AN INVESTIGATION OF THE ONCOGENIC POTENTIAL AND FUNCTION OF THE DUAL SPECIFICITY PHOSPHATASE 12

by

ERICA L. CAIN

B.A., Kansas State University, 2007

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Division of Biology
College of Arts and Sciences

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Abstract

Large-scale genomic approaches have demonstrated many atypical dual specificity phosphatases (DUSPs) are differentially expressed or mutated in cancer. DUSPs are proteins predicted to have the ability to dephosphorylate Ser/Thr and Tyr residues, and the atypical DUSP subgroup contains at least 16 members with diverse substrates that include proteins, nucleic acids, and sugars, and some of the atypical DUSPs function in the cell not as phosphatases but as scaffolds in signal transduction pathways. Of the atypical DUSPs, DUSP12 is one of the most evolutionarily conserved with homologs found in organisms ranging from yeast to humans. DUSP12 is of particular interest as it has been identified to be one of only two candidate genes for the target of a genetic amplification found in liposarcomas. Furthermore, DUSP12 may be an oncogene in that over-expression of *dusp12* in cell culture promotes apoptosis resistance, cell motility, and the up-regulation of two established oncogenes, the hepatocyte growth factor receptor (*c-met*) and integrin alpha 1 (*itga1)*. Additionally, DUSP12 may protect from apoptosis by functioning as a regulator of stress-induced translation repression and stress granule formation that may be due to its interaction with the DEAD Box RNA Helicase, DDX3.

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Approved by:

Major Professor
Dr. Alexander Beeser

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Large-scale genomic approaches have demonstrated many atypical dual specificity phosphatases (DUSPs) are differentially expressed or mutated in cancer. DUSPs are proteins predicted to have the ability to dephosphorylate Ser/Thr and Tyr residues, and the atypical DUSP subgroup contains at least 16 members with diverse substrates that include proteins, nucleic acids, and sugars, and some of the atypical DUSPs function in the cell not as phosphatases but as scaffolds in signal transduction pathways. Of the atypical DUSPs, DUSP12 is one of the most evolutionarily conserved with homologs found in organisms ranging from yeast to humans. DUSP12 is of particular interest as it has been identified to be one of only two candidate genes for the target of a genetic amplification found in liposarcomas. Furthermore, DUSP12 may be an oncogene in that over-expression of *dusp12* in cell culture promotes apoptosis resistance, cell motility, and the up-regulation of two established oncogenes, the hepatocyte growth factor receptor (*c-met*) and integrin alpha 1 (*itga1)*. Additionally, DUSP12 may protect from apoptosis by functioning as a regulator of stress-induced translation repression and stress granule formation that may be due to its interaction with the DEAD Box RNA Helicase, DDX3.

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Dedication

This work is dedicated to my two children, Victor and Vivian, who arrived in the final year of my graduate studies and have proved to be an incredible source of joy and laughter. I hope that throughout their life, they always shoot for the stars. I love you both.

Chapter 1 - Introduction

As of 2010, cancer is the number two cause of death in the United States (Murphy *et al.* 2012), and the most recent statistics available from the National Cancer Institute and Centers for Disease Control reveal that since 1978, cancer mortality rates have only dropped fourteen percent (Figure 1.1). "Cancer" describes a diverse collection of neoplastic diseases that arise from aberrant cell signaling produced by multiple genomic alterations that result in the activation and inactivation of oncogenes and tumor suppressors, respectively [Reviewed in Hanahan and Weinberg, 2011]. One means to reduce cancer mortality rates would be discovery of new cancer therapies. The identification of new oncogenes and tumor suppressors that drive cancer development and/or progression can reveal new potential drug targets for cancer therapy. By examining tumor genomes for genomic alterations such as amplifications, deletions, and translocations, new cancer relevant genes may be identified. The atypical dual specificity phosphatase 12, encoded by the *dusp12* gene, is amplified and/or over-expressed in multiple cancers (Hirai *et al.,* 1999; Gratias *et al.,* 2005; Kresse *et al.,* 2005; Mendryzk *et al.,* 2006). The amplification and over-expression of *dusp12* in various cancers suggests that DUSP12 might promote the development and/or progression of cancer. As the function of DUSP12 in normal and cancer cell biology is unclear, whether *dusp12* functions as an oncogene remains to be determined.

DUSP12: an atypical dual specificity phosphatase

DUSP12 belongs to the dual specificity subfamily of the protein tyrosine phosphatases (PTPs) (Guan *et al.*, 1992). DUSPs can be further divided into subgroups based on substrate specificity and sequence homology [Reviewed in (Patterson *et al.*, 2009)], but all DUSPs, including DUSP12, contain a highly conserved catalytic domain that is homologous to the first DUSP identified encoded by the VH1 gene of Vaccinia virus (Guan *et al.*, 1991). DUSPs share the consensus D…HC(X)$_5$RS/T catalytic cysteine sequence (Denu and Dixon, 1995) of class I PTPs (PTP catalytic mechanism overviewed in Figure 1.2), but unlike other PTPs (Zhang *et al.*, 1994), the DUSPs do not contain the phospho-tyrosine recognition domain. This results in a

shallower catalytic clef, which most likely enables DUSPs to dephosphorylate not only phosphorylated tyrosine, but phosphorylated serine and threonine residues as well (Denu and Dixon, 1995).

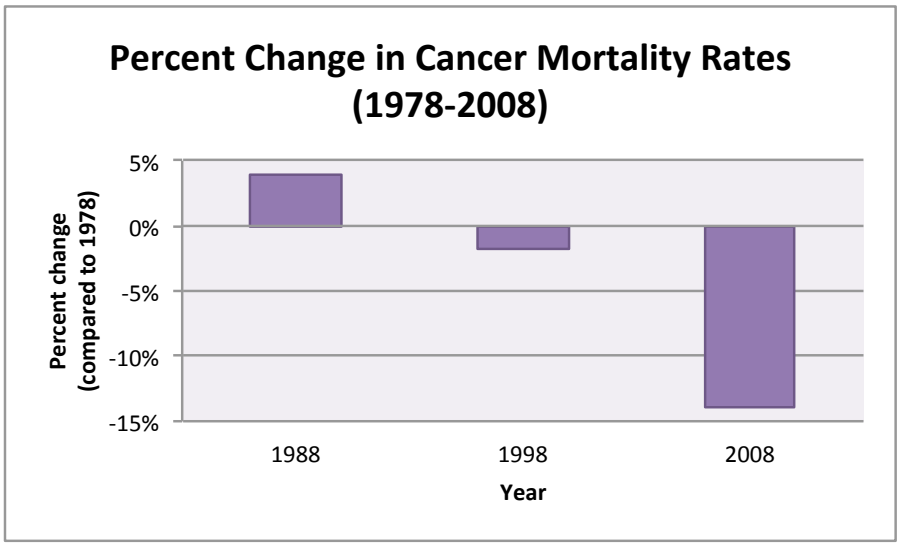

Figure 1.1 Percent change in cancer mortality rates (1978-2008)

The percent change in cancer mortality rates for 1988, 1998, and 2008 were compared to 1978 were calculated from data available from the National Cancer Institute's Fast Stats program. Data from the U.S. age-adjusted mortality rates for all cancer sites, age groups, races, and sexes were used.

Fast Stats: An interactive tool for access to SEER cancer statistics. Surveillance Research Program, National Cancer Institute. http://seer.cancer.gov/faststats. (Accessed on 3-21-2012)

DUSP12 belongs to the atypical subgroup of the DUSP subfamily (Patterson *et al.,* 2009*)*. Atypical DUSPs are classified as such due to a lack of sequence similarity to better-characterized DUSPs and/or due to differences in substrate specificity compared to other DUSP subgroups (Patterson *et al.*, 2009). The atypical DUSP subgroup contains members that dephosphorylate proteins (Liu *et al.*, 2006; Todd *et al.*, 1999; Todd *et al.*, 2002; Sekine *et al.*, 2006; Sekine *et al.*, 2007), nucleic acids (Yuan *et al.*, 1998), and sugars (Worby *et al.*, 2006). Additionally, some atypical DUSPs function independently of their phosphatase activity and instead function as scaffolds in signal transduction pathways (Zama *et al.,* 2002; Takagaki *et al.*, 2004; Park *et al.,* 2010). As the atypical DUSPs regulate diverse cellular processes such as

proliferation and apoptosis, they are often deregulated in diseases such as cancer (Patterson *et al.* 2009).

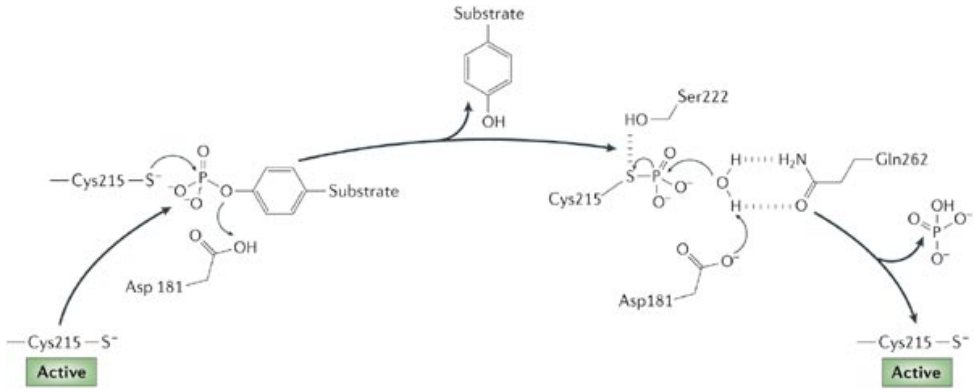

Figure 1.2 An overview of the catalytic mechanism of class I protein tyrosine phosphatases. The catalytic mechanism for protein tyrosine phosphatase 1B (PTP1B) is shown. All class I PTPs use this same basic catalytic mechanism. Catalysis proceeds through a two-step mechanism that involves the production of a cysteinyl-phosphate intermediate. In the first step, there is a nucleophlic attack on the phosphate by the sulphur atom of the thiolate ion of the essential cysteine residue. This is coupled with protonation of the tyrosyl leaving group of the substrate by the conserved aspartic acid residue. The second step involves the hydrolysis of the phosphoenzyme intermediate mediated by a glutamine residue, which coordinates a water molecule, and the aspartic acid residue, which now functions as a general base, culminating in the release of phosphate. Reprinted and adapted by permission from Macmillan Publishers Ltd: Nature Reviews Molecular Cell Biology (Tonks, 2006), copyright (2006).

