo sapiens | 1 | 1 | 1 | 2 | 0.0036 | Down |
| 0.0021 | Up |
| cDNA FLJ54170, highly similar to Cytosolic nonspecific dipeptidase | Homo sapiens | 1 | 2 | 2 | 1 | Homo sapiens | cDNA, FLJ96465, highly similar to Homo sapiens solute carrier family 25 (mitochondrial carrier;phosphate carrier), member 3 (SLC25A3), nuclear gene encodingmitochondrial protein, transcript variant 1b... | Homo sapiens | 1 | 1 | 2 | 1 | 0.0037 | Down |
| 0.0025 | Up |
| Tumor protein D52 | Homo sapiens | 2 | 1 | 1 | 2 | Homo sapiens | Cluster of Histone H2A type 3 | Homo sapiens | 1 | 1 | 3 | 1 | 0.0037 | Down |
| 0.0032 | Up |
| Serine-threonine kinase receptor-associated protein | Homo sapiens | 1 | 2 | 1 | 1 | Homo sapiens | Cluster of Histone H3.3 | Homo sapiens | 2 | 1 | 1 | 2 | 0.0038 | Down |
| 0.0027 | Up |
| Cluster of T-complex protein 1 subunit gamma | Homo sapiens | 1 | 2 | 2 | 1 | Homo sapiens | Cluster of NPC-A-16 | Homo sapiens | 1 | 1 | 2 | 1 | 0.0039 | Down |
| 0.003 | Up |
| Gene Name | Description | Up/Down | Log2 Fold Change |
|-----------|-------------|---------|----------------|
| Cluster of Nucleosome assembly protein 1-like 4, isoform CRA_b | OS=Homo sapiens GN=NAP1L4 PE=3 SV=1 (A0A024RCC9_HUMAN) | Down | 0.0039 |
| Eukaryotic translation initiation factor 3 subunit M | OS=Homo sapiens GN=EIF3M PE=1 SV=1 | Down | 0.0042 |
| Cluster of 14-3-3 protein zeta/delta | OS=Homo sapiens GN=YWHAZ PE=1 SV=1 (1433Z_HUMAN) | Down | 0.0043 |
| Costars family protein ABRACL | OS=Homo sapiens GN=ABRACL PE=1 SV=1 | Down | 0.0043 |
| Cluster of cDNA, FLJ95650, highly similar to Homo sapiens karyopherin (importin) beta 1 (KPNB1), mRNA OS=Homo sapiens PE=2 SV=1 (B2RBR9_HUMAN) | Down | 0.0045 |
| Cluster of cDNA FLJ53116, highly similar to T-complex protein 1 subunit epsilon OS=Homo sapiens PE=2 SV=1 (B4DZTS_HUMAN) | Down | 0.0048 |
| Solute carrier family 2, facilitated glucose transporter member 1 OS=Homo sapiens GN=SLC2A1 PE=1 SV=2 | Down | 0.0048 |
| Myotrophin | OS=Homo sapiens GN=MTPN PE=1 SV=2 | Down | 0.0055 |
| Profilin-1 | OS=Homo sapiens GN=PFN1 PE=1 SV=2 | Down | 0.0059 |
| Gene Name                        | OS          | PE  | SV  | Description                                                                 | OS          | PE  | SV  | Description                                                                 |
|---------------------------------|-------------|-----|-----|------------------------------------------------------------------------------|-------------|-----|-----|------------------------------------------------------------------------------|
| Testicular tissue protein Li 75 | Homo sapiens| 2   | 1   | Down                           | Homo sapiens| 2   | 1   | Cluster of Histone deacetylase                                               |
|                                  |             |     |     |                                |             |     |     | OS=Homo sapiens PE=2 SV=1 (Q6IT96_HUMAN)                                    |
| Drebrin-like protein             | Homo sapiens| 2   | 1   | Down                           | Homo sapiens| 2   | 1   | Cluster of G protein-binding protein                                          |
|                                  |             |     |     |                                |             |     |     | OS=Homo sapiens PE=2 SV=1 (Q53GS0_HUMAN)                                    |
| Cluster of HSPA1L                | Homo sapiens| 3   | 1   | Down                           | Leucine-rich repeat-containing protein 59           |
|                                  |             |     |     |                                | OS=Homo sapiens PE=2 SV=1 (A0A1U9X7X4_HUMAN)        |
| Niban-like protein 1             | Homo sapiens| 2   | 3   | Down                           | Asparagine synthetase [glutamine-hydrolyzing]     |
|                                  |             |     |     |                                | OS=Homo sapiens PE=1 SV=1 (FAM129B_HUMAN)          |
| Cluster of Nucleolar and coiled-body phosphoprotein 1 | Homo sapiens | 1 | 2 | Down                           | Cluster of Calnexin                               |
|                                  |             |     |     |                                | OS=Homo sapiens PE=1 SV=2 (NOLC1_HUMAN)            |
| Serine/arginine repetitive matrix 1 isoform 2 (Fragment) | Homo sapiens | 1 | 1 | Down                           | Septin-2 OS=Homo sapiens                          |
|                                  |             |     |     |                                | OS=Homo sapiens PE=1 SV=1 (SRRM1_HUMAN)            |
| Malignant T-cell-amplified sequence 1 | Homo sapiens | 1 | 1 | Down                           | Lon protease homolog, mitochondrial                |
|                                  |             |     |     |                                | OS=Homo sapiens PE=1 SV=1 (MCTS1_HUMAN)           |
| Cluster of cDNA FLJ32482 fis, clone SKMNC2001324, highly similar to Importin-4 | Homo sapiens | 2 | 1 | Down                           | Cluster of RPS4X protein (Fragment)               |
|                                  |             |     |     |                                | OS=Homo sapiens PE=2 SV=1 (B3KPY9_HUMAN)           |
| Cluster of Hsp70-binding protein 1 OS=Homo sapiens | Homo sapiens | 1 | 1 | Down                           | Cluster of cDNA                                   |
|                                  |             |     |     |                                | OS=Homo sapiens PE=1 SV=1 (HPBP1_HUMAN)           |
| Gene Name | Fold Change | Regulation | Gene Name | Fold Change | Regulation |
|-----------|-------------|------------|-----------|-------------|------------|
| Spectrin alpha chain, non-erythrocytic 1 | 0.0069 | Down | Topoisomerase (DNA) I | 0.0057 | Up |
| OS=Homo sapiens GN=SPTAN1 PE=1 SV=1 (B3KQQ3_HUMAN) | | | OS=Homo sapiens GN=TOP1 PE=2 SV=1 | | |
| Cluster of Neuroblast differentiation-associated protein AHNAK | 0.0075 | Down | Ribosome-binding protein 1 | 0.0058 | Up |
| OS=Homo sapiens GN=AHNAK PE=1 SV=2 (AHNK_HUMAN) | | | OS=Homo sapiens GN=RRBP1 PE=1 SV=1 | | |
| Nascent polypeptide-associated complex subunit alpha, muscle-specific form | 0.0073 | Down | Peptidyl-prolyl cis-trans isomerase B | 0.0064 | Up |
| OS=Homo sapiens GN=NACA PE=1 SV=1 | | | OS=Homo sapiens GN=PPIB PE=1 SV=2 | | |
| Chromobox protein homolog 1 | 0.0075 | Down | Cluster of Small nuclear ribonucleoprotein-associated protein N | 0.0064 | Up |
| OS=Homo sapiens GN=CBX1 PE=1 SV=1 | | | OS=Homo sapiens GN=SNRPN PE=1 SV=1 (RSMN_HUMAN) | | |
| Isocitrate dehydrogenase [NADP] | 0.0078 | Down | Cluster of Chromodomain-helicase-DNA-binding protein 4 | 0.0064 | Up |
| OS=Homo sapiens | | | OS=Homo sapiens GN=CHD4 PE=1 SV=1 (A0A0C4DGG9_HUMAN) | | |
| Cluster of Calmodulin-1 | 0.0079 | Down | 60S ribosomal protein L35 | 0.0066 | Up |
| OS=Homo sapiens GN=CALM1 PE=1 SV=1 (CALM1_HUMAN) | | | OS=Homo sapiens GN=RPL35 PE=1 SV=2 | | |
| Cluster of Heterogeneous nuclear ribonucleoprotein L | 0.0084 | Down | Trifunctional enzyme subunit beta, mitochondrial | 0.0068 | Up |
| OS=Homo sapiens GN=HNRNPL PE=1 SV=2 (HNRPL_HUMAN) | | | OS=Homo sapiens GN=HADHB PE=1 SV=3 | | |
| Endoribonuclease | 0.0089 | Down | Cluster of cDNA FLJ61290, highly similar to Neutral alpha-glucosidase AB | 0.0071 | Up |
| LACTB2 OS=Homo sapiens GN=LACTB2 PE=1 SV=2 | | | OS=Homo sapiens PE=2 SV=1 (B4DJ30_HUMAN) | | |
| Description                                                                 | Ratio | Direction | Description                                                                                     | Ratio |
|----------------------------------------------------------------------------|-------|-----------|-----------------------------------------------------------------------------------------------|-------|
| Cluster of Barrier to autointegration factor 1, isoform CRA_a               | 0.0091| Down      | Deoxynucleoside triphosphate triphosphohydrolase SAMHD1 OS=Homo sapiens                        | 0.0074| Up        |
| (A0A024R5H0_HUMAN)                                                         |       |           |                                                                                               |       |
| Cluster of Epididymis secretory protein Li 85 OS=Homo sapiens GN=PCBP1 PE=2| 0.0092| Down      | Testis derived transcript (3 LIM domains) OS=Homo sapiens GN=TES PE=4                        | 0.0075| Up        |
| SV=1                                                                      |       |           |                                                                                               |       |
| (Q53SS8_HUMAN)                                                             |       |           |                                                                                               |       |
| Cluster of Calponin (Fragment) OS=Homo sapiens PE=2 SV=1                   | 0.0094| Down      | Cluster of ADP/ATP translocase 2 OS=Homo sapiens GN=SLC25A5 PE=1                             | 0.0079| Up        |
| (Q53GK7_HUMAN)                                                             |       |           |                                                                                               |       |
| BAG6 OS=Homo sapiens GN=BAT3 PE=1 SV=1                                    | 0.01  | Down      | Cluster of GTP-binding nuclear protein Ran (Fragment) OS=Homo sapiens GN=RAN PE=1            | 0.0082| Up        |
|                                                                            |       |           |                                                                                               |       |
| Cluster of Keratin, type II cytoskeletal 8 OS=Homo sapiens GN=KRT8 PE=1    | 0.011 | Down      | Voltage-dependent anion-selective channel protein 1 OS=Homo sapiens GN=VDAC1 PE=1           | 0.0088| Up        |
| SV=7 (K2C8_HUMAN)                                                          |       |           |                                                                                               |       |
| Fatty acid synthase OS=Homo sapiens GN=FASN PE=1 SV=3                     | 0.011 | Down      | Adenylate kinase 1 variant (Fragment) OS=Homo sapiens PE=2 SV=1                               | 0.0088| Up        |
|                                                                            |       |           |                                                                                               |       |
| Cluster of Cofilin-1 OS=Homo sapiens GN=CFL1 PE=1 SV=3                   | 0.011 | Down      | Nodal modulator 3 OS=Homo sapiens GN=NOMO3 PE=1                                               | 0.0089| Up        |
| (COF1_HUMAN)                                                               |       |           |                                                                                               |       |
| Cluster of Transgelin-2 OS=Homo sapiens GN=TAGLN2 PE=1 SV=3               | 0.011 | Down      | 60S ribosomal protein L11 OS=Homo sapiens GN=RPL11 PE=1                                       | 0.009 | Up        |
| (TAGL2_HUMAN)                                                              |       |           |                                                                                               |       |
| Splicing factor U2AF 65 kDa subunit OS=Homo sapiens GN=U2AF2 PE=1 SV=4    | 0.011 | Down      | PGRMC1 protein OS=Homo sapiens GN=PGRMC1 PE=2                                                | 0.0095| Up        |
|                                                                            |       |           |                                                                                               |       |
| HDCMB21P OS=Homo sapiens PE=2 SV=1                                         | 0.011 | Down      | Eukaryotic translation initiation factor 2 beta OS=Homo sapiens                               | 0.0096| Up        |
|                                                                            |       |           |                                                                                               |       |
| cDNA FLJ75699, highly similar to Homo sapiens                             | 0.011 | Down      | Cluster of Basigin OS=Homo sapiens                                                           | 0.0097| Up        |
| Gene Name                                      | OS         | GN             | PE | SV | Fold Change | Expression | Functional Description                                                                 |
|-----------------------------------------------|------------|----------------|----|----|-------------|------------|-----------------------------------------------------------------------------------------|
| osteoclast stimulating factor 1 (OSTF1), mRNA | Homo sapiens | BSG            | 2  | 1  | 0.012       | Down       | 60S ribosomal protein L24                                                                 |
| Cluster of Annexin A1                        | Homo sapiens | ANXA1          | 1  | 2  | 0.012       | Down       | Putative peripheral benzodiazepine receptor-related protein (Fragment)                   |
| Cluster of BolA-like protein 2               | Homo sapiens | BOLA2          | 1  | 2  | 0.013       | Down       | NADPH--cytochrome P450 reductase                                                          |
| Cluster of Glyceraldehyde-3-phosphate        | Homo sapiens | GAPDH          | 3  | 1  | 0.013       | Down       | Cluster of Transketolase variant (Fragment)                                             |
| Cluster of Protein disulfide-isomerase       | Homo sapiens | P4HB           | 1  | 2  | 0.013       | Down       | Cluster of 2'-5'-oligoadenylate synthetase 3, 100kDa, isoform CRA_a                     |
| Cluster of Heat shock 70kDa protein 4         | Homo sapiens | OAS3           | 4  | 1  | 0.013       | Down       | Multifunctional methyltransferase subunit TRM112-like protein                           |
| Cluster of Tumor protein D54                  | Homo sapiens | TP53           | 1  | 2  | 0.013       | Down       | Cluster of Glutamine-fructose-phosphate aminotransferase [isomerizing] 1 [OS=Homo sapiens]  |
| Gene Name | Fold Change | Change Direction | Description |
|-----------|-------------|------------------|-------------|
| Testicular tissue protein | 0.013 | Down | cDNA, FLJ93510, highly similar to Homo sapiens JTV1 gene (JTV1), mRNA |
| Synaptogyrin OS=Homo sapiens GN=SYNGR2 | 0.014 | Down | Lysine-tRNA ligase OS=Homo sapiens GN=KARS |
| Cluster of ELAV-like protein 1 OS=Homo sapiens GN=ELAVL1 | 0.015 | Down | E3 ubiquitin-protein ligase RNF213 OS=Homo sapiens GN=RNF213 |
| Cluster of Cellular retinoic acid-binding protein 2 OS=Homo sapiens GN=CRABP2 | 0.015 | Down | Cluster of Cytochrome b-c1 complex subunit 2, mitochondrial OS=Homo sapiens GN=UQCRC2 |
| cDNA FLJ52068, highly similar to Microtubule-associated protein RP/EB family member 1 | 0.015 | Down | Cluster of cDNA FLJ54020, highly similar to Heterogeneous nuclear ribonucleoprotein U |
| Cluster of SET OS=Homo sapiens GN=SET | 0.015 | Down | 40S ribosomal protein S3a OS=Homo sapiens GN=RPS3A |
| Cluster of Nuclease sensitive element binding protein-1 | 0.015 | Down | Cluster of Core histone macro-H2A.1 OS=Homo sapiens GN=H2AFY |
| Cluster of La-related protein 1 OS=Homo sapiens GN=LARP1 | 0.015 | Down | X-ray repair cross-complementing protein 6 OS=Homo sapiens GN=XRCC6 |
| Cluster of T-complex protein 1 subunit beta OS=Homo sapiens GN=CCT2 | 0.016 | Down | Mitochondrial carrier homolog 2 OS=Homo sapiens GN=MTCH2 |
| Cluster of Histone-binding protein RBBP7 OS=Homo sapiens GN=RBBP7 | 0.016 | Down | Cluster of Isocitrate dehydrogenase [NAD] subunit, mitochondrial OS=Homo sapiens GN=IDH3G |
| Gene Name                                    | Expression | Fold Change | Description                                                                 | Species | Accession | Peptide Count | Up/Down |
|---------------------------------------------|------------|-------------|-----------------------------------------------------------------------------|---------|-----------|---------------|----------|
| Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1 OS=Homo sapiens GN=PIN1 PE=1 SV=1 | Down       | 0.016       | Prohibitin-2 OS=Homo sapiens GN=PHB2 PE=1 SV=1                             | 0.016   | Up        |
| Calreticulin variant (Fragment) OS=Homo sapiens PE=2 SV=1 | Down       | 0.017       | Cluster of Interferon-induced protein with tetratricopeptide repeats 1 OS=Homo sapiens GN=IFIT1 PE=1 SV=2 (IFIT1_HUMAN) | 0.016   | Up        |
| Cluster of CSTB protein OS=Homo sapiens GN=CSTB PE=2 SV=1 (Q76LA1_HUMAN) | Down       | 0.017       | Ubiquitin carboxyl-terminal hydrolase 14 OS=Homo sapiens GN=USP14 PE=1 SV=2 | 0.016   | Up        |
| Cluster of Far upstream element-binding protein 2 OS=Homo sapiens GN=KHSRP PE=1 SV=1 (A0A087WTP3_HUMAN) | Down       | 0.017       | Cluster of Tubulin alpha chain OS=Homo sapiens GN=TUBA1C PE=1 SV=1 (F5H5D3_HUMAN) | 0.017   | Up        |