DUSP12-from yeast to man

Conserved domains of the DUSP12 protein

DUSP12 is one of the most evolutionarily conserved atypical DUSPs, with DUSP12 homologs in species such as, the yeasts, *Saccharomyces cerevisiae* (Guan *et al.*, 1992) and *Candida albicans* (Hanaoka *et al.*, 2005), the malaria parasite, *Plasmodium falciparum* (Kumar *et al.*, 2004), the fruit fly, *Drosophila melanogaster* (Sun *et al.*, 2008), and in the mammals (rats (Munoz-Alonso *et al.*, 2000), mice (Zhang *et al.*, 2000), and humans (Muda *et al.*, 1999)). Importantly, in budding yeast, DUSP12 (designated Yvh1p) can be substituted with human DUSP12, to suppress the phenotypes of *yvh1Δ* yeast strains (Muda *et al.*, 1999). As DUSP12 can

functionally substitute its yeast counterpart, they are not only homologs, but functional orthologs as well (Muda *et al.*, 1999). Therefore, we can gain insights into the function of human DUSP12 by examining DUSP12 function in model organisms.

All DUSP12 homologs contain two distinct domains (Figure 1.2). The N-terminal domain, as previously described, is the DUSP domain, which is shared among all dual specificity phosphatases, and predicts the ability of DUSP12 to dephosphorylate phospho-serine, phospho-threonine, and phospho-tyrosine residues. While no substrates for human DUSP12 have been identified, DUSP12 does demonstrate phosphatase activity towards the artificial substrates, *p*-nitrophenyl phosphate, 3-*O*-methylfluorescein phosphate (Muda *et al.*, 1999), and 6,8-difluoro-4-methylumbelliferyl phosphate (Bonham and Vacratsis, 2009), and the budding yeast DUSP12 ortholog (designated Yvh1p), demonstrates activity towards phospho-tyrosine containing casein, but not phospho-serine containing casein *in vitro* (Guan *et al.*, 1992). In addition to the DUSP domain, all DUSP12 homologs contain an extended cysteine rich C-terminus (Muda *et al.*, 1999; Guan *et al.*, 1992; Zhang *et al.*, 2000; Kumar *et al.*, 2004; Munoz-Alonso *et al.*, 2000; Hanaoka *et al.*, 2005; Sun *et al.*, 2008)(Figure 1.2) which is unique to DUSP12 homologs. While the CRD has been demonstrated to bind zinc (Muda *et al.*, 1999; Kumar *et al.*, 2004), the function of the CRD is unclear.

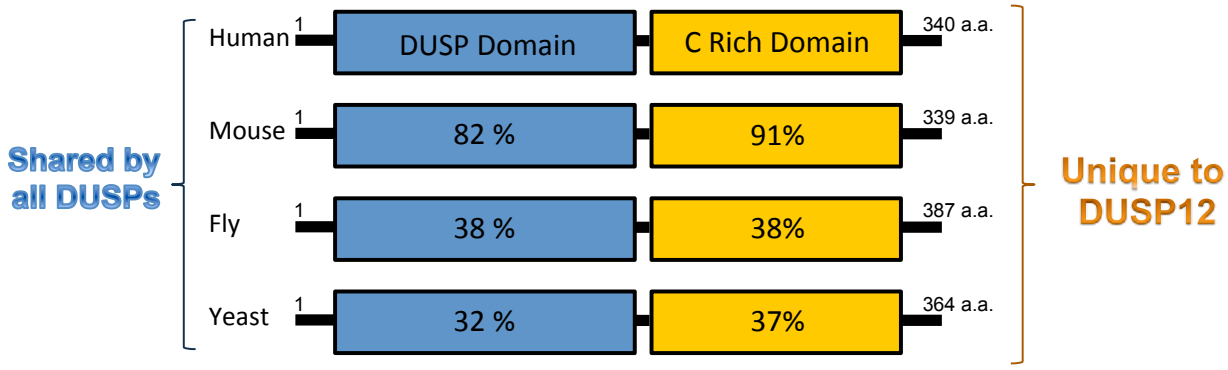

Figure 1.3 Percent identity of DUSP12 homologs compared to human DUSP12.
Using the NCBI Blast alignment tool, the two domains of the DUSP12 homologs in mice, flies, and baker's yeast were compared to human DUSP12. The DUSP domain (amino acids 26-166 in human DUSP12) is found in all dual specificity phosphatases. The cysteine rich domain (CRD)

(amino acids 220-313 in human DUSP12) is unique to DUSP12 homologs and has been demonstrated to bind zinc (Muda *et al.*, 1999).

The knowledge of DUSP12 function prior to 2008

Here I present the literature concerning DUSP12 that existed at the beginning of this thesis work in 2008, as this strongly shaped the original objectives of this research. During the course of this work, several reports, in addition to mine, came out concerning DUSP12 function in both yeast and humans, and a review of this literature is presented in the section on DUSP12 in chapter two of this thesis.

Prior to 2008, relatively few studies existed on DUSP12 or DUSP12 homologs in other species, and only one of these studies focused specifically on the human DUSP12 protein (Muda *et al.* 1999). Human DUSP12 is homologous to Yvh1p in yeast, which was first identified twenty years ago (Guan *et al.*, 1992). Most insights into DUSP12 function come from studies of Yvh1p. Importantly, human DUSP12 is a functional ortholog of yeast Yvh1p, as human *dusp12* could functionally complement *YVH1* in *yvh1Δ* yeast (Muda *et al.,* 1999), supporting the use of *S. cerevisiae,* as a model organism to study DUSP12 function.

Disruption of the *YVH1* gene results in a diverse set of phenotypes including slow growth (Guan *et al.*, 1992), and defects in glycogen accumulation (Beeser and Cooper, 2000), meiosis, and sporulation (Park *et al.,* 1996). *YVH1* also appears to function in sub-optimal growth conditions as its transcription is induced by nitrogen starvation (Guan *et al.,* 1992) and low temperatures (Sakumoto *et al.,* 1999), and *yvh1Δ* strains have impaired stress response element mediated gene expression (Beeser and Cooper, 2000). To examine whether the phosphatase activity is required for Yvh1p function, Muda *et al.* inactivated the phosphatase activity of Yvh1p by altering the catalytic cysteine (C117) to a serine (Muda *et al.,* 1999). The essential catalytic cysteine of PTPs acts as a nucleophile to attack the phosphorus atom of the phosphorylated residue during dephosphorylation (Barford, *et al.,* 1994), and alteration of the catalytic cysteine to a serine blocks the nucleophilic attack and hence inactivates the phosphatase activity, but does not stop substrate binding (Jia *et al.,* 1995). Expression of the catalytically inactive variant of Yvh1p in *yvh1Δ* yeast strains, suppressed the growth defect in yeast lacking *YVH1* (Muda *et al.*, 1999). However, as the Yvh1p C117S variant most likely does not inhibit substrate binding (Jia *et al.,* 1995), it is possible that the Yvh1p C117S could be complexing with

its substrate and thereby inhibiting it (Flint *et al.,* 1997), thus the requirement of the phosphatase activity of Yvh1p for it's function could not yet be ruled out. A year later, Beeser and Cooper demonstrated that not only was the function of Yvh1p independent of it's phosphatase activity, but expression of a *YVH1* mutant containing only the cysteine rich domain (CRD) (amino acids 206-364) could suppress all the *yvh1Δ* phenotypes. This demonstrated that the CRD of unknown function, and not the catalytic phosphatase domain, is sufficient for Yvh1p function in yeast (Beeser and Cooper, 2000). Additionally, Yvh1p may directly regulate the cyclic AMP-dependent protein kinase A cascade, as reduction in cAMP levels partially suppressed the sporulation and glycogen accumulation defects (but not the slow growth phenotype) of *yvh1Δ* yeast (Beeser and Cooper, 2000).

As the identification of interacting proteins can often provide insights into the function of a protein, Sakumoto *et al.* sought to identify Yvh1p interacting proteins via a yeast two-hybrid experiment, where they identified Nop7p as an Yvh1p interacting protein (Sakumoto *et al.*, 2001). Nop7p is a regulator of both DNA replication (Du and Stillman, 2002) and ribosome biogenesis (Harnpicharnchai *et al.*, 2001). Nop7p is a component of the origin of recognition complex (ORC), which is the platform for the assembly of different protein complexes at origins of DNA replication, and loss of the *NOP7* gene is lethal in yeast (Du and Stillman, 2002)

Ribosome biogenesis is a complex process that is regulated spatially and temporally [Reviewed in (Kressler *et al.*, 2010)]. Ribosomal RNA is produced and processed in the nucleolus and the two subunits, 40S and 60S subunits, are first assembled with ribosomal proteins in the nucleus (Kressler *et al.*, 2010). These pre-40S and pre-60S subunits are then exported into the cytoplasm where further maturation occurs that eventually results in the binding of the 40S and 60S to mRNA in the mature translation competent 80S ribosomal complex (Kressler *et al.*, 2010) (Figure 1.3). During translation of an mRNA, multiple ribosomes bind and translate simultaneously, and this is described as the polysome. Free 40S, 60S, and 80S ribosomal subunits can be separated by centrifugation in a sucrose density gradient (Rotenberg *et al.*, 1988). Analysis of the polysome profile of yeast with a Nop7p degron containing variant, which at non-permissive temperatures is degraded, revealed a lack of free 60S subunits, reduced polysome levels, and an increase in 80S ribosomes at non-permissive temperatures, suggesting that Nop7p is important for the production and stability of the 60S subunit (Du and Stillman, 2002). This hypothesis was consistent with the previous work demonstrating that Nop7p

cosediments with pre-ribosomal subunits (Harnpicharnchai *et al.*, 2001). As ribosomes are necessary to produce proteins that drive cellular processes such as proliferation, ribosome biogenesis and proliferation are linked (Sollner-Webb and Tower, 1986). As Nop7p is a component of the origin of replication, and involved in ribosome biogenesis, it is hypothesized to function as a bridge between these two processes (Du and Stillman, 2002).