| Eukaryotic translation initiation factor 3 subunit A OS=Homo sapiens GN=EIF3A PE=1 SV=1 | Down       | 0.017       | Cluster of Proteasome subunit alpha type OS=Homo sapiens GN=PSMA6 PE=1 SV=1 (G3V295_HUMAN) | 0.017   | Up        |
| Proteasome subunit alpha type OS=Homo sapiens PE=2 SV=1 | Down       | 0.017       | DNA-dependent protein kinase catalytic subunit OS=Homo sapiens GN=PRKDC PE=1 SV=3 | 0.017   | Up        |
| EF-hand domain-containing protein D2 OS=Homo sapiens GN=EFHD2 PE=1 SV=1 | Down       | 0.017       | Apoptosis-inducing factor 1, mitochondrial OS=Homo sapiens GN=AIFM1 PE=1 SV=1 | 0.018   | Up        |
| cDNA FLJ56566, highly similar to Small glutamine-rich tetratricopeptide repeat-containing protein A OS=Homo sapiens PE=2 SV=1 | Down       | 0.017       | Eukaryotic translation initiation factor 3 subunit D OS=Homo sapiens GN=EIF3D PE=2 SV=1 | 0.018   | Up        |
| Cluster of Prosaposin (Variant Gaucher disease and variant metachromatic leukodystrophy), isoform CRA_b OS=Homo sapiens GN=PSAP PE=4 SV=1 (A0A024QZQ2_HUMAN) | Down       | 0.017       | Bifunctional glutamate/proline-tRNA ligase OS=Homo sapiens GN=EPRS PE=1 SV=5 | 0.019   | Up        |
| Gene Name | Expression | Fold Change |
|-----------|------------|-------------|
| Cluster of cDNA FLJ55694, highly similar to Dipeptidyl-peptidase 1 (EC 3.4.14.1) OS=Homo sapiens PE=2 SV=1 (B4DJQ8_HUMAN) | Down | 0.018 |
| Cluster of Myosin regulatory light chain 12A OS=Homo sapiens GN=MYL12A PE=1 SV=2 (ML12A_HUMAN) | Down | 0.018 |
| Drug-sensitive protein 1 OS=Homo sapiens GN=YA61 PE=2 SV=1 | Down | 0.018 |
| Cluster of Myeloid-derived growth factor OS=Homo sapiens GN=MYDGF PE=1 SV=1 (MYDGF_HUMAN) | Down | 0.018 |
| Cluster of Polypyrimidine tract binding protein 1, isoform CRA_b OS=Homo sapiens GN=PTBP1 PE=1 SV=4 (A6NLN1_HUMAN) | Down | 0.019 |
| Nuclear migration protein nudC OS=Homo sapiens GN=NUDC PE=1 SV=1 | Down | 0.019 |
| Integrin alpha-6 OS=Homo sapiens GN=ITGA6 PE=1 SV=5 | Down | 0.019 |
| Gamma-glutamylcyclotransferase OS=Homo sapiens GN=GGCT PE=1 SV=1 | Down | 0.019 |
| Annexin A10 OS=Homo sapiens GN=ANXA10 PE=1 SV=3 | Down | 0.019 |
| Cluster of Elongation factor 1-delta (Fragment) OS=Homo sapiens | Down | 0.02 |

Histone H1x OS=Homo sapiens GN=H1FX PE=1 SV=1 | Up | 0.019 |
Cluster of 40S ribosomal protein S16 OS=Homo sapiens GN=RPS16 PE=1 SV=2 (RS16_HUMAN) | Up | 0.02 |
60S ribosomal protein L34 OS=Homo sapiens GN=RPL34 PE=1 SV=3 | Up | 0.02 |
EPHX1 protein (Fragment) OS=Homo sapiens GN=EPHX1 PE=2 SV=1 | Up | 0.02 |
60S ribosomal protein L30 (Fragment) OS=Homo sapiens GN=RPL30 PE=1 SV=1 | Up | 0.02 |
Flap endonuclease 1 OS=Homo sapiens GN=FEN1 PE=2 SV=1 | Up | 0.02 |
Cluster of 40S ribosomal protein S19 OS=Homo sapiens GN=RPS19 PE=2 SV=1 (B0ZBD0_HUMAN) | Up | 0.024 |
Cluster of 60S ribosomal protein L17 OS=Homo sapiens GN=RPL17 PE=1 SV=3 (RL17_HUMAN) | Up | 0.024 |
Cluster of Serine hydroxymethyltransferase, mitochondrial (Fragment) OS=Homo sapiens GN=SHMT2 PE=1 SV=1 (G3V4W5_HUMAN) | Up | 0.025 |
Regulator of chromosome condensation 2, isoform CRA_a OS=Homo | Up | 0.025 |
| Gene Name | OS | PE | SV | Fold Change | Regulation | Gene Name | OS | PE | SV | Fold Change | Regulation |
|-----------|----|----|----|-------------|------------|-----------|----|----|----|-------------|------------|
| EEF1D     | Homo sapiens | 1 | 1 | 0.02 | Down | RCC2 | Homo sapiens | 4 | 1 | 0.025 | Up |
| BTF3      | Homo sapiens | 1 | 1 | 0.02 | Down | Glutamate dehydrogenase | Homo sapiens | 2 | 1 | 0.025 | Up |
| STAMN1    | Homo sapiens | 2 | 1 | 0.02 | Down | Histone H1.0 | Homo sapiens | 1 | 3 | 0.025 | Up |
| TPT1      | Homo sapiens | 1 | 1 | 0.02 | Down | Phosphoserine aminotransferase | Homo sapiens | 2 | 1 | 0.025 | Up |
| SNRPF     | Homo sapiens | 1 | 1 | 0.02 | Down | 60S ribosomal protein L13 | Homo sapiens | 1 | 1 | 0.026 | Up |
| PLEC      | Homo sapiens | 1 | 1 | 0.021 | Down | Ribosomal protein L19 | Homo sapiens | 1 | 1 | 0.027 | Up |
| OLA1      | Homo sapiens | 1 | 1 | 0.021 | Down | Peroxisome proliferator activated receptor interacting complex protein | Homo sapiens | 1 | 1 | 0.027 | Up |
| RANGAP1   | Homo sapiens | 1 | 1 | 0.021 | Down | cDNA FLJ45395 fis, clone | Homo sapiens | 1 | 1 | 0.028 | Up |
| PEF1      | Homo sapiens | 1 | 1 | 0.021 | Down | cDNA FLJ76789, highly similar to Homo sapiens methionine-tRNA synthetase (MARS), mRNA | Homo sapiens | 2 | 1 | 0.028 | Up |
| Gene/Protein Name                                                                 | Fold Change | Regulation | Description                                                                 | Fold Change |
|----------------------------------------------------------------------------------|-------------|------------|----------------------------------------------------------------------------|-------------|
| cDNA FLJ78041, highly similar to Homo sapiens NADH dehydrogenase (ubiquinone)    | 0.022       | Down       | flavoprotein 2, 24kDa (NDUFV2), mRNA OS=Homo sapiens PE=2 SV=1               |             |
| SH3 domain binding glutamic acid-rich protein like 3, isoform CRA_a (Fragment)  | 0.023       | Down       | OS=Homo sapiens GN=SH3BGRL3 PE=4 SV=1                                      |             |
| Cluster of Ran-binding protein 6 OS=Homo sapiens GN=RANBP6 PE=1 SV=2 (RNP6_HUMAN)| 0.023       | Down       | Cluster of 60S ribosomal protein L7a OS=Homo sapiens GN=RPL7A PE=1 SV=2 (RL7A_HUMAN) | 0.029       |
| Cluster of CMP kinase OS=Homo sapiens GN=CMPK1 PE=1 SV=3 (KCY_HUMAN)             | 0.024       | Down       | Cluster of CTNND1 protein (Fragment) OS=Homo sapiens GN=CTNND1 PE=2 SV=2 (Q96FS1_HUMAN) | 0.029       |
| Sorting nexin 1 isoform a variant (Fragment) OS=Homo sapiens PE=2 SV=1            | 0.024       | Down       | 39S ribosomal protein L4, mitochondrial OS=Homo sapiens GN=MRPL4 PE=1 SV=1    | 0.029       |
| Cytochrome c oxidase subunit 5A, mitochondrial OS=Homo sapiens GN=COX5A PE=1 SV=2| 0.025       | Down       | Cluster of Ribosomal protein L23, isoform CRA_b OS=Homo sapiens GN=RPL23 PE=3 SV=1 (A0A024R1Q8_HUMAN) | 0.03        |
| Leucine-rich repeat-containing protein 47 OS=Homo sapiens GN=LRRC47 PE=1 SV=1     | 0.025       | Down       | Leucine-rich PPR-motif containing OS=Homo sapiens GN=LRPPRC PE=4 SV=1        | 0.03        |
| Acyl-CoA-binding protein OS=Homo sapiens GN=DBI PE=1 SV=2                        | 0.025       | Down       | ATP synthase subunit g, mitochondrial OS=Homo sapiens GN=ATP5L PE=1 SV=3     | 0.03        |
| Splicing factor 3B subunit 5 OS=Homo sapiens GN=SF3B5 PE=1 SV=1                  | 0.025       | Down       | Cluster of Signal recognition particle subunit SRP72 OS=Homo sapiens         | 0.03        |
| Gene Name | Expression | Regulation | Description |
|-----------|------------|------------|-------------|
| Ribosomal protein, large, P1, isoform CRA_a | 0.026 | Down | Protein NipSnap homolog 2 OS=Homo sapiens GN=NIPSAP2 PE=1 SV=1 |
| HCG26477 OS=Homo sapiens GN=RPS28 PE=2 SV=1 | 0.026 | Down | 40S ribosomal protein S11 OS=Homo sapiens GN=RPS11 PE=1 SV=3 |
| Asparagine-tRNA ligase, cytoplasmic OS=Homo sapiens GN=NARS PE=1 SV=1 | 0.027 | Down | Cluster of Peptidyl-prolyl cis-trans isomerase OS=Homo sapiens PE=2 SV=1 (B2R6X6_HUMAN) |
| Cluster of Epiplakin OS=Homo sapiens GN=EPPK1 PE=1 SV=1 (A0A087X1U6_HUMAN) | 0.027 | Down | Cluster of Citrate synthase OS=Homo sapiens GN=CS PE=1 SV=1 (B4DJV2_HUMAN) |
| Alanine-tRNA ligase, cytoplasmic OS=Homo sapiens GN=AARS PE=1 SV=2 | 0.029 | Down | Heterochromatin protein 1-binding protein 3 OS=Homo sapiens GN=HP1BP3 PE=1 SV=1 |
| Cluster of cDNA FLJ60127, highly similar to Ubiquilin-2 OS=Homo sapiens PE=2 SV=1 (B4DM19_HUMAN) | 0.03 | Down | Cluster of RPL26 protein OS=Homo sapiens GN=RPL26 PE=2 SV=1 (Q6IBH6_HUMAN) |
| SARS protein OS=Homo sapiens GN=SARS PE=2 SV=1 | 0.031 | Down | Regulation of nuclear pre-mRNA domain-containing protein 1B OS=Homo sapiens GN=RPRD1B PE=1 SV=1 |
| cDNA FLJ76387, highly similar to Homo sapiens splicing factor, arginine/serine-rich 9 (SFRS9), mRNA OS=Homo sapiens PE=2 SV=1 | 0.031 | Down | Mago nashi protein OS=Homo sapiens GN=FLJ10292 PE=2 SV=1 |

**Note:** The table includes various genes and their corresponding expression levels, indicating whether they are upregulated (Up) or downregulated (Down) in the context of biological processes or disease states. The expression levels are quantified, and the table also notes the organism (OS=Homo sapiens) and specific gene identifiers (e.g., GN=HEL103 PE=2 SV=1).
| Gene          | P-value | Direction | Description                                                                 | P-value | Direction |
|--------------|---------|-----------|-----------------------------------------------------------------------------|---------|-----------|
| Splicing factor arginine/serine-rich 3 | 0.032   | Down      | Tryptophan–tRNA ligase, cytoplasmic OS=Homo sapiens GN=WARS PE=1 SV=2         | 0.034   | Up        |
| Cluster of Epididymis secretory protein Li 102 | 0.032   | Down      | Calcium load-activated calcium channel OS=Homo sapiens GN=TMCO1 PE=2 SV=1    | 0.034   | Up        |
| Methionine aminopeptidase 2 | 0.032   | Down      | Cluster of Protein PML OS=Homo sapiens GN=PML PE=1 SV=1 (H3BT57_HUMAN)       | 0.035   | Up        |
| Cluster of Epithelial-splicing regulatory protein 1 (Fragment) | 0.032 | Down      | Cluster of 4OS ribosomal protein S2 (Fragment) OS=Homo sapiens GN=RPS2 PE=1 SV=1 (E9PMM9_HUMAN) | 0.036   | Up        |
| Na(+)/H(+) exchange regulatory cofactor NHERF1 | 0.033   | Down      | Cluster of DNA helicase (Fragment) OS=Homo sapiens PE=2 SV=1 (Q53FG5_HUMAN)  | 0.036   | Up        |
| Cluster of Calpain small subunit 1 | 0.034   | Down      | cDNA FLJ54723, highly similar to Poly (ADP-ribose) polymerase 9 (EC 2.4.2.30) OS=Homo sapiens PE=2 SV=1 | 0.036   | Up        |
| Ran-specific GTPase activating protein | 0.035   | Down      | Cluster of 2-oxoglutarate dehydrogenase, mitochondrial OS=Homo sapiens GN=OGDH PE=1 SV=1 (A0A0D9SFS3_HUMAN) | 0.037   | Up        |
| Cluster of DNA damage Einding protein 1 | 0.035   | Down      | Delta-1-pyrroline 5-carboxylate synthase OS=Homo sapiens GN=ALDH18A1 PE=1 SV=2 | 0.038   | Up        |
| Cluster of Pyruvate kinase OS=Homo sapiens | 0.036   | Down      | cDNA FLJ76962, highly similar to Homo sapiens nucleolar protein 5A (56kDa with KKE/D) | 0.038   | Up        |
| Gene Name                                                                 | Start | Direction | Description                                                                                           | FDR   | Significance |
|---------------------------------------------------------------------------|-------|-----------|-------------------------------------------------------------------------------------------------------|-------|--------------|
| Adenylosuccinate synthetase isozyme 2                                    | 0.036 | Down      | mRNA OS=Homo sapiens PE=2 SV=1                                                                      | 0.039 | Up           |
| Cluster of T-complex protein 1 subunit delta                             | 0.037 | Down      | 4OS ribosomal protein S8 OS=Homo sapiens PE=2 SV=1                                                   | 0.04  | Up           |
| Cluster of Serine/threonine protein phosphatase 2A 65 kDa regulatory subunit A alpha isomorf OS=Homo sapiens GN=PPP2R1A PE=1 SV=4 (2AAA_HUMAN) | 0.037 | Down      | Splicing factor 3B subunit 1 OS=Homo sapiens GN=SF3B1 PE=1 SV=3                                     | 0.04  | Up           |
| Protein kinase C substrate 80K-H, isoform CRA_a OS=Homo sapiens GN=PRKCSH PE=4 SV=1 | 0.037 | Down      | 4OS ribosomal protein S24 OS=Homo sapiens PE=2 SV=1                                                 | 0.041 | Up           |
| Cluster of Methylene tetrahydrofolate dehydrogenase (NADP+ dependent) 1, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase, isoform CRA_a OS=Homo sapiens GN=MTHFD1 PE=3 SV=1 (A0A024R652_HUMAN) | 0.038 | Down      | Cluster of Protein FAM162A OS=Homo sapiens GN=FAM162A PE=1 SV=2 (F162A_HUMAN)                     | 0.042 | Up           |
| RcNSEP1 (Fragment) OS=Homo sapiens PE=4 SV=1                              | 0.038 | Down      | Procollagen galactosyltransferase 1 OS=Homo sapiens GN=COLGALT1 PE=1 SV=1                           | 0.042 | Up           |
| Cluster of S-methyl-5'-thioadenosine phosphorylase OS=Homo sapiens GN=MTAP PE=2 SV=1 (Q6FH1T1_HUMAN) | 0.038 | Down      | Catenin beta-1 OS=Homo sapiens GN=CTNNB1 PE=1 SV=1                                                | 0.043 | Up           |
| Cluster of Dimethylarginine dimethylaminohydrolase                       | 0.038 | Down      | Cluster of elf2AK2 protein OS=Homo sapiens GN=Elf2AK2                                             | 0.045 | Up           |
| Gene Name                                      | Peptide Information | Expression | Fold Change |
|-----------------------------------------------|---------------------|------------|-------------|
| 1, isoform CRA_b                              | OS=Homo sapiens    | PE=2 SV=1 | (B7ZKK7_HUMAN) |
| Cluster of Adenylyl cyclase associated protein| OS=Homo sapiens    | PE=2 SV=1 | (B2RDY9_HUMAN) |
| Prefoldin subunit 2                           | OS=Homo sapiens    | PE=2 SV=1 | (B7ZKK7_HUMAN) |
| Testicular tissue protein Li 198              | OS=Homo sapiens    | PE=2 SV=1 | |
| Thioredoxin domain-containing protein 17      | OS=Homo sapiens    | PE=2 SV=1 | |
| Ubiquitin-conjugating enzyme E2 variant 2    | OS=Homo sapiens    | PE=2 SV=1 | |
| Cluster of Tubulin beta-4A chain              | OS=Homo sapiens    | PE=2 SV=1 | |
| Pyridoxal kinase                              | OS=Homo sapiens    | PE=2 SV=1 | |
| Cluster of cDNA, FLJ96580, highly similar    | OS=Homo sapiens    | PE=2 SV=1 | |
| Cluster of cDNA, FLJ96580, highly similar    | OS=Homo sapiens    | PE=2 SV=1 | |

Cluster of cDNA, FLJ96580, highly similar to Homo sapiens hepatoma-derived growth factor (high-mobility group protein 1-like) (HDGF), mRNA OS=Homo sapiens PE=2 SV=1 (B2RDE8_HUMAN)
| Gene Name                                      | Fold Change | Direction | Gene Name                                      | Fold Change | Direction |
|-----------------------------------------------|-------------|-----------|-----------------------------------------------|-------------|-----------|
| Heterogeneous nuclear ribonucleoprotein D0   | 0.042       | Down      | Cluster of Reticulon-4                        | 0.05        | Up        |
| (Fragment) OS=Homo sapiens GN=HNRNPD PE=1 SV=8|             |           | OS=Homo sapiens GN=RTN4 PE=1 SV=2 (RTN4_HUMAN)|             |           |
Table 11 Proteome response of HT29 cells to treatment with cycloheximide from the perspective of a gene ontological (GO) analysis

| Cycloheximide UPs |  |  |
|-------------------|------------------|------------------|
| source            | term name                                   | adjusted p value | term size |
| GO:BP             | RNA splicing, via transesterification reactions with bulged adenosine as nucleophile | 0.045679         | 334       |
| GO:BP             | mRNA splicing, via spliceosome               | 0.045679         | 334       |
| GO:BP             | RNA splicing, via transesterification reactions | 0.048032         | 337       |
| GO:CC             | ribonucleoprotein complex                    | 1.53 X 10^-05    | 862       |
| GO:CC             | catalytic step 2 spliceosome                 | 6.10 X 10^-05    | 85        |
| GO:CC             | spliceosomal complex                         | 8.82 X 10^-05    | 176       |

| Cycloheximide DOWNs |  |  |
|---------------------|------------------|------------------|
| source              | term name                                   | adjusted p value | term size |
| GO:MF               | ubiquitin protein ligase binding             | 0.011285         | 286       |
| GO:MF               | ubiquitin-like protein ligase binding        | 0.015028         | 301       |
| GO:MF               | peptidase activator activity                | 0.031093         | 39        |
| GO:MF               | ubiquitin-like protein conjugating enzyme activity | 0.036163       | 41        |
| GO:BP               | protein catabolic process                    | 3.72 X 10^-05    | 911       |
| GO:BP               | regulation of protein catabolic process       | 0.000107         | 372       |
| GO:BP               | positive regulation of protein modification by small protein conjugation or removal | 0.000463        | 127       |
| GO:BP               | regulation of protein modification by small protein conjugation or removal | 0.000619        | 220       |
| GO:BP               | proteolysis involved in cellular protein catabolic process | 0.002204       | 696       |
| GO:BP               | cellular protein catabolic process           | 0.004378         | 751       |
| GO:BP               | proteasomal protein catabolic process        | 0.00669          | 451       |
| GO:BP               | regulation of proteolysis involved in cellular protein catabolic process | 0.007841      | 206       |
| GO:BP               | regulation of catabolic process              | 0.016192         | 870       |
| GO:BP               | regulation of cellular protein catabolic process | 0.017517       | 237       |
| GO:BP               | proteasome x 10-mediated ubiquitin-dependent protein catabolic process | 0.027965       | 391       |
| GO:CC               | proteasome complex                           | 0.002105         | 67        |
| GO:CC               | endopeptidase complex                        | 0.002233         | 68        |
| GO:CC               | peptidase complex                            | 0.006792         | 90        |
Table 12: Proteome response of HT29 cells to treatment with ribavirin from the perspective of a GO analysis

| Ribavirin UPs | term name | adjusted p value | term size |
|--------------|-----------|-----------------|-----------|
| GO:MF        | cadherin binding involved in cell-cell adhesion | 0.001339 | 19        |
| GO:MF        | cadherin binding                          | 0.004298 | 323       |
| GO:MF        | cell-cell adhesion mediator activity         | 0.026171 | 50        |
| GO:MF        | cell adhesion mediator activity              | 0.042976 | 59        |
| GO:MF        | cell adhesion molecule binding               | 0.043532 | 489       |
| GO:CC        | cell-cell adherens junction                  | 0.005655 | 112       |
| GO:CC        | adherens junction                           | 0.032212 | 537       |
| GO:CC        | proteasome complex                          | 0.033012 | 67        |
| GO:CC        | endopeptidase complex                        | 0.034499 | 68        |
| GO:CC        | anchoring junction                           | 0.037428 | 552       |

| Ribavirin DOWNs | term name | adjusted p value | term size |
|-----------------|-----------|-----------------|-----------|
| GO:MF           | structural constituent of ribosome          | 0.004931 | 168       |
| GO:MF           | helicase activity                           | 0.039684 | 150       |
| GO:BP           | protein targeting to ER                     | 6.04E-05 | 108       |
| GO:BP           | establishment of protein localization to endoplasmic reticulum | 7.78E-05 | 112       |
| GO:BP           | mRNA metabolic process                      | 9.37E-05 | 784       |
| GO:BP           | ribonucleoprotein complex biogenesis        | 0.000134 | 447       |
| GO:BP           | nuclear-transcribed mRNA catabolic process, nonsense mediated decay | 0.000141 | 122       |
| GO:BP           | protein localization to endoplasmic reticulum | 0.000296 | 136       |
| GO:BP           | SRP-dependent cotranslational protein targeting to membrane | 0.000772 | 96        |