In addition, to the of Yvh1p and Nop7p interaction by yeast two-hybrid there is a genetic interaction between *YVH1* and *NOP7.* Multicopy *NOP7* was able to rescue the slow growth defect and recover transcript levels of sporulation-specific genes associated with *YVH1* disruption mutants (Sakumoto *et al.*, 2001). The interaction between Yvh1p and Nop7p appears to be evolutionarily conserved. Yeast two hybrid and phage display approaches revealed that the *Plasmodium falciparum* homolog of DUSP12 interacts with the Nop7p homolog in *Plasmodium* (Kumar *et al.,* 2004). An interaction of Yvh1p with Nop7p suggests a role for Yvh1p in ribosome biogenesis, which was further supported a year later when Yvh1p was found to be associated with purified pre-60S ribosomal particles (Nissan *et al.*, 2002). As ribosome biogenesis is coupled with processes such as proliferation and the stress response, regulation of ribosome biogenesis by Yvh1p could explain the diverse phenotypes seen in yeast lacking *YVH1*. Importantly, if human DUSP12 is involved in ribosome biogenesis, this can have important implications for DUSP12 in cancer, where ribosome biogenesis is frequently deregulated (Silvera *et al.*, 2010). DUSP12 homologs were also identified and studied in the rat (Munoz-Alonso *et al.,* 2000) and mouse (Zhang *et al.,* 2000). In a yeast two-hybrid screen the glucokinase interacting protein (GKAP) was identified in a screen searching for gluckinase interactants in rats (Munoz-Alonso *et al.,* 2000). Based on protein sequence similarity, GKAP is the rat homolog of DUSP12, and was predicted to have phosphatase activity. Indeed, GKAP demonstrated phosphatase activity against the tyrosine analog *p*-nitrophenyl phosphate (Munoz-Alonso *et al.,* 2000). Additionally, a phosphatase assay using purified GKAP and purified phosphorylated glucokinase demonstrated that glucokinase was a substrate for GKAP *in vitro,* and glucokinase activity was reduced upon dephosphorylation by GKAP (Munoz-Alonso *et al.,* 2000). The identification of a substrate for the DUSP12 homolog in rat raises the interesting possibility that unlike in yeast, DUSP12's phosphatase activity may have importance in mammals. As in rats, little work into the function of DUSP12 in mice has been done. *dusp12,* designated *mVH1* in mice*,* is widely expressed in mouse tissue with the highest

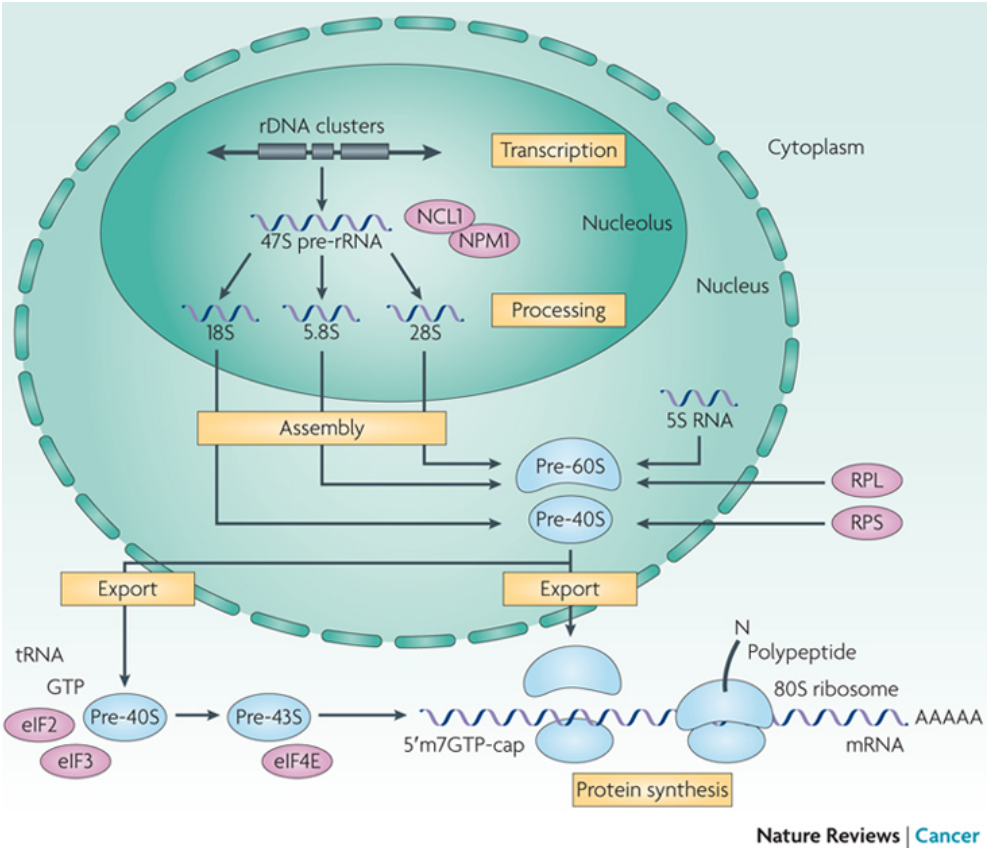

Figure 1.4 A brief overview of ribosome biogenesis.

The biogenesis of the ribosome of the ribosome machinery is a highly coordinated process, which is composed of the synthesis and import of ribosomal proteins from the nucleus, synthesis and processing of ribosomal RNA (rRNA), assembly of ribosomal proteins, and subsequent transport of the mature subunits into the cytoplasm. Most of these events take place in the nucleolus, expect for 5S rRNA synthesis (which occurs in the nucleoplasm) and synthesis of ribosomal proteins (which occurs in the cytoplasm). The basic translation machinery is composed of ribosomal subunits, mRNAs, transfer RNAs (tRNAs), and translational initiation and elongation factors. First, the initiation factors eIF2, eIF3, tRNA and GTP are incorporated into a 40S ribosomal subunit to forma 43S complex. Second, eIF4E is recruited into the 43S complex to form a 48S complex with mRNA. Finally, a 60S ribosomal subunit and the 48S subunit form the final 80S complex. rDNA, ribosomal DNA; NCL1, nucleolin; NPM1, nucleophosmin; RPS, proteins of the small ribosomal subunit; RPL, proteins of the large ribosomal subunit. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Cancer (Van Riggelen *et al*., 2010), copyright (2010).

expression in the liver, spleen, and lung (Zhang *et al.* 2000). The expression of *mVH1* may also be regulated by the cell cycle since transcription could be induced by the addition of serum and the highest accumulation of transcripts were at the G1/S phase (Zhang *et al.* 2000).

The one report investigating human DUSP12 specifically prior to 2008, was produced in 1999 (Muda *et al.,* 1999). This report was the first to clone and identify the human *dusp12* gene and demonstrated that human DUSP12 and a catalytically inactive DUSP12 C115S variant could replace Yvh1p in yeast and suppress the *yvh1Δ* growth defect (Muda *et al.,* 1999). Additionally, the subcellular localization of DUSP12 was described (Muda *et al.,* 1999). Transiently expressed *dusp12* with a C-terminal green fluorescence protein (GFP) tag in African green monkey fibroblast-like kidney (COS7) cells localized primarily to the nucleus, but could also be found in the cytoplasm (Muda *et al.,* 1999). The same localization pattern of DUSP12 was also seen when monitoring endogenous DUSP12 by immunofluorescence in HeLa cells, a cervical cancer cell line (Muda *et al.,* 1999). Finally, as the spacing of the conserved cysteines and the presence of a histidine in the cysteine rich domain (CRD) of DUSP12 appear similar to that found in zinc fingers, it was hypothesized that the CRD of DUSP12 bound zinc (Muda *et al.,* 1999). This hypothesis was supported by inductively coupled plasma (ICP) experiments using recombinant glutathione S-transferase (GST) tagged DUSP12 and a GST tagged DUSP12 variant lacking the CRD (Muda *et al.,* 1999). This experiment revealed that full-length DUSP12 bound zinc while the DUSP12 variant lacking the CRD did not (Muda *et al.,* 1999) which may be conserved in other organisms as the CRD of the DUSP12 homolog found in *Plasmodium falciparum* can also bind zinc (Kumar *et al,,* 2004).

Finally, in 2005, DUSP12 was proposed to be a pro-survival phosphatase (MacKeigan *et al.*, 2005). In order to identify new phosphatases that regulate apoptosis (programmed cell death), *MacKeigan et al.* used a siRNA library that contained two siRNAs per gene to knock-down over 200 phosphatases in HeLa cells and examined the induction of apoptosis by a DNA fragmentation enzyme-linked immunosorbent assay (ELISA) (MacKeigan *et al.*, 2005). Knock-down of *dusp12* induced spontaneous apoptosis in this siRNA screen. They confirmed that *dusp12* promoted cell survival by using pooled siRNAs and examining caspase activation (MacKeigan *et al.*, 2005). One important hallmark of cancer is the ability to resist apoptosis (Hanahan and Weinberg, 2011). Therefore, demonstrating that *dusp12* over-expression can protect from apoptosis could suggest a role for DUSP12 in cancer biology.

Research Aims

Although *dusp12* is amplified in multiple cancers (Gratias *et al.*, 2005; Hirai *et al.*, 1999; Kresse *et al.*, 2005; Mendrzyk *et al.*, 2006), whether *dusp12* is an oncogene is not known. As amplified genomic regions can easily contain hundreds of genes, identification of the gene that is the target of the genetic amplification can be problematic. Importantly, *Kresse et al*. were able to use fluorescence *in situ* hybridization and comparative genomic hybridization to narrow the candidate genes for the target of the genetic amplification of the 1q21-1q23 amplicon in liposarcomas to two genes, *atf6* and *dusp12* (Kresse *et al.*, 2005). The activating transcription factor 6 (*atf6)* is a transcription factor of the unfolded protein response (UPR) a signaling pathway that is activated by the presence of unfolded/misfolded proteins and drives the cellular response to endoplasmic reticulum stress (Haze *et al.*, 1999). While the up-regulation of the UPR has been demonstrated to both promote and inhibit tumor growth [Reviewed in (Ma and Hendershot, 2004)], no report conclusively demonstrates that *atf6* functions as an oncogene. *dusp12* is thought to be the more likely target for the genetic amplification as it is more highly expressed (Kresse *et al.*, 2005). In contrast to *atf6*, relatively little is known about the function of *dusp12* in both normal and cancer cell biology.

If *dusp12* is the target for the genetic amplification, it may be an oncogene. Oncogenes can promote cancer development and progression in a variety of ways. If DUSP12 is indeed an oncogene one would expect for it to contribute to one or more cancer phenotypes, such as, sustained proliferation, resistance to apoptosis, tumor angiogenesis (growth of blood vessels to a tumor), invasion of cancer cells to surrounding tissues, or metastasis (the ability to establish growth in a new part of the body) (Hanahan and Weinberg, 2011). DUSP12 may regulate proliferation because loss of the yeast DUSP12 ortholog caused a growth defect (Guan *et al.,* 1992). Additionally, as silencing *dusp12* with RNA interference caused spontaneous apoptosis (MacKiegan *et al.,* 2005), DUSP12 may be a negative regulator of apoptosis. Therefore, I hypothesize that *dusp12* is an oncogene that may promote proliferation and apoptosis resistance in cells.

The first aim of this thesis work was to test whether DUSP12 can promote proliferation, apoptosis resistance, or other cancer relevant properties. I sought to mimic the pathological state by establishing a non-tumorigenic cell line that stably over-expressed *dusp12*, and examined the

cell phenotype. I measured characteristics such as proliferation rates and susceptibility to apoptosis.

The second aim of this work was to investigate the cellular function of DUSP12. This was done by identifying a protein that interacts with DUSP12, and by examining signaling altered by *dusp12* over-expression. By investigating DUSP12 protein interactions, and the cell processes and signaling impacted by human DUSP12, the role of DUSP12 in both normal and cancer cell biology can be better understood. Indeed, this thesis work, in addition to work reported by other research teams in the last five years (reviewed in Chapter 2) have provided evidence for *dusp12* being a cancer relevant gene.