| GO:BP           | cotranslational protein targeting to membrane | 0.001043 | 101       |
| GO:BP           | Nucleobase containing compound catabolic process | 0.001124 | 553       |
| GO:BP           | RNA catabolic process                       | 0.001463 | 345       |
| GO:BP                              | Description                                               | Score   | IDs  |
|-----------------------------------|-----------------------------------------------------------|---------|------|
| GO:BP                             | heterocycle catabolic process                             | 0.002584| 602  |
| GO:BP                             | cellular nitrogen compound catabolic process              | 0.002626| 603  |
| GO:BP                             | protein targeting to membrane                             | 0.002847| 190  |
| GO:BP                             | aromatic compound catabolic process                       | 0.003232| 616  |
| GO:BP                             | RNA processing                                            | 0.00351 | 902  |
| GO:BP                             | rRNA processing                                           | 0.004007| 200  |
| GO:BP                             | organic cyclic compound catabolic process                 | 0.005355| 649  |
| GO:BP                             | nuclear-transcribed mRNA catabolic process                | 0.005538| 210  |
| GO:BP                             | mRNA catabolic process                                    | 0.007079| 311  |
| GO:BP                             | rRNA metabolic process                                    | 0.012281| 237  |
| GO:BP                             | regulation of mRNA metabolic process                     | 0.025407| 265  |
| GO:BP                             | maturation of LSU-rRNA                                    | 0.0314  | 17   |
| GO:BP                             | ribosome biogenesis                                       | 0.037944| 282  |
| GO:CC                             | ribonucleoprotein complex                                 | 2.81E-08| 862  |
| GO:CC                             | cytosolic ribosome                                        | 0.000273| 119  |
| GO:CC                             | nucleolus                                                 | 0.000371| 934  |
| GO:CC                             | polysome                                                  | 0.000582| 75   |
| GO:CC                             | cytosolic part                                            | 0.001415| 244  |
| GO:CC                             | ribosomal subunit                                         | 0.004585| 194  |
| GO:CC                             | ribosome                                                  | 0.017723| 247  |
| KEGG                              | Ribosome                                                  | 0.002587| 149  |
| CORUM                             | Nop56p-associated pre-rRNA complex                        | 0.001674| 104  |
| CORUM                             | Ribosome, cytoplasmic                                     | 0.040959| 80   |
**Table 13 Proteome response of HT29 cells to treatment with 4E1RCat from the perspective of a GO analysis**

| 4E1RCat UPs | source | term name                                                                 | adjusted p value | term size |
|-------------|--------|---------------------------------------------------------------------------|------------------|-----------|
|             | GO:MF  | structural constituent of ribosome                                        | 5.88 X 10^{-08}  | 168       |
|             | GO:MF  | structural molecule activity                                              | 0.010018         | 809       |
|             | GO:BP  | SRP-dependent cotranslational protein targeting to membrane               | 2.46 X 10^{-07}  | 96        |
|             | GO:BP  | cotranslational protein targeting to membrane                             | 3.53 X 10^{-07}  | 101       |
|             | GO:BP  | peptide metabolic process                                                | 4.34 X 10^{-07}  | 884       |
|             | GO:BP  | protein targeting to ER                                                  | 5.68 X 10^{-07}  | 108       |
|             | GO:BP  | establishment of protein localization to endoplasmic reticulum           | 7.35 X 10^{-07}  | 112       |
|             | GO:BP  | peptide biosynthetic process                                             | 7.36 X 10^{-07}  | 727       |
|             | GO:BP  | translational initiation                                                 | 1.13 X 10^{-06}  | 198       |
|             | GO:BP  | nuclear-transcribed mRNA catabolic process, nonsens x 10-mediated decay   | 1.34 X 10^{-06}  | 122       |
|             | GO:BP  | mRNA catabolic process                                                   | 1.52 X 10^{-06}  | 311       |
|             | GO:BP  | protein localization to endoplasmic reticulum                            | 2.89 X 10^{-06}  | 136       |
|             | GO:BP  | RNA catabolic process                                                    | 3.79 X 10^{-06}  | 345       |
|             | GO:BP  | amide biosynthetic process                                               | 4.80 X 10^{-06}  | 857       |
|             | GO:BP  | translation                                                              | 8.83 X 10^{-06}  | 704       |
|             | GO:BP  | protein targeting to membrane                                            | 2.97 X 10^{-05}  | 190       |
|             | GO:BP  | heterocycle catabolic process                                             | 3.03 X 10^{-05}  | 602       |
|             | GO:BP  | cellular nitrogen compound catabolic process                             | 3.07 X 10^{-05}  | 603       |
|             | GO:BP  | establishment of protein localization to membrane                         | 3.47 X 10^{-05}  | 306       |
|             | GO:BP  | aromatic compound catabolic process                                       | 3.76 X 10^{-05}  | 616       |
|             | GO:BP  | nuclear-transcribed mRNA catabolic process                               | 5.91 X 10^{-05}  | 210       |
| GO:BP                          | organic cyclic compound catabolic process | 6.17 X 10^{-05} | 649 |
|-------------------------------|-------------------------------------------|-----------------|-----|
| GO:BP                         | nucleobas x 10-containing compound catabolic process | 0.000224        | 553 |
| GO:BP                         | protein localization to membrane           | 0.000367        | 586 |
| GO:BP                         | protein targeting                          | 0.000384        | 418 |
| GO:BP                         | establishment of protein localization to organelle | 0.002354        | 531 |
| GO:BP                         | mRNA metabolic process                      | 0.0042          | 784 |
| GO:BP                         | ribosomal large subunit biogenesis         | 0.009208        | 71  |
| GO:BP                         | protein localization to organelle           | 0.010942        | 881 |
| GO:CC                         | cytosolic large ribosomal subunit           | 1.26 X 10^{-09} | 64  |
| GO:CC                         | large ribosomal subunit                     | 2.16 X 10^{-09} | 122 |
| GO:CC                         | ribosome                                   | 1.82 X 10^{-08} | 247 |
| GO:CC                         | ribosomal subunit                          | 8.98 X 10^{-08} | 194 |
| GO:CC                         | cytosolic ribosome                         | 1.07 X 10^{-07} | 119 |
| GO:CC                         | ribonucleoprotein complex                   | 4.62 X 10^{-07} | 862 |
| GO:CC                         | cytosolic part                             | 1.57 X 10^{-05} | 244 |
| GO:CC                         | polysome                                   | 2.18 X 10^{-05} | 75  |
| GO:CC                         | polysomal ribosome                         | 2.72 X 10^{-05} | 30  |
| KEGG                          | Ribosome                                   | 1.68 X 10^{-06} | 149 |
| CORUM                         | 60S ribosomal subunit, cytoplasmic          | 5.48 X 10^{-07} | 47  |
| CORUM                         | Ribosome, cytoplasmic                       | 2.55 X 10^{-05} | 80  |
| CORUM                         | Nop56p-associated pr x 10-rRNA complex      | 0.000161        | 104 |

| 4E1RCat DOWNs                     | term name          | adjusted p value | term size |
|-----------------------------------|--------------------|------------------|-----------|
| source                            | term name          | adjusted p value | term size |
| GO:CC                             | nucleoid           | 0.001483         | 43        |
| GO:CC                             | mitochondrial nucleoid | 0.001483      | 43        |
| GO:CC                             | nuclear replisome  | 0.042695         | 25        |
Table 14 Proteome response of HT29 cells to treatment with DMSO from the perspective of a GO analysis

| DMSO UPS | term name                                                                 | adjusted p value | term size |
|----------|---------------------------------------------------------------------------|------------------|-----------|
| GO:BP    | SRP-dependent cotranslational protein targeting to membrane              | 3.20 X 10^-25    | 96        |
| GO:BP    | cotranslational protein targeting to membrane                             | 1.24 X 10^-24    | 101       |
| GO:BP    | protein targeting to ER                                                   | 7.24 X 10^-24    | 108       |
| GO:BP    | establishment of protein localization to endoplasmic reticulum           | 1.87 X 10^-23    | 112       |
| GO:BP    | protein localization to endoplasmic reticulum                            | 9.38 X 10^-23    | 136       |
| GO:BP    | nuclear-transcribed mRNA catabolic process, n 10-mediated decay          | 1.71 X 10^-22    | 122       |
| GO:BP    | translational initiation                                                  | 7.82 X 10^-20    | 198       |
| GO:BP    | translation                                                               | 4.04 X 10^-19    | 704       |
| GO:BP    | peptide biosynthetic process                                              | 1.34 X 10^-18    | 727       |
| GO:BP    | protein targeting to membrane                                             | 1.07 X 10^-17    | 190       |
| GO:BP    | cellular nitrogen compound catabolic process                             | 1.14 X 10^-17    | 603       |
| GO:BP    | aromatic compound catabolic process                                       | 2.35 X 10^-17    | 616       |
| GO:BP    | nucleobas x 10-containing compound catabolic process                     | 5.73 X 10^-17    | 553       |
| GO:BP    | RNA catabolic process                                                     | 6.68 X 10^-17    | 345       |
| GO:BP    | amide biosynthetic process                                                | 7.67 X 10^-17    | 857       |
| GO:BP    | heterocycle catabolic process                                             | 9.86 X 10^-17    | 602       |
| GO:BP    | nuclear-transcribed mRNA catabolic process                               | 1.19 X 10^-16    | 210       |
| GO:BP    | peptide metabolic process                                                 | 2.43 X 10^-16    | 884       |
| GO:BP    | organic cyclic compound catabolic process                                 | 1.15 X 10^-15    | 649       |
| GO:BP    | mRNA catabolic process                                                    | 8.20 X 10^-15    | 311       |
| GO:BP    | establishment of protein localization to membrane                         | 6.63 X 10^-14    | 306       |
| GO:BP | Description | p-value | Count |
|-------|-------------|---------|-------|
| GO:BP | establishment of protein localization to organelle | $1.10 \times 10^{-13}$ | 531 |
| GO:BP | protein localization to organelle | $2.96 \times 10^{-12}$ | 881 |
| GO:BP | protein targeting | $1.08 \times 10^{-11}$ | 418 |
| GO:BP | protein localization to membrane | $4.14 \times 10^{-09}$ | 586 |
| GO:BP | ncRNA metabolic process | $4.89 \times 10^{-09}$ | 544 |
| GO:BP | mRNA metabolic process | $2.31 \times 10^{-08}$ | 784 |
| GO:BP | rRNA metabolic process | $6.98 \times 10^{-06}$ | 237 |
| GO:BP | DNA conformation change | $8.04 \times 10^{-06}$ | 278 |
| GO:BP | ribosome biogenesis | $9.86 \times 10^{-06}$ | 282 |
| GO:BP | ribonucleoprotein complex biogenesis | $3.80 \times 10^{-05}$ | 447 |
| GO:BP | rRNA processing | $5.93 \times 10^{-05}$ | 200 |
| GO:BP | generation of precursor metabolites and energy | $6.66 \times 10^{-05}$ | 463 |
| GO:BP | positive regulation of gene expression, epigenetic | $0.000119$ | 60 |
| GO:BP | DNA metabolic process | $0.000122$ | 999 |
| GO:BP | nucleoside triphosphate metabolic process | $0.000125$ | 294 |
| GO:BP | purine nucleoside triphosphate metabolic process | $0.000363$ | 275 |
| GO:BP | tRNA aminoacylation for protein translation | $0.000379$ | 47 |
| GO:BP | ncRNA processing | $0.000401$ | 368 |
| GO:BP | tRNA aminoacylation | $0.000588$ | 50 |
| GO:BP | cellular amino acid metabolic process | $0.000629$ | 333 |
| GO:BP | amino acid activation | $0.000676$ | 51 |
| GO:BP | drug metabolic process | $0.000719$ | 770 |
| GO:BP | aerobic respiration | $0.000781$ | 76 |
| GO:BP | nucleobase x 10-containing small molecule metabolic process | $0.001094$ | 728 |
| GO:BP | nucleosome assembly | $0.001275$ | 143 |
| GO:BP | carboxylic acid metabolic process | $0.001329$ | 989 |
| GO:BP | purine ribonucleoside triphosphate metabolic process | $0.001757$ | 268 |
| GO:BP | ribonucleoside triphosphate metabolic process | $0.002253$ | 274 |
| GO:BP | nucleotide metabolic process | $0.002259$ | 640 |
| GO:BP | nucleoside phosphate metabolic process | $0.002547$ | 645 |
| GO:BP | ATP metabolic process | $0.003151$ | 238 |
| GO:BP                                      | Description                                           | p-value   | Count |
|-------------------------------------------|-------------------------------------------------------|-----------|-------|
| GO:BP cytoplasmic translation             |                                                       | 0.003416  | 92    |
| GO:BP tricarboxylic acid metabolic process|                                                       | 0.00381   | 42    |
| GO:BP chromatin assembly                  |                                                       | 0.004222  | 163   |
| GO:BP chromosome condensation             |                                                       | 0.005052  | 44    |
| GO:BP DNA packaging                       |                                                       | 0.005259  | 207   |
| GO:BP cellular respiration                |                                                       | 0.005546  | 168   |
| GO:BP interspecies interaction between organisms |                                                 | 0.00649  | 811   |
| GO:BP telomere organization               |                                                       | 0.006503  | 171   |
| GO:BP ribosomal large subunit biogenesis  |                                                       | 0.006694  | 71    |
| GO:BP RNA processing                      |                                                       | 0.010605  | 902   |
| GO:BP nucleosome organization             |                                                       | 0.010802  | 181   |
| GO:BP chromatin assembly or disassembly   |                                                       | 0.015838  | 189   |
| GO:BP nucleosome positioning              |                                                       | 0.015975  | 15    |
| GO:BP oxidative phosphorylation           |                                                       | 0.01604   | 113   |
| GO:BP chromatin organization involved in regulation of transcription | | 0.01604   | 113   |
| GO:BP doubl x 10-strand break repair via nonhomologous end joining | | 0.022388  | 85    |
| GO:BP tricarboxylic acid cycle            |                                                       | 0.024778  | 34    |
| GO:BP purine nucleotide metabolic process |                                                       | 0.025024  | 506   |
| GO:BP telomere maintenance                |                                                       | 0.025356  | 158   |
| GO:BP ribose phosphate metabolic process  |                                                       | 0.025643  | 507   |
| GO:BP negative regulation of gene expression, epigenetic | | 0.028211  | 122   |
| GO:BP non-recombinational repair           |                                                       | 0.037721  | 92    |
| GO:BP regulation of DNA metabolic process |                                                       | 0.037729  | 409   |
| GO:BP citrate metabolic process           |                                                       | 0.043431  | 38    |
| GO:BP negative regulation of chromatin silencing | | 0.044117  | 19    |
| GO:BP energy derivation by oxidation of organic compounds | | 0.045598  | 260   |
| GO:BP purine ribonucleotide metabolic process | | 0.04707   | 474   |
| GO:CC cytosolic ribosome                  |                                                       | 1.21 X 10-25 | 119   |
| GO:CC ribosome                            |                                                       | 5.07 X 10-25 | 247   |
| GO:CC ribonucleoprotein complex           |                                                       | 5.01 X 10-22 | 862   |
| GO:CC ribosomal subunit                   |                                                       | 2.06 X 10-21 | 194   |
| GO:CC cytosolic part                      |                                                       | 8.23 X 10-19 | 244   |
| GO:CC cytosolic large ribosomal subunit   |                                                       | 5.75 X 10-14 | 64    |
| GO:CC large ribosomal subunit             |                                                       | 1.97 X 10-12 | 122   |
| GO:CC                     | Description                                      | Value         | Count |
|---------------------------|--------------------------------------------------|---------------|-------|
| GO:CC                     | mitochondrial matrix                             | 4.27 X 10^-10 | 466   |
| GO:CC                     | cytosolic small ribosomal subunit                | 2.23 X 10^-09 | 48    |
| GO:CC                     | nucleoid                                         | 2.48 X 10^-08 | 43    |
| GO:CC                     | mitochondrial nucleoid                           | 2.48 X 10^-08 | 43    |
| GO:CC                     | protein-DNA complex                              | 6.31 X 10^-08 | 203   |
| GO:CC                     | organelle inner membrane                         | 1.03 X 10^-07 | 506   |
| GO:CC                     | small ribosomal subunit                          | 2.33 X 10^-07 | 75    |
| GO:CC                     | focal adhesion                                   | 5.03 X 10^-07 | 404   |
| GO:CC                     | cell-substrate adherens junction                 | 5.69 X 10^-07 | 407   |
| GO:CC                     | cell-substrate junction                          | 6.68 X 10^-07 | 411   |
| GO:CC                     | mitochondrial envelope                           | 6.75 X 10^-07 | 723   |
| GO:CC                     | mitochondrial membrane                           | 9.85 X 10^-07 | 680   |
| GO:CC                     | chromosomal part                                 | 1.22 X 10^-06 | 924   |
| GO:CC                     | mitochondrial inner membrane                     | 2.83 X 10^-06 | 449   |
| GO:CC                     | polysome                                         | 4.38 X 10^-06 | 75    |
| GO:CC                     | nucleolus                                        | 6.48 X 10^-06 | 934   |
| GO:CC                     | pigment granule                                  | 6.51 X 10^-06 | 105   |
| GO:CC                     | melanosome                                        | 6.51 X 10^-06 | 105   |
| GO:CC                     | nucleosome                                       | 9.35 X 10^-06 | 109   |
| GO:CC                     | adherens junction                                | 9.47 X 10^-06 | 537   |
| GO:CC                     | anchoring junction                                | 1.49 X 10^-05 | 552   |
| GO:CC                     | nuclear chromosome                               | 1.51 X 10^-05 | 608   |
| GO:CC                     | DNA packaging complex                            | 1.85 X 10^-05 | 117   |
| GO:CC                        | term name                                                                 | adjusted p value | term size |
|------------------------------|----------------------------------------------------------------------------|------------------|-----------|
| nuclear chromosome, telomeric region | 3.21 X 10-05                                                             | 124              |
| GO:CC                        | polysomal ribosome                                                         | 5.28 X 10-05     | 30        |
| mitochondrial protein complex | 0.000108                                                                  | 260              |
| nuclear chromosome part      | 0.000119                                                                  | 570              |
| chromosome, telomeric region | 0.00029                                                                   | 157              |
| aminoacyl-tRNA synthetase multienzyme complex | 0.000727                                                                 | 12               |
| chromatin                    | 0.001508                                                                  | 553              |
| GO:CC                        | chromosomal region                                                         | 0.002642         | 347       |
| GO:CC                        | organelle outer membrane                                                  | 0.02339          | 209       |
| GO:CC                        | outer membrane                                                            | 0.025162         | 211       |
| catalytic step 2 spliceosome  | 0.026484                                                                  | 85               |