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Chapter 2 - Atypical Dual Specificity Phosphatases and their Potential Roles in Cancer

Abstract

While kinases have a well-established role in cancer and are often targeted for drug therapies, research in the last decade has revealed that phosphatases may be equally, if not more, attractive targets for drug therapy. The dual specificity phosphatase (DUSP) subfamily, contains a variety of proteins important in cancer development and/or progression. The DUSPs can be subdivided into seven distinct subgroups with the mitogen activated protein kinase phosphatase (MKP), phosphatase and tensin homolog (PTEN), and phosphatase of regenerating liver (PRL) subgroups being most clearly associated with oncogenesis. Large-scale genomic approaches have demonstrated that many DUSP's are differentially expressed in a variety of different cancers. For some phosphatases, the pathways they regulate can be inferred from the identity of their substrates, but for phosphatases whose substrates have not been identified (including members of the atypical DUSP subgroup), determining if and how these proteins contribute to carcinogenesis is decidedly more difficult. Here, I provide a review of the atypical DUSPs' known function(s) and cancer associations.

Introduction

The dual specificity phosphatases (DUSPs) are a subfamily within the protein tyrosine phosphatase (PTP) family of protein phosphatases, which unlike other PTP's they can remove phosphate groups from serine and threonine residues in addition to tyrosine residues (Denu and Dixon, 1995). DUSPs share the class I PTP consensus D…HC(X)$_5$RS/T catalytic cysteine sequence, but unlike other PTPs, the DUSPs do not contain the phospho-tyrosine recognition domain. They have a shallower catalytic clef, which most likely enables DUSPs to dephosphorylate all three phosphorylated residues (serine/threonine/tyrosine) (Zhang *et al.,* 1994; Denu and Dixon, 1995). In addition, the DUSP subfamily contains members that have non-protein substrates, such as lipids (Alonso *et al.* 2004), nucleic acids (Yuan *et al.* 1998), and sugars (Worby *et al.* 2006).

DUSPs are important regulators of multiple signaling pathways that regulate fundamental cell processes such as proliferation, apoptosis, and migration, and they are often deregulated in

disease (Patterson *et al.*, 2009). DUSPs can be divided into subgroups based on substrate specificity and sequence homology, but all DUSPs contain a highly conserved prototypical DUSP domain first characterized in the Vaccinia Virus's VH1 gene (Guan *et al.*, 1991). Within the DUSP superfamily, the best-characterized DUSPs are the mitogen activated protein kinase phosphatases (MKP's), which directly antagonize the activating dual phosphorylation of mitogen activated protein kinases (MAPK) (Patterson *et al.*, 2009). As deregulated MAPK signaling is implicated in cancer initiation and progression, it is not surprising that respective MKP also functionally contribute to disease progression (Patterson *et al.*, 2009).

In addition to MKPs, the DUSP subfamily contains a distinct subgroup described as the atypical DUSPs (Patterson *et al.*, 2009). There are 16 DUSPs (Table 2.1) that are commonly classified as atypical based on the lack of sequence similarity to better-characterized DUSPs and/or due to their substrate specificity (Patterson *et al.*, 2009; Bayon and Alonso, 2010). The substrates for many atypical DUSPs are unknown or debated. The characterized substrates include proteins (such as MAPKs) (Patterson *et al.*, 2009), nucleic aids (RNA) (Yuan *et al.*, 1998), and sugars (glycogen) (Worby *et al.*, 2006). In addition, several atypical DUSPs appear to function independently of their phosphatase activity and instead function as scaffolds in signal transduction pathways where they bind with multiple members of a signaling pathway to promote their interactions (Takagaki *et al.*, 2004; Park *et al.*, 2010; Zama *et al.*, 2002).

Several atypical DUSPs have been found to regulate important cellular processes such as apoptosis and proliferation (Patterson *et al.*, 2009), but how the DUSPs contribute to these processes is largely unknown. Potential roles for specific atypical DUSPs are beginning to be elucidated from high throughput sequencing/genomic approaches, suggesting that like MKPs the atypical DUSPs may also contribute to cancer initiation and progression (Patterson *et al.*, 2009). The following sections present a current synthesis of what is known about how the atypical DUSPs function, and will focus specifically on how these proteins may contribute to cancer initiation and progression by functioning as oncogenes and/or tumor suppressors. Current gaps in knowledge on the function of these proteins in both normal and cancer cell biology is highlighted to inspire new research on these poorly understood proteins.

Atypical DUSPs	
Name	**Aliases**
Laforin	EPM2A (epilepsy, progressive myoclonus type 2A)
STYX	Serine/threonine/tyrosine interacting protein
DUSP3	VHR (VH1-related)
DUSP11	PIR1 (phosphatase that interacts with RNA-ribonucleoprotein complexes)
DUSP12	hYVH1 (human YVH1)
DUSP13A	MDSP (muscle-restricted DUSP), SKRP4 (stress activated protein kinase pathway-regulating phosphatase-4), BEDP (branching-enzyme interacting DUSP)
DUSP13B	TMDP (testis-and skeletal muscle-specific DUSP)
DUSP14	MKP-L (MKP-1 like protein tyrosine phosphatases), MKP6
DUSP15	VHY (VH1-like member Y)
DUSP18	DUSP20, LMWDSP20 (low-molecular-weight DUSP20)
DUSP19	DUSP17, SKRP1
DUSP21	LMWDSP21
DUSP22	LMWDSP2, JSP1 (JNK-stimulating phosphatases 1), JKAP (JNK pathway-associated phosphatase), MKPX, VHX
DUSP23	DUSP25, LDP3 (low molecular mass DUSP3), VHZ
DUSP26	DUSP24, LDP4, MKP8, NEAP (neuroendrocrine associated phosphatase), SKRP3
DUSP27	DUPD1 (DUSP and pro-isomerase domain containing 1

Table 2.1 The atypical DUSPs and their associated aliases.

Atypical DUSPs currently implicated in cancer.

Laforin

Laforin, is most well known for its role in a fatal form of progressive myoclonus epilepsy known as Lafora disease, which results from loss of function mutations in the Laforin encoding gene, *epma2* (Ganesh *et al.*, 2006). The development of Lafora's disease is thought to partly occur from defects in glycogen metabolism because Laforin dephosphorylates glycogen (Wang *et al.*, 2002) and functions as an adapter protein for the E3 ubiquitin ligase, Malin (Worby *et al.,*

2008). As an adaptor, Laforin promotes the ubiquitination and subsequent degradation of glycogen metabolism regulators. However, over the last several years, studies have revealed potential role(s) for Laforin in cancer development and/or progression with Laforin functioning as both an oncogene and tumor suppressor in a cell type- and cell context- dependent manner (Wang *et al.*, 2006; Wang *et al.*, 2008). Due to alternative splicing, at least two isoforms of Laforin have been described that differ in their subcellular localization and phosphatase activity (Dubey *et al.*, 2012). The major isoform encodes for a 331 amino acid protein that localizes to the cytoplasm, and loss of function mutations of this isoform can cause Lafora's disease (Ganesh *et al.*, 2002; Singh *et al.*, 2008). Here, we discuss the potential roles of the major isoform in cancer. It is the most studied, however, the role of the other isoforms, if any, in cancer remains to be determined.

A potential tumor suppressive function for Laforin was derived primarily from observations that it was down-regulated in many murine and human lymphomas (Wang *et al.*, 2006). This role has been confirmed experimentally, as immunocompromised mice lacking Laforin develop spontaneous tumors (Wang *et al.*, 2006). Although, Laforin is a known phosphatase of glycogen (Worby *et al.*, 2006), recent work has identified protein substrates for Laforin as well (Liu *et al.*, 2006). At the molecular level, Laforin most likely inhibits cancer formation via glycogen synthase kinase 3 (GSK3β) by removing the inhibitory phosphate at Ser9 (Liu *et al.*, 2006). Consistent with being a regulator of GSK3β, cells lacking Laforin display altered signaling downstream of GSK3β, such as decreased GSK3β activity, increased cyclin D1 stability, and increased WNT signaling which promotes proliferation (Liu *et al.*, 2006; Liu *et al.*, 2008).

Laforin additionally possesses pro-survival attributes and may be a potential therapeutic target. Lymphomas with low Laforin expression are susceptible to apoptosis induced by energy deprivation caused by the addition of 2-deoxyglucose, while lymphomas with high Laforin expression are resistant (Wang *et al.*, 2008). The molecular mechanism by which Laforin regulates the cellular response to energy deprivation remains unclear, but may be due to its indirect regulation of the WNT signaling pathway via GSK3β (Wang *et al.*, 2006; Wang *et al.*, 2008). Whether Laforin's regulation of glycogen metabolism plays a role in the cellular response to energy depravation remains to be determined experimentally.

One of the first research reports to characterize Laforin identified the protein as a potential regulator of translation since it associates with polyribosomes (Ganesh *et al.*, 2000). Whether Laforin directly affects translation by binding to polyribosomes is unknown, however, recent work has demonstrated that Laforin can protect from stresses that perturb proteostasis by functioning as a regulator of autophagy, the heat shock response, and possibly the proteasome (Liu *et al.*, 2009; Vernia *et al.*, 2009; Aguado *et al.*, 2010; Sengupta *et al.*, 2011). For example, Laforin was silenced by RNA interference during times of ER stress, human embryonic kidney (HEK293) cells and the neuroblastoma cell line, SH-SY5Y, were more susceptible to apoptosis (Vernia *et al.*, 2009). Laforin may also affect proteasome activity because the proteasome activity was reduced in the livers of mice lacking Laforin (Vernia *et al.,* 2009). Later, it was found that Laforin protects cells from ER stress caused by the accumulation of unfolded and/or misfolded proteins by promoting autophagy by inhibition of the mammalian target of rapamycin (mTOR) pathway by a currently unknown mechanism (Aquado *et al.,* 2010). In addition, Laforin interacts with heat shock factor 1 (HSF1), a transcription factor that induces the transcription of genes that are critical in the heat shock response (Sengupta *et al.*, 2011). Although the mechanism by which Laforin regulates HSF1 is unclear, Laforin clearly is important in the heat shock response because Laforin is necessary for African green monkey fibroblast-like kidney (COS7) cells to up-regulate the transcription of HSF1-dependent genes and to be fully protected from thermal stress (Sengupta *et al.*, 2011). As a positive regulator of proteostatic pathways, up-regulation of Laforin transcription and protein synthesis may allow cancer cells to survive in conditions where proteostasis has been perturbed. Further work is required to examine the cell type and context by which Laforin may function to promote or inhibit cancer.

DUSP3

Despite studie*s* demonstrating that DUSP3 (alternatively named VHR for VH-1 related) dephosphorylates the MAPKs, extracellular signal-regulated kinase 1/2 (ERK1/2) and c-Jun N-terminal kinase (JNK), it is not classified in the MKP DUSP subfamily because it lacks the typical MAPK binding domain (Todd *et al.*, 1999; Todd *et al.*, 2002; Patterson *et al.*, 2009). As a regulator of MAPKs, it is not surprising that DUSP3 is implicated in cancer. It has been described as having both oncogenic (Henkens *et al.*, 2008; Wu *et al.*, 2009; Arnoldussen *et al.*,

2008) and tumor suppressive (Hao and ElShamy, 2007; Wang *et al.*, 2011) roles in a manner that is most likely cell type dependent.