| inner mitochondrial membrane protein complex | 0.037937                                                                 | 130              |
| Ribosome                     | 3.51 X 10-16                                                              | 149              |
| KEGG                         | Biosynthesis of amino acids                                               | 0.000168         | 73        |
| KEGG                         | Citrate cycle (TCA cycle)                                                 | 0.000521         | 30        |
| KEGG                         | Carbon metabolism                                                         | 0.001097         | 115       |
| KEGG                         | Parkinson disease                                                         | 0.003001         | 129       |
| KEGG                         | Non-homologous end-joining                                                | 0.003257         | 13        |
| KEGG                         | Aminoacyl-tRNA biosynthesis                                               | 0.005164         | 44        |
| KEGG                         | Huntington disease                                                        | 0.013718         | 185       |
| Ribosome, cytoplasmic        | 1.70 X 10-16                                                              | 80               |
| Nop56p-associated pr x 10-rRNA complex | 7.48 X 10-15                                                             | 104              |
| 60S ribosomal subunit, cytoplasmic | 1.38 X 10-09                                                              | 47               |
| 40S ribosomal subunit, cytoplasmic | 1.49 X 10-05                                                              | 31               |
| 40S ribosomal subunit, cytoplasmic | 2.74 X 10-05                                                              | 33               |
| TRBP containing complex (DICER, RPL7A, EIF6, MOV10 and subunits of the 60S ribosomal particle) | 3.71 X 10-05 | 25 |
| Multisynthetase complex      | 0.037748                                                                  | 11               |
| prohibitin 2 complex, mitochondrial | 0.049881                                                                 | 5                |

| DMSO UPs                     | source term name                                          | adjusted p value | term size |
|------------------------------|-----------------------------------------------------------|------------------|-----------|
| mRNA metabolic process       | GO:BP                                                     | 6.35 X 10-10     | 784       |
| GO:BP                  | Description                                                                 | Value       | Count |
|-----------------------|-----------------------------------------------------------------------------|-------------|-------|
| GO:BP                 | peptide metabolic process                                                  | 2.09 X 10^-08 | 884   |
| GO:BP                 | peptide biosynthetic process                                               | 4.72 X 10^-08 | 727   |
| GO:BP                 | RNA splicing                                                               | 1.11 X 10^-07 | 422   |
| GO:BP                 | amide biosynthetic process                                                 | 1.72 X 10^-07 | 857   |
| GO:BP                 | RNA splicing, via transesterification reactions                            | 3.61 X 10^-07 | 337   |
| GO:BP                 | protein folding                                                            | 4.19 X 10^-07 | 211   |
| GO:BP                 | translation                                                                | 4.68 X 10^-07 | 704   |
| GO:BP                 | negative regulation of mRNA metabolic process                             | 5.71 X 10^-07 | 70    |
| GO:BP                 | nucleobas x 10-containing compound catabolic process                       | 1.06 X 10^-06 | 553   |
| GO:BP                 | regulation of mRNA metabolic process                                       | 1.95 X 10^-06 | 265   |
| GO:BP                 | mRNA splicing, via spliceosome                                             | 2.01 X 10^-06 | 334   |
| GO:BP                 | RNA splicing, via transesterification reactions with bulged adenosine as nucleophile | 2.01 X 10^-06 | 334   |
| GO:BP                 | regulated exocytosis                                                       | 2.10 X 10^-06 | 750   |
| GO:BP                 | aromatic compound catabolic process                                        | 2.22 X 10^-06 | 616   |
| GO:BP                 | RNA catabolic process                                                      | 3.52 X 10^-06 | 345   |
| GO:BP                 | mRNA catabolic process                                                     | 3.86 X 10^-06 | 311   |
| GO:BP                 | heterocycle catabolic process                                              | 6.31 X 10^-06 | 602   |
| GO:BP                 | cellular nitrogen compound catabolic process                               | 6.54 X 10^-06 | 603   |
| GO:BP                 | organic cyclic compound catabolic process                                  | 6.82 X 10^-06 | 649   |
| GO:BP                 | neutrophil degranulation                                                   | 8.91 X 10^-06 | 483   |
| GO:BP                 | neutrophil activation involved in immune response                          | 1.00 X 10^-05 | 486   |
| GO:BP                 | symbiont process                                                           | 1.26 X 10^-05 | 762   |
| GO:BP                 | exocytosis                                                                 | 1.43 X 10^-05 | 864   |
| GO:BP                                      | Description                                                | Fold Change | p-value |
|-------------------------------------------|------------------------------------------------------------|-------------|---------|
| GO:BP neutrophil activation               |                                                           | 1.47 X 10^-05 | 496     |
| GO:BP neutrophil mediated immunity        |                                                           | 1.53 X 10^-05 | 497     |
| GO:BP granulocyte activation              |                                                           | 1.91 X 10^-05 | 503     |
| GO:BP viral process                       |                                                           | 2.99 X 10^-05 | 696     |
| GO:BP leukocyte degranulation             |                                                           | 4.86 X 10^-05 | 529     |
| GO:BP interspecies interaction between organisms |                                                   | 4.99 X 10^-05 | 811     |
| GO:BP myeloid cell activation involved in immune response |                                      | 6.63 X 10^-05 | 538     |
| GO:BP protein localization to organelle   |                                                           | 8.29 X 10^-05 | 881     |
| GO:BP nuclear transport                   |                                                           | 8.35 X 10^-05 | 337     |
| GO:BP mRNA processing                     |                                                           | 8.40 X 10^-05 | 501     |
| GO:BP interleukin-12-mediated signaling pathway |                                             | 8.47 X 10^-05 | 47      |
| GO:BP myeloid leukocyte mediated immunity |                                                           | 8.68 X 10^-05 | 546     |
| GO:BP negative regulation of mRNA splicing, via spliceosome |                               | 0.000115     | 20      |
| GO:BP cellular response to interleukin-12 |                                                           | 0.000119     | 49      |
| GO:BP response to interleukin-12          |                                                           | 0.000141     | 50      |
| GO:BP regulation of mRNA stability        |                                                           | 0.000173     | 119     |
| GO:BP regulation of catabolic process     |                                                           | 0.000226     | 870     |
| GO:BP establishment of protein localization to organelle |                       | 0.000232     | 531     |
| GO:BP regulation of RNA stability         |                                                           | 0.000339     | 127     |
| GO:BP nucleocytoplasmic transport         |                                                           | 0.000401     | 334     |
| GO:BP RNA processing                      |                                                           | 0.000482     | 902     |
| GO:BP negative regulation of RNA splicing |                                                           | 0.000501     | 25      |
| GO:BP drug metabolic process              |                                                           | 0.000856     | 770     |
| GO:BP regulation of mRNA catabolic process |                                                       | 0.000978     | 141     |
| GO:BP myeloid leukocyte activation        |                                                           | 0.001267     | 635     |
| GO:BP negative regulation of mRNA processing |                                                   | 0.001295     | 29      |
| GO:BP protein localization to nucleus     |                                                           | 0.001379     | 247     |
| GO:BP leukocyte activation involved in immune response |                       | 0.001623     | 694     |
| GO:BP cytoplasmic translation             |                                                           | 0.001634     | 92      |
| GO:BP negative regulation of programmed cell death |                           | 0.001733     | 960     |
| GO:BP cell activation involved in immune response |                       | 0.001797     | 698     |
| GO:BP                                      | Description                                                        | p-value | Count |
|-------------------------------------------|--------------------------------------------------------------------|---------|-------|
| GO:BPcellular amino acid metabolic process | cell biological processes                                          | 0.001986| 333   |
| GO:BP regulation of cellular catabolic process | cell biological processes                                          | 0.002288| 759   |
| GO:BP positive regulation of protein localization to Cajal body | biological regulation                                           | 0.003443| 9     |
| GO:BP regulation of protein localization to Cajal body | biological regulation                                           | 0.003443| 9     |
| GO:BP RNA localization                     | biological regulation                                             | 0.003762| 198   |
| GO:BP negative regulation of apoptotic process | biological regulation                                             | 0.004064| 946   |
| GO:BP translational initiation             | biological regulation                                             | 0.004373| 134   |