DUSP3 is implicated to be an oncogene because it is up-regulated in a several cancers (Henkens *et al.,* 2008; Arnoldussen *et al.,* 2008; Wu *et al.,* 2009). It also promotes proliferation (Henkens *et al.*, 2008; Wu *et al.*, 2009;). For example, in cervical cancer, DUSP3 may promote proliferation by tempering ERK1/2 and JNK activities by dephosphorylation (Henkens *et al.*, 2008; Wu *et al.*, 2009). DUSP3 also may be an inhibitor of apoptosis. In the prostate cancer cells, LNCaP, DUSP3 protected cells from apoptosis induced by thapsigargin or 12-*0*-tetradecanoylophorbol-13-acetate by inhibiting JNK activity (Arnoldussen *et al.*, 2008). Although DUSP3 is up-regulated in cervical and prostate cancers (Henkens *et al.*, 2008; Arnoldussen *et al.*, 2008), it is still unclear whether DUSP3 indeed promotes cancer development and/or progression.

In addition to being a potential oncogene, recently, DUSP3 has been reported to display tumor suppressor properties. Over-expression of the oncogene *brca1-iris* increases cell proliferation by up-regulating cyclin D1 in breast cancer cell lines. Over-expression of *dusp3* (which is down-regulated by BRCA1-IRIS) suppresses the expression of cylclin D1, even in cells over-expressing *brca1-iris* (Hao and ElShamy 2007). Additionally, in non-small cell lung cancer (NSCLC), where DUSP3 transcript levels are low, in both a mouse xenograft model and in the NSCLC H1299 cell line, over-expression of *dusp3* suppressed growth of tumors and cells, respectively (Wang *et al.*, 2011). Recently, the growth receptor, ErbB, was identified as a DUSP3 substrate. DUSP3 removed the activating phosphorous from Y992, thereby inhibiting ErbB activity (Wang *et al.*, 2011). As ErbB can function as an oncoprotein and is deregulated in many cancers (Howe and Brown, 2011), the ability of DUSP3 to inhibit its activity has important implications for cancer therapies. The identification of regulators of DUSP3 can reveal potential therapeutic targets.

DUSP3 is activated by the pseudokinase, Vaccinia-related kinase 3 (VRK3) (Kang and Kim, 2006). VRK3 inhibits ERK1/2 activity in a kinase independent manner by promoting its dephosphorylation by DUSP3 (Kang and Kim, 2006). VRK3 co-immunoprecipitates with DUSP3 and ERK1/2 and is thought to function as a scaffold to promote the DUSP3-ERK1/2 interaction (Kang and Kim, 2006). In addition, VRK3 is down-regulated in colorectal cancer (Hennig *et al.*, 2011). It would be interesting to examine whether tumors with down-regulated

VRK3 also have decreased DUSP3 activity. Since VRK3 promotes DUSP3's activity towards ERK1/2, one would expect these tumors to have high ERK1/2 activity, which is known to promote cancer growth (Dhillon *et al.*, 2007), and would further support the role of DUSP3 as a tumor suppressor.

In addition to VRK3, DUSP3's catalytic activity is increased by the tyrosine kinase, TYK2, in a manner that is dependent on TYK2's kinase activity (Hoyt *et al.*, 2007). TYK2 phosphorylates DUSP3 at Y138, and this phosphorylation is needed for DUSP3 to dephosphorylate the signal transducer and activator of transcription 5 (STAT5) (Hoyt *et al.*, 2007). The ability of DUSP3 to inhibit STAT5 may have consequences in cancer cell biology, since STAT5 activity is known to promote cancer development and progression (Koptyra *et al.*, 2011). Therefore the loss of DUSP3 activity could possibly increase STAT5 activity and promote cancer development. If DUSP3 does indeed function as a tumor suppressor in some cell types, identification of negative regulators of DUSP3 would be beneficial by exposing new potential cancer drug targets.

DUSP11

DUSP11 is hypothesized to regulate splicing due to interactions with RNA splicing factors and its ability to dephosphorylate RNA trinucleotides (Yuan *et al.*, 1998). Recently, DUSP11 has been described as a potential tumor suppressor because over-expression in the osteosarcoma cell line, U2OS, and in mouse embryonic fibroblasts resulted in inhibition of proliferation dependent on DUSP11's phosphatase activity (Caprara *et al.*, 2009). Additionally, knock-down of *dusp11* increased proliferation in both normal and DNA damaged U20S cells (Caprara *et al.*, 2009).

Additionally, *dusp11* was identified to be a transcriptional target of the p53 tumor suppressor (Caprara *et al.*, 2009). p53 is a transcription factor that can promote cell cycle arrest, senescence, and apoptosis in response to DNA damage and oncogene activation, and is one of the most commonly mutated genes in cancer (Suzuki and Matsubara, 2011). In many cell lines lacking p53, *dusp11* expression is reduced; furthermore, in U2OS cells, *dusp11* is up-regulated in a p53 dependent manner (Caprara *et al.*, 2009; Yuan *et al.*, 1998). The ability of p53 to up-regulate DUSP11 provides new insights into p53 function, in particular, the proposed function of

p53 in the regulation of splicing by providing a link for p53 to the splicing machinery (Caprara *et al.*, 2009).

DUSP11 also associates with SAM68 (SRC-associated protein in mitotic cells), a splicing factor that when phosphorylated by ERK1/2, promotes the alternative splicing of CD44 variant exon v5, which is suggested to promote metastatic cancers (Matter *et al.*, 2002; Arch *et al.*, 1992). Although DUSP11 is mainly known as a RNA phosphatase, it does have some activity against protein substrates *in vitro* (Yuan *et al.*, 1998; Deshpande *et al.*, 1999). Whether, DUSP11 functions as a tumor suppressor by counteracting the ERK1/2 dependent phosphorylation of SAM68 remains to be determined experimentally. As many oncogenes and tumor suppressors are impacted by alternative splicing (Blair and Zi, 2011), DUSP11's role in splicing has important implications and thus needs to be examined further.

DUSP12

DUSP12 is evolutionarily conserved in organisms ranging from the budding yeast to humans, and is one of only two candidate genes for the target of a genetic amplification found in invasive liposarcomas (Guan *et al.*, 1992; Muda *et al.*, 1999; Kresse *et al.*, 2005). In addition, *dusp12* is up-regulated or amplified in other cancers including, neuroblastoma, retinoblastoma, intracranial ependymoma, and chronic myelogenous leukemia (Hirai *et al.*, 1999; Gratias *et al.*, 2005; Mendrzyk *et al.*, 2006; Biernacki *et al.*, 2010). The amplification of *dusp12* in a variety of cancers has lead to the hypothesis that *dusp12* is an oncogene. However, its function in normal and cancer cell biology remains unclear.

All DUSP12 homologs contain the highly conserved DUSP domain, but DUSP12 also contains a unique cysteine rich C-terminus that is found in all DUSP12 homologs (Muda *et al.*, 1999). Insights into the function of DUSP12 in human cells can be gained by examining the function of the DUSP12 ortholog in budding yeast, Yvh1p, one of the first eukaryotic DUSPs identified (Muda *et al.*, 1999). The transcription of *YVH1* can be up-regulated by nitrogen starvation and low temperatures and *yvh1Δ* yeast strains exhibit slow growth, and defects in sporulation, glycogen accumulation, and ribosome biogenesis (Guan *et al.*, 1992; Sakumoto *et al.*, 1999; Beeser and Cooper, 2000; Liu and Chang, 2009; Kemmler *et al.*, 2009). Interestingly, the C-terminus, not the N-terminus containing the phosphatase domain, of Yvh1p is able to suppress all the mutant phenotypes of *yvh1Δ* strains, suggesting that the phosphatase activity of

Yvh1p is not required for its cellular function in yeast (Beeser and Cooper, 2000). The cysteine-rich domain of the C-terminus in human DUSP12 is able to incorporate zinc (Muda *et al.* 1999) and to function as a redox sensor to protect the phosphatase activity during oxidative conditions (Bonham and Vacratsis 2009), but how the cysteine rich domain alone can suppress the phenotypes in *yvh1Δ* yeast remains unknown. Furthermore, as expression of human DUSP12 (in a phosphatase independent manner) is able to suppress all the known *yvh1Δ* phenotypes (Muda *et al.* 1999), DUSP12 and Yvh1p are functional orthologs, which suggests that investigations into Yvh1p function in yeast can provide valuable insights into the function of DUSP12 in human cells.

Recent work has revealed that Yvh1p is a critical factor in ribosome biogenesis (Lo *et al.*, 2009; Liu and Chang, 2009; Kemmler *et al.*, 2009). Ribosome biogenesis is a complex process that is regulated spatially and temporally (Kressler *et al.*, 2010). Ribosomal RNA is produced and processed in the nucleolus and the two subunits, 40S and 60S subunits, are first assembled with ribosomal proteins in the nucleus (Kressler *et al.*, 2010). These pre-40S and pre-60S subunits are then exported into the cytoplasm where further maturation occurs that eventually results in the binding of the 40S and 60S to mRNA in the mature translation competent 80S ribosomal complex (Kressler *et al.*, 2010).

Pma1-10 encodes for a misfolded plasma membrane ATPase that undergoes rapid degradation (Liu and Chang, 2006). Interestingly, loss of *YVH1,* or the ribosome biogenesis factors *RPL19A* and *RPL35A* (Song *et al.,* 1996; Planta and Mager, 1998), results in stabilization of *pma1-10* (Liu and Chang, 2006). During translation of an mRNA, multiple ribosomes bind and translate simultaneously, and this is described as the polysome, and free 40S, 60S, and 80S ribosomal subunits can be separated by centrifugation in a sucrose density gradient (Rotenberg *et al.*, 1988). Separation of polysomes, extracted from *yvh1Δ* strains, by centrifugation in a sucrose density gradient revealed a decrease in free 60S and 80S ribosomes and an increase in 40S subunits (Liu and Chang, 2009). Additionally, polysome profiles from *yvh1Δ* had halfmer polysomes (Liu and Chang, 2009) which can form as a result of stalled initiation complexes due to either a lack of mature 60S subunits or defective subunit binding (Rotenberg *et al.*, 1988). Importantly, the polysome profile could be restored to a wild-type phenotype by expressing the Yvh1p cysteine rich domain alone (Liu and Chang, 2009), which is the same domain that suppresses all previously known *yvh1Δ* phenotypes (Beeser and Cooper, 2000). Yeast lacking

YVH1 also showed mislocalization of the 60S subunit, as monitored using GFP-tagged Rpl25a (Gadal *et al.*, 2001), to the nucleus, suggesting *YVH1* is important in the nuclear export of the maturing 60S subunit (Liu and Chang, 2009).

Two independent research teams further elucidated the role of Yvh1p in pre-60S maturation in 2009 (Lo *et al.*, 2009; Kemmler *et al.*, 2009). The maturation of the pre-60S begins in the nucleus and is regulated by the sequential addition and removal of several protein factors (Kressler *et al.,* 2010). During late pre-60S maturation, the pre-60S subunit is exported to the cytoplasm where the ribosomal stalk, an essential component required for the recruitment of translation factor to the 60S, is added (Kressler *et al.,* 2010). Additionally, fusion of Yvh1p to an exclusively cytoplasmic protein found that exclusively cytoplasmic Yvh1p suppressed all *yvh1Δ* phenotypes including the exportation of pre-60S subunits from the nucleus, suggesting that Yvh1p functions in the late maturation of the pre-60S subunit that occurs in the cytoplasm (Kemmler *et al.*, 2009). The isolation of spontaneous fast-growing colonies that arose from *yvh1Δ* strains identified suppressors of the *yvh1Δ* phenotype (Kemmler *et al.,* 2009). From one of these colonies, a gain of function allele of *MRT4* (mRNA turnover 4) was found to suppress the *yvh1Δ* slow growth defect (Kemmler *et al.*, 2009). Mrt4p associates with the pre-60S subunit (Harnpicharnchai *et al.*, 2001), localizes to the nucleus and plays a role in mRNA turnover (Zuk *et al.*, 1999). Mrt4p is released from the pre-60S during export to the cytoplasm, and cycled back to the nucleus (Kemmler *et al.*, 2009). Without Yvh1p, there was an accumulation of pre-60S ribosomes bound to Mrt4p in the cytoplasm, suggesting that Yvh1p is critical for the release of Mrt4p from the pre-60S ribosomes (Kemmler *et al.*, 2009).

In addition, the continued presence of Mrt4p on the pre-60S subunits in the cytoplasm blocks the addition of the ribosomal factor P0 and the ribosomal stalk, one of last steps in the maturation of the 60S subunit (Lo *et al.*, 2009; Kressler *et al.,* 2010). Furthermore, Rpl12 binding to the pre-60S subunit is a prerequisite for Yvh1p binding to the pre-60S and subsequent displacement of Mrt4p (Lo *et al.*, 2009). Later, it was demonstrated that P0 binding to the pre-60S was required for the removal of Yvh1p from the pre-60S subunit (Lo *et al.*, 2010). Interestingly, Yvh1p with a nuclear localization signal to Yvh1p, as with the exclusively cytoplasmic Yvh1p (Kemmler *et al.*, 2009), suppressed all mutant phenotypes of *yvh1Δ* yeast (Lo *et al.*, 2009), suggesting that Yvh1p can function in ribosome biogenesis in both the nucleus and cytoplasm (Lo *et al.*, 2009; Kemmler *et al.*, 2009).

Recently, northern blot analysis revealed yeast lacking *YVH1* had decreased total RNA levels, and decreases in ribosomal RNAs of both the small 40S subunit and large 60S subunit of the ribosome (Sugiyama *et al.,* 2011). Furthermore, the species of ribosomal RNA that exist prior or during the ribosomal RNA processing steps of ribosome biogenesis were also decreased, suggesting that *YVH1* also plays a role in early ribosome biogenesis (Sugiyama *et al.,* 2011). As *MRT4* was originally identified in a screen to identify factors involved in mRNA decay (Zuk *et al.*, 1999), investigations into whether Yvh1p also functions in mRNA decay were made by examining the half lives of the short lived *URA5* transcript and the long lived transcript of the *RPL28* gene (Herrick *et al.*, 1990) by northern blotting (Sugiyama *et al.,* 2011). Yeast lacking *YVH1* had decreased mRNA decay rates of these two genes, implicating a role for Yvh1p in the regulation of mRNA decay (Sugiyama *et al.*, 2011).

Finally, the genetic interaction of *YVH1* and *MRT4* were demonstrated to affect not only ribosome biogenesis and proliferation, but glycogen accumulation and sporulation, as expression of the gain of function allele of *MRT4* in *yvh1Δ* yeast, was able to suppress all the *yvh1*Δ phenotypes previously described (Sugiyama *et al.*, 2011). Furthermore, microarray data revealed that *YVH1* expression clusters with ribosome biogenesis factors (Wade *et al.* 2001) and as ribosome biogenesis genes respond to environmental changes (Gasch *et al.*, 2000), the up-regulation of *YVH1* during nitrogen starvation (Guan *et al.*, 1992) and cold temperatures (Sakumoto *et al.*, 1999) also can be explained by the role of Yvh1p in ribosome biogenesis. Collectively, these findings suggest that the diverse phenotypes associated with *yvh1Δ* strains may be a result of defective ribosome maturation, since alterations in ribosome composition can greatly impact the translation of proteins involved in many signaling pathways and cellular processes (Silvera *et al.*, 2010). Furthermore, the function of Yvh1p as a ribosome maturation factor may be conserved in humans since knock-down of human DUSP12 in HeLa cells resulted in mislocalization of the ribosome factors MRTO4 and eIF6 (Lo *et al.*, 2010). In addition, knock-down of the ribosome factor P0 in HeLa resulted in exclusion of DUSP12 from the nucleus (Lo *et al.*, 2010). The potential function of human DUSP12 in ribosome biogenesis would have important consequences in cancer cell biology. Ribosomes are essential for protein translation, and when ribosome biogenesis and/or translation are deregulated, diseases such as cancer can arise (Silvera *et al.*, 2010). However, further work to clarify the role of human DUSP12 in ribosome biogenesis and the translatome affected by DUSP12, is necessary.

Several reports in the last five years have demonstrated that human DUSP12 may regulate cellular processes such as cell cycle progression (Kozarova *et al.*, 2011) and apoptosis (MacKeigan *et al.*, 2005; Sharda *et al.*, 2009; Cain *et al.*, 2011), in a phosphatase independent (Kozarova *et al.*, 2011) and dependent manner (Sharda *et al.*, 2009) respectively, suggesting that in humans, DUSP12 may have multiple functions that can differentially require the phosphatase domain, the cysteine rich domain (CRD), or both.

Over-expression of *dusp12* in human embryonic kidney cells (HEK293) resulted in an increase percent of cells in the G2/M phase and an increase in polyploidy in a manner dependent on the CRD (Kozarova *et al.*, 2011). Further supporting a role of DUSP12 in the cell cycle, RNA interference of *dusp12* in HEK293 and cervical cancer cells (HeLa), resulted in the induction of cellular senescence, where cells are permanently arrested in the cell cycle (Kozarova *et al.*, 2011). Additionally, cells over-expressing *dusp12* treated with nocodazole to induce the mitotic checkpoint (Wang and Burke, 1995) had an increase proportion of cells exhibiting polyploidy, suggesting that over-expression of *dusp12* could over-ride the mitotic checkpoint (Kozarova *et al.*, 2011) Collectively, these experiments demonstrate that DUSP12 can affect cellular DNA content, which could have important consequences as polyploidy and genomic instability can promote cancer development (Hanahan and Weinberg, 2011).

Using mass spectrometry, three phosphorylated residues of DUSP12, Ser14, Thr252, and Ser335 were identified (Kozarova *et al.*, 2011). DUSP12 has been described as having a nuclear (Muda *et al.*, 1999), perinuclear (Lo *et al.*, 2009), and cytoplasmic (Sharda *et al.*, 2009) subcellular localization. To examine whether phosphorylation at these sites altered DUSP12 localization, a series of single point mutants of DUSP12 were produced (Kozarova *et al.*, 2011). Ser14, Ser335, or Thr252 were altered to an alanine, which prevents phosphorylation of DUSP12 at these amino acids, or the residues were mutated to glutamic acid, which mimics the phosphorylated state (Thorsness and Koshland, 1987). Phosphorylation variants of Ser14 and Thr252 did not alter the subcellular localization of DUSP12, but DUSP12 variants with altered Ser335 exhibited a change in localization (Kozarova *et al.*, 2011). The DUSP12 S335A (non-phosphorylatable) variant displayed a more nuclear localization than wild-type DUSP12, while the DUSP12 S335E (phospho-mimetic) variant localized mainly to the cytoplasm (Kozarova *et al.*, 2011). Additionally, the cellular DNA content of cells over-expressing the DUSP12 S335E variant had an increase in the proportion of cells in the G2/M phase, suggesting that the

localization of DUSP12 to the cytoplasm and phosphorylation at S335 are important in promoting the progression of the cell cycle (Kozarova *et al.*, 2011). The effect on DUSP12 subcellular localization by S335 phosphorylation may be unique to mammalian DUSP12's. This site is conserved in mice and rats, but not in the DUSP12 homologs of non-mammalian species, including the better characterized DUSP12 in yeast (Kozarova *et al.*, 2011). A question that remains to be resolved is whether the regulation of the cell cycle by DUSP12 is actually due to its proposed role in ribosome biogenesis, as the cell cycle progression and ribosome biogenesis are normally linked as the availability of translating ribosomes is critical for the production of proteins that drive cellular processes such as the cell cycle.

A role for DUSP12 in cancer was further indicated by our work where we evaluated the oncogenic potential of DUSP12 in a cell culture model (Cain *et al.*, 2011). To simulate the pathological state in cancer, we over-expressed *dusp12* in a non-tumorigenic human embryonic kidney cell line (HEK293), and examined the cells for cancer associated properties (Cain *et al.*, 2011). Although, DUSP12 has been reported to alter the cell cycle (Kozarova *et al.*, 2011), we observed no increase in cell proliferation as monitored by ATP levels using Promega's Cell Titer Glo assay (Cain *et al.*, 2011). We also demonstrated, for the first time, that DUSP12 promotes cell motility as demonstrated by a scratch wound and transmigration assay (Cain *et al.*, 2011).

DUSP12 also protected cells from apoptosis induced by staurosporine and thapsigargin (Cain *et al.,* 2011), a non-specific kinase inhibitor and a disrupter of intercellular calcium levels, respectively (He *et al.*, 2003). This is consistent with the reports of a pro-survival function of DUSP12 previously reported in 2005 and 2009 (MacKeigan *et al.,* 2005; Sharda *et al.,* 2009). *dusp12* was first identified as a pro-survival gene in a siRNA screen against cellular kinases and phosphatases, where knock-down of *dusp12* expression in HeLa cells induced spontaneous apoptosis (MacKeigan *et al.,* 2005). Additional work revealed that transient over-expression of *dusp12*, but not the catalytically inactive DUSP12 (C115S) or a DUSP12 variant lacking the cysteine rich domain (CRD), in HeLa cells promoted resistance to a variety of apoptotic stimuli such as heat shock, oxidative stress, and activation of the FAS death receptor, but not due to cisplatin, which induces apoptosis by DNA damage (Sharda *et al.*, 2009). This suggests that both DUSP12's phosphatase activity and CRD are required for it's anti-apoptotic function (Sharda *et al.,* 2009). Additionally, purified DUSP12 and purified heat shock protein 70 (HSP70) bind *in vitro* (Sharda *et al.* 2009). The interaction between these two proteins required the CRD of

DUSP12 as demonstrated by immunoprecipitation experiments and DUSP12 interacted with the ATPase domain of HSP70 suggesting that this is not a chaperone:substrate interaction (Sharda *et al.* 2009). Finally, *in vitro* phosphatase assays demonstrated that the addition of HSP70 promoted the phosphatase activity of DUSP12, while protein folding assays revealed that DUSP12 did not affect HSP70's chaperone function (Sharda *et al.* 2009). Whether the HSP70-DUSP12 interaction alone is responsible for the anti-apoptotic affects of DUSP12 in the cell remains to be determined.