| GO:BP ribonucleoprotein complex assembly   | biological regulation                                             | 0.00455 | 235   |
| GO:BP regulation of RNA splicing           | biological regulation                                             | 0.004889| 166   |
| GO:BP protein stabilization                | biological regulation                                             | 0.004922| 10    |
| GO:BP ATP metabolic process                | biological regulation                                             | 0.005235| 238   |
| GO:BP chaperon x 10-mediated protein folding | biological regulation                                             | 0.005321| 56    |
| GO:BP protein localization to Cajal body   | biological regulation                                             | 0.005688| 10    |
| GO:BP positive regulation of establishment of protein localization to telomere | biological regulation                                             | 0.005688| 10    |
| GO:BP protein localization to nuclear body | biological regulation                                             | 0.005688| 10    |
| GO:BP positive regulation of organelle organization | biological regulation                                             | 0.007231| 602   |
| GO:BP cofactor metabolic process           | biological regulation                                             | 0.007491| 554   |
| GO:BP ribonucleoprotein complex subunit organization | biological regulation                                             | 0.008596| 249   |
| GO:BP regulation of establishment of protein localization to telomere | biological regulation                                             | 0.008861| 11    |
| GO:BP carboxylic acid metabolic process    | biological regulation                                             | 0.0095  | 989   |
| GO:BP nucleoside triphosphate metabolic process | biological regulation                                             | 0.010643| 294   |
| GO:BP protein export from nucleus          | biological regulation                                             | 0.01261 | 183   |
| GO:BP regulation of establishment of protein localization to chromosome | biological regulation                                             | 0.013176| 12    |
| GO:BP positive regulation of protein localization to chromosome, telomeric region | biological regulation                                             | 0.013176| 12    |
| GO:BP protein localization to nucleoplasm  | biological regulation                                             | 0.013176| 12    |
| GO:BP proteasomal ubiquitin-independent protein catabolic process | biological regulation                                             | 0.013791| 25    |
| GO:BP ribonucleotidet metabolic process     | biological regulation                                             | 0.01796 | 489   |
| GO:BP purine ribonucleoside triphosphate metabolic process | biological regulation                                             | 0.019033| 268   |
| GO:BP regulation of translation            | biological regulation                                             | 0.019468| 398   |
| GO:BP purin x 10-containing compound metabolic process | biological regulation                                             | 0.019549| 541   |
| GO:BP ribonucleoside triphosphate metabolic process | biological regulation                                             | 0.024107| 274   |
| GO:BP purine nucleoside triphosphate metabolic process | biological regulation                                             | 0.02506 | 275   |
| GO:BP sulfur compound metabolic process     | biological regulation                                             | 0.02604 | 362   |
| GO:BP regulation of protein localization to chromosome, telomeric region | biological regulation                                             | 0.026184| 14    |
| GO:BP                                      | Description                                                | p-value | Count |
|--------------------------------------------|-------------------------------------------------------------|---------|-------|
| GO:BP                                      | nuclear export                                             | 0.026583| 198   |
| GO:BP                                      | positive regulation of catabolic process                    | 0.027289| 409   |
| GO:BP                                      | regulation of mRNA splicing, via spliceosome                | 0.028151| 99    |
| GO:BP                                      | purine nucleotide metabolic process                        | 0.028473| 506   |
| GO:BP                                      | negative regulation of protein polymerization               | 0.029089| 72    |
| GO:BP                                      | ribose phosphate metabolic process                         | 0.029236| 507   |
| GO:BP                                      | supramolecular fiber organization                          | 0.029339| 660   |
| GO:BP                                      | generation of precursor metabolites and energy              | 0.033165| 463   |
| GO:BP                                      | cellular aldehyde metabolic process                        | 0.034877| 74    |
| GO:BP                                      | positive regulation of telomerase RNA localization to Cajal body | 0.035395| 15    |
| GO:BP                                      | cytoplasmic translational initiation                       | 0.041777| 31    |
| GO:BP                                      | purine ribonucleotide metabolic process                    | 0.044737| 474   |
| GO:CC                                      | cytoplasmic vesicle lumen                                  | 5.76E-12| 336   |
| GO:CC                                      | vesicle lumen                                              | 6.17E-12| 337   |
| GO:CC                                      | secretory granule lumen                                    | 1.58E-11| 319   |
| GO:CC                                      | adherens junction                                          | 1.49E-10| 537   |
| GO:CC                                      | anchoring junction                                         | 2.96E-10| 552   |
| GO:CC                                      | ribonucleoprotein complex                                  | 7.94E-09| 862   |
| GO:CC                                      | cell-substrate junction                                    | 2.42E-08| 411   |
| GO:CC                                      | focal adhesion                                             | 1.09E-07| 404   |
| GO:CC                                      | cell-substrate adherens junction                           | 1.26E-07| 407   |
| GO:CC                                      | ficolin-1-rich granule lumen                               | 2.10E-07| 123   |
| GO:CC                                      | ficolin-1-rich granule                                     | 2.10E-07| 123   |
| GO:CC                                      | cytosolic part                                             | 2.45E-06| 244   |
| GO:CC                                      | chaperone complex                                          | 7.91E-06| 18    |
| GO:CC                                      | chaperonin-containing T-complex                             | 1.12E-05| 10    |
| GO:CC                                      | proteasome complex                                         | 1.23E-05| 67    |
| GO:CC                                      | endopeptidase complex                                      | 1.41E-05| 68    |
| GO:CC                        | Description                                | Score   | P-value  |
|------------------------------|--------------------------------------------|---------|----------|
| contractile fiber part       |                                            | 2.59 X 10^-05 | 216     |
| contractile fiber            |                                            | 6.28 X 10^-05 | 232     |
| supramolecular fiber         |                                            | 6.60 X 10^-05 | 941     |
| actin cytoskeleton           |                                            | 6.82 X 10^-05 | 482     |
| supramolecular polymer       |                                            | 7.71 X 10^-05 | 948     |
| supramolecular complex       |                                            | 7.88 X 10^-05 | 949     |
| secretory vesicle            |                                            | 0.000144 | 977     |
| zona pellucida receptor complex |                                        | 0.000155 | 7       |
| peptidase complex            |                                            | 0.000165 | 90      |
| myofibril                    |                                            | 0.000224 | 220     |
| secretory granule            |                                            | 0.000232 | 831     |
| cell-cell junction           |                                            | 0.000491 | 450     |
| spliceosomal complex         |                                            | 0.000983 | 176     |
| cytoplasmic stress granule   |                                            | 0.001033 | 59      |
| proteasome core complex      |                                            | 0.001678 | 24      |
| sarcomere                    |                                            | 0.003199 | 199     |
| cell cortex part             |                                            | 0.003342 | 163     |
| cytoplasmic region           |                                            | 0.004385 | 476     |
| cell cortex                  |                                            | 0.004522 | 289     |
| cortical cytoskeleton        |                                            | 0.004924 | 103     |
| perinuclear region of cytoplasm |                                        | 0.004951 | 693     |
| hemidesmosome                |                                            | 0.014924 | 7       |
| myelin sheath                |                                            | 0.025036 | 166     |
| polymeric cytoskeletal fiber |                                            | 0.026699 | 719     |
| ribonucleoprotein granule    |                                            | 0.040086 | 218     |
| Spliceosome                  |                                            | 0.00028  | 45      |
| Proteasome                   |                                            | 0.000299 | 132     |
| 26S proteasome               |                                            | 0.000344 | 22      |
| 20S proteasome               |                                            | 0.006425 | 14      |
| CCT complex (chaperonin containing TCP1 complex) | | 0.009222 | 8      |
| PA28gamma-20S proteasome     |                                            | 0.009421 | 15      |
| PA700-20S-PA28 complex       |                                            | 0.012117 | 36      |
| PA28-20S proteasome          |                                            | 0.013395 | 16      |
| PABPC1-HSPA8-HNRPD-EIF4G1 complex |                        | 0.020393 | 4       |
| BBS-chaperonin complex       |                                            | 0.026472 | 10      |
| Multiprotein complex (mRNA turnover) |                        | 0.049927 | 5       |
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