DUSPs can be inactivated by oxidation of the catalytic cysteine that is responsible for nucleophilic attack on the phosphorous atom during dephosphorylation (Denu and Tanner, 1998). DUSP12 protected cells from apoptosis induced by redox-sensitive signaling pathways (Clement *et al.*, 1998; Shen and Pervaiz, 2006; Zhao *et al.*, 2006), but not via DNA damage pathways (Siddik, 2003). Therefore, it was examined whether the zinc binding domain of the CRD promoted DUSP12's phosphatase activity from inactivation by oxidization (Bonham and Vacratsis, 2009) *In vitro* analysis of DUSP12's phosphatase activity against the artificial substrate, 6,8-difluoro-4-methylumbelliferyl phosphate, demonstrated that DUSP12 variants lacking the zinc binding domain had reduced phosphatase activity during oxidative conditions. This suggests that the CRD functions as a redox sensor to protect DUSP12's phosphatase catalytic cysteine from inactivation during oxidative stress (Bonham and Vacratsis, 2009).

To gain insights into how DUPS12 could promote cancer properties such as increased cell motility and apoptosis resistance, we investigated whether over-expression of *dusp12* altered the expression of cancer relevant genes using a Cancer Gene PCR array (SABiosciences) (Cain *et al.* 2011). We identified and confirmed that DUSP12 up-regulated two integrin alpha 1 (*itga1*) and the prominent oncogene, hepatocyte growth factor receptor, *c-met* (Cain *et al.*, 2011). ITGA1 promotes cancer proliferation, motility, invasion, and angiogenesis (Lochter *et al.*, 1999; Pozzi *et al.*, 1998; Senger *et al.*, 2002), but whether it is responsible for the DUSP12 dependent motility remains to be examined. Although the oncogene c-MET is known to regulate growth, survival, and migration signaling (Birchmeier *et al.*, 2003), it is unlikely that c-MET is responsible for the pro-survival and migration function of DUSP12 we observed in our stable cell lines since active c-MET signaling was not present in cells over-expressing *dusp12* (Cain *et al.*, 2011). This was not surprising because c-MET often requires its ligand, hepatocyte growth factor (HGF), to be supplied in an autocrine or paracrine manner to function in cancer (Fukuda

et al., 1998). However, the ability of DUSP12 to up-regulate c-MET has important implications as in a tumor microenvironment where HGF is supplied, DUSP12 could promote the development and/or progression of cancer.

Collectively, various research teams have demonstrated that *dusp12* has oncogenic potential; most notably due to its anti-apoptotic abilities (MacKiegan *et al.,* 2005; Sharda *et al.,* 2009; Cain *et al.,* 2011). However, DUSP12 may also promote cancer by affecting the cell cycle and promoting genomic instability (Kozarova *et al.*, 2011), increasing cell motility, and by the up-regulation of the cancer relevant genes, *itga1* and *c-met* (Cain *et al.,* 2011). Whether DUSP12 promotes these diverse phenotypes due to its proposed role in ribosome biogenesis (Lo *et al.*, 2009; Lo *et al.*, 2010) or a currently unknown mechanism, remains to be seen.

DUSP18

DUSP18 is highly expressed in the testis, liver, brain, and ovary, but can be found in lower levels throughout the remaining tissues. Interestingly it is detected in a variety of primary tumors and cancer cell lines (Hood *et al.*, 2002; Wu *et al.*, 2006; Wu *et al.*, 2003). DUSP18 contains a unique C-terminal domain of unknown function and displays catalytic activity *in vitro* and *in vivo* to phosphorylated c-Jun N-terminal kinase (JNK) but not other mitogen activated protein kinases (MAPKs) (Hood *et al.*, 2002; Wu *et al.*, 2003; Wu *et al.*, 2006).

In COS7 cells DUSP18 has nuclear and cytoplasmic subcellular localization, and can be found in the intermembrane space of the mitochondria, as well (Hood *et al.*, 2002; Rardin *et al.*, 2008). Rardin *et al.* were able to demonstrate that DUSP18 normally localizes to the mitochondria and refuted the previous reports of DUSP18 localizing to the cytoplasm and nucleus (Hood *et al.* 2002) because over-expression of DUSP18 with N-terminal tags disrupted localization to the mitochondria (Rardin *et al.* 2008). Inducing apoptosis can also alter the mitochondrial localization of DUSP18. Like cytochrome c, it is released from the mitochondria into the cytoplasm (Rardin *et al.*, 2008). Since JNK does not localize to the mitochondria, DUSP18 cannot interact with it (Rardin *et al.*, 2008), however it is possible that upon the release of DUSP18 from the mitochondria, it may be able to interact and regulate JNK. Whether this is indeed the case and/or if DUSP18 has unidentified mitochondrial substrate remains to be determined. As a JNK regulator, DUSP18 has the potential to regulate processes such as apoptosis, which has significant implications in cancer.

DUSP22

DUSP22 is widely expressed in human tissues (Shen *et al.*, 2001; Alonso *et al.*, 2002). It may be a tumor suppressor in that it is down-regulated in breast cancer and lymphomas (Bernard-Pierrot *et al.*, 2008; Feldman *et al.*, 2011), and is a positive prognostic marker for B cell chronic lymphatic leukemia (Jantus Lewintre *et al.*, 2009). In anaplastic lymphoma kinase (ALK)-negative anaplastic large cell lymphomas, the commonly found t(6;7)(p25.3;q32.3) translocation disrupts the *dusp22* gene (Feldman *et al.*, 2011). However, the translocation also results in the up-regulation of the microRNA *mir29* (Feldman *et al.*, 2011). While MIR29 is mainly thought of having tumor suppressor qualities (Han *et al.*, 2010; Gebeshuber *et al.*, 2009), further work is needed to examine whether disruption of DUSP22 without MIR29 up-regulation can promote cancer.

Although down-regulated in many cancers, the real function of DUSP22 especally as a tumor suppressor is unclear. It may be cell type and context specific. There are conflicting reports concerning the ability of DUSP22 to dephosphorylate the mitogen activated protein kinases (MAPKs) (Alonso *et al.*, 2002; Aoyama *et al.*, 2001; Chen *et al.*, 2002; Shen *et al.*, 2001). However, most studies indicate DUSP22 as a regulator of c-Jun N-terminal kinase (JNK) (Aoyama *et al.*, 2001; Shen *et al.*, 2001; Chen *et al.*, 2002). Over-expression of both *jnk* and *dusp22* in COS7 cells demonstrated that JNK phosphorylation is inhibited by DUSP22 (Aoyama *et al.*, 2001). Nevertheless, several reports have demonstrated that DUSP22 can actually promote JNK activity in a phosphatase dependent manner (Chen *et al.*, 2002; Shen *et al.*, 2001). First, DUSP22 can bind the JNK kinase, map kinase kinase 7 (MKK7), but not JNK itself (Chen *et al.*, 2002). Over-expression of *dusp22* enhances MKK7 activity resulting in increased JNK phosphorylation (Chen *et al.*, 2002). Exactly how DUSP22 promotes MKK7 and JNK activity is unclear but the requirement of DUSP22 for their activation was confirmed in mouse embryonic stem cells lacking DUSP22. These cells were unable to activate JNK in response to cytokines (Chen *et al.*, 2002).

DUSP22 can also dephosphorylate STAT3 (signal transducer and activator of transcription 3) (Sekine *et al.*, 2006). STAT3 is activated by cytokines and growth factors and in some cancers, is constitutively active (Lavecchia *et al.*, 2011). Over-expression of *dusp22* reduces STAT3 phosphorylation and activity in the tumorigenic human embryonic kidney (HEK293T) cells while knock-down of *dusp22* enhanced STAT3 activity (Sekine *et al.*, 2006).

Since STAT3 activity is associated with cancer (Lavecchia *et al.*, 2011), the ability of DUSP22 to negatively regulate STAT3 further implicates it as a tumor suppressor.

Another reported substrate of DUSP22 is the estrogen receptor (ERα) (Sekine *et al.*, 2007). DUSP22 most likely functions in a negative feedback loop to regulate ERα. Activation of the estrogen receptor induces *dusp22* transcription and DUSP22 dephosphorylates and inhibits ERα in cells (Sekine *et al.*, 2007). DUSP22 is down-regulated in breast cancer, in particular, DUSP22 is down-regulated in breast cancers containing the 8p11-12 amplicon (Bernard-Pierrot *et al.*, 2008). This amplicon contains the potential oncogene, *ppapdc1b,* which is thought to be responsible for DUSP22 down-regulation. Knock-down of *ppapdc1b* increased *dusp22* transcription (Bernard-Pierrot *et al.*, 2008). The oncogenic activity of *ppapdc1b,* in part, may be due to its ability to down-regulate *dusp22* transcription to allow for increased estrogen receptor activity. It was reported that when 11 tumor samples were analyzed they all contained the 8p11-12 amplicon and all had ER-positive statuses (Bernard-Pierrot *et al.*, 2008).

Finally, DUSP22 may also function as a tumor suppressor by inhibiting cell migration and invasion (Li *et al.*, 2010). DUSP22 is myristoylated, indicating that it is targeted to the plasma membrane (Schwertassek *et al.*, 2010). While loss of myristoylation does not affect its catalytic activity *in vitro,* over-expression of this *dusp22* mutant in cells caused cell detachment and apoptosis, which was not observed in cells over-expressing wild-type *dusp22* that was capable of being myristoylated (Schwertassek *et al.*, 2010). The ability to be myristoylated and targeted to the plasma membrane may be important to the finding that the focal adhesion kinase (FAK) serves as a DUSP22 substrate (Li *et al.*, 2010). FAK promotes cell adhesion and inhibition of FAK caused detachment and apoptosis in some cell lines (Martin, 2003). DUSP22 localizes with FAK at actin filament enriched regions of lamelapodia and *dusp22* over-expression inhibited cell migration and reduced FAK phosphorylation while knock-down promoted cell migration and FAK phosphorylation in H1299 cells (Li *et al.*, 2010)

DUSP22 is indicated to have a variety of substrates and to be down-regulated in a variety of cancers. Further work is necessary to unravel the function of DUSP22 in various cell types and contexts to better understand its role in preventing cancer formation and progression. Furthermore, identification of negative regulators of DUSP22, such as PPAPDC1B in breast cancer, will identify new targets to be exploited in cancer therapies.

DUSP23

In vitro ERK1/2 is a DUSP23 substrate, however cotransfection experiments in COS7 cells demonstrated that DUSP23 was an activator of the stress activated MAPKs, JNK and p38 (Takagaki *et al.*, 2004). This activity is not dependent on its phosphatase activity and MKK4 and 6 also had increased activation, suggesting that DUSP23 may act as a scaffold to promote MKK binding to JNK and p38 (Takagaki *et al.*, 2004). However, further work is needed to see if this is indeed the case.

DUSP23 is highly methylated and has decreased mRNA expression in neuroblastoma, suggesting tumor suppressor qualities (Caren *et al.*, 2011). Interestingly, DUSP23 levels were lower in tumors from deceased patients than from those of patients exhibiting no clinical symptoms, suggesting that DUSP23 levels could be a prognostic marker for neuroblastomas (Caren *et al.*, 2011). In contrast, DUSP23 may be an oncogene since it is amplified in many cancers, including breast, colon, lung, squamous carcinoma, pancreatic, brain, esophageal, stomach, bladder, kidney, skin, ovary, prostate, and testicular cancers (Tang *et al.*, 2010). Over-expression of *dusp23* in the MCF7 breast cancer cell line increased proliferation while knock-down of *dusp23* decreased proliferation (Tang *et al.*, 2010). As DUSP23 is differentially expressed in a variety of cancers, whether DUSP23 is an oncogene or tumor suppressor requires further investigation, and whether its ability to promote JNK and p38 activation is important in regulating the cell cycle in MCF7 cells or whether DUSP23 is acting on some currently unknown substrate remains to be determined.

DUSP26

In cancer, DUSP26 may be a tumor suppressor or oncogene depending on cell type. It is differentially expressed in a variety of cancers. In glioblastoma tissues, neuroblastoma, brain, and ovarian cancer cell lines, *dusp26* is transcriptionally down-regulated (Patterson *et al.*, 2010; Tanuma *et al.*, 2009), which is consistent with *dusp26* functioning as a tumor suppressor. Similarly, over-expression of *dusp26* in immortalized breast epithelial, MCF10A, cells suppressed colony formation and acinar growth in 3D culture, while knock-down of DUSP26 in immortalized ovarian epithelia, HOSE17.1, cells increased colony formation and proliferation (Patterson *et al.*, 2010).

Dusp26 has also been implicated to be an oncogene because it was over-expressed and located on the 8p12 amplicon found in anaplastic thyroid carcinoma tissue (Yu *et al.*, 2007). Furthermore, in anaplastic thyroid carcinoma primary tumors and cell lines, over-expression of *dusp26* promoted colony formation, while knock-down of *dusp26* transcripts reduced proliferation (Yu *et al.*, 2007). DUSP26 was identified as a p53 phosphatase and is over-expressed in neuroblastoma cell lines and tumor samples (Shang *et al.*, 2010). DUSP26 can dephosphorylate the tumor suppressor p53 at S20 and S37, inhibiting p53 and p53-mediated apoptosis induced by genotoxic stress (Shang *et al.*, 2010). As the pro-apoptotic tumor suppressor p53 is inactivated in many cancers (Suzuki and Matsubara, 2011), the identification of new negative regulators of p53 revealed new potential drug targets for cancer therapy.

Finally, in addition to p53, other substrates have been described for DUSP26, making it difficult to determine the cellular function of DUSP26. There are conflicting reports of whether it is a MAPK phosphatase. *In vitro,* p38 served as a DUSP26 substrate (Yu *et al.*, 2007). This was confirmed by cell culture experiments where in HEK293T cells, over-expression of *dusp26* resulted in reduction of p38 activity and p38-mediated apoptosis, while knock-down of *dusp26* transcripts increased p38 activity (Vasudevan *et al.*, 2005). Similarly, in COS7 cells, over-expression of *dusp26* increased both JNK and p38 phosphorylation levels (Takagaki *et al.*, 2004). However, in the rat neuronal cell line, PC12, DUSP26 had no affect on MAPKs (Wang *et al.*, 2006). Furthermore, in epithelial cells, over-expression of *dusp26* resulted in reduced proliferation that was not mediated by MAPK inhibition (Patterson *et al.*, 2010). Therefore, these discrepancies must be resolved.

In addition to MAPKs, DUSP26 regulates the kinesin superfamily 3 (KIF3) motor complex by dephosphorylating the kinesin-associated protein 3 (KAP3) (Tanuma *et al.*, 2009). Consistent with being a negative regulator of KIF3, over-expression of *dusp26* increased cell-to-cell adhesion and intracellular transport (Tanuma *et al.*, 2009). Clearly, more work is needed to identify and verify DUSP26 substrates. As the role of DUSP26 in normal and cancer cell biology remains murky at best, more research is required to further our understanding of DUSP26 in various cells. The scientific community would also greatly benefit from the production of a DUSP26 knock-out mouse to further clarify the physiological role of this enigmatic protein.

The least studied atypical DUSPs.

The previous sections discussed the atypical DUSPs described in the literature with tumor suppressive and/or oncogenic potential. We compared the expression of all the atypical DUSPs in tumors of the prostate to normal prostate tissue using the cBio Cancer Genomics Portal (http://www.cbioportal.org/) and microarray data deposited by the Memorial Sloan-Kettering Cancer (MSKCC) Center's Prostate Oncogenome Project (Taylor *et al.*, 2010). Comparison of 85 tumors to normal prostate tissue revealed that atypical DUSPs, including atypical DUSPs which have not been previously associated with cancer, have aberrant expression in prostate cancer (Table 2.2). The following sections describe what is currently known about the remaining atypical DUSPs because they, too, may have cancer relevance, but require more extensive investigations into their function as relatively little is known about them.

STYX

The prototypical pseudophosphatase, STYX, contains a substitution of the catalytic cysteine in the DUSP catalytic domain for a glycine, inactivating the phosphatase (Wishart and Dixon, 2002). Since STYX, and other proteins containing the STYX domain, can still bind phosphorylated proteins, they could function as antagonists to prevent dephosphorylation of the target protein (Wishart and Dixon, 1998). In mice, *styx* expression is restricted to the testis and is essential for spermatogenesis. Male *styx* knock-out mice are infertile (Wishart and Dixon, 2002). STYX protects CRHSP-24, a protein implicated in translational repression of histone mRNAs, from dephosphorylation. Therefore, mouse infertility is attributed to lack of CRHSP-24 activity (Wishart and Dixon, 2002). It is unknown whether STYX can be up-regulated and function in other cell types.

Gene Expression in Prostate Cancer			
Gene	**Down**	**Up**	**Total**
EPM2A	13%	0%	**13%**
STYX	2%	7%	**9%**
DUSP3	40%	0%	**40%**
DUSP11	4%	5%	**8%**
DUSP12	1%	15%	**16%**
DUSP13	0%	5%	**5%**
DUSP14	6%	11%	**16%**
DUSP15	4%	0%	**4%**
DUSP18	40%	4%	**44%**
DUSP19	1%	1%	**2%**
DUSP21	0%	7%	**7%**
DUSP22	15%	2%	**18%**
DUSP23	11%	8%	**19%**
DUSP26	1%	2%	**4%**
DUSP27	2%	5%	**7%**
AR	2%	7%	**9%**

Table 2.2 Differential expression of atypicla DUSPs in prostate cancer.

Using the cBio Cancer Genomics Portal (http://www.cbioportal.org/), and microarray data deposited by MSKCC Prostate Oncogenome Project (Taylor *et al.* 2010), we compared the expression of atypical DUSPs in tumors to normal prostate tissue using a Z score threshold of +/- 2. The androgen receptor (AR) is included for comparison as a gene already implicated to be differentially expressed or altered in prostate cancer (Taylor *et al.,* 2010)

DUSP13A/B

The *dusp13* gene is unusual in that it encodes for two similar protein products transcribed by alternative reading frames (Alonso *et al.*, 2004; Chen *et al.*, 2004). Designated DUSP13A

and DUSP13B, the proteins are expressed in the muscle and testis, respectively (Chen *et al.*, 2004; Nakamura *et al.*, 1999). Both proteins have catalytic phosphatase activity *in vitro,* but DUSP13B exhibits significantly higher activity than DUSP13A (Nakamura *et al.*, 1999). Interestingly, both proteins have been implicated to regulate apoptosis, but in different fashions. Knock-down of *dusp13a* in cells reduces apoptosis signal regulating kinase 1 (ASK1) activity and ASK1 mediated apoptosis (Park *et al.*, 2010). Furthermore, DUSP13A binds ASK1 and promotes its activity in a phosphatase-independent manner (Park *et al.*, 2010). It is unclear how DUSP13A promotes ASK1 activity, but it can either prevent ASK1 phosphorylation by blocking AKT (an inhibitor of ASK1) binding or act as a scaffold to promote its activation (Park *et al.*, 2010).

While DUSP13A promotes apoptosis (Park *et al.*, 2010), DUSP13B appears to be anti-apoptotic (Katagiri *et al.*, 2011). In contrast to DUSP13A, over-expression of *dusp13b* in COS7 cells caused reduced phosphorylation of the stress activated MAPKs, JNK and p38, suggesting that it is a MAPK phosphatase. Over-expression of *dusp13b*, in a phosphatase-dependent manner, reduced the activity of the down-stream JNK effector, activator protein 1 (AP-1), which is thought to promote apoptosis in spermatogenesis (Katagiri *et al.*, 2011). The anti-apoptotic role of DUSP13B makes it easy to speculate that if up-regulated in different cell types, it could promote cell survival and possible cancer formation, but this remains to be demonstrated.

DUSP14

In vitro, DUSP14 has phosphatase activity against all three MAPKs, which is why it has also been designated MAPK phosphatase 1-like (MKP-L) and MKP-6 (Marti *et al.*, 2001). However, DUSP14 is commonly classified as an atypical DUSP because it lacks the typical MAPK binding domain found in the MKP DUSP subfamily (Patterson *et al.*, 2009). In cell culture, expression of a catalytically inactive version of DUSP14 only enhances ERK1/2 and JNK phosphorylation, suggesting that *in vivo,* p38 is not a DUSP14 substrate (Marti *et al.*, 2001). While *dusp14* is located on a chromosomal region that is amplified in gastric cancer, it is unlikely to be the target for the genetic amplification in that *dusp14* expression is not increased (Varis *et al.*, 2002). However, in β pancreatic cells inhibition of DUSP14 increased cell proliferation and ERK1/2 phosphorylation (Klinger *et al.*, 2008). If DUSP14 has similar activity

in other cell types, loss of DUSP14 may promote cancer development due to increased proliferative ability.

DUSP15

DUSP15 contains an N-terminal consensus sequence that signals for myristoylation and is localized to the mitochondria (Hood *et al.*, 2002). The DUSP15 crystal structure revealed that DUSP15 contains unique structures and lacks the domain known for substrate recognition found in other DUSPs, suggesting that DUSP15 has unique substrate recognition mechanisms (Yoon *et al.*, 2005). DUSP15 displays phosphatase activity *in vitro* against artificial substrates (Alonso *et al.*, 2004), but currently the substrates for DUSP15 in the cell are unknown. DUSP15 also has been identified as a candidate gene in a quantitative trait locus that is thought to harbor genes that control for the predisposition to growth and fatness in mice (Jerez-Timaure *et al.*, 2005). Due to a lack of studies of DUSP15 function, it is unknown whether DUSP15 has any role in cancer development.

DUSP19

DUSP19 is an interesting atypical DUSP in that it appears to be able to both positively and negatively regulate JNK activation, most likely in a phosphatase independent manner (Zama *et al.*, 2002; Zama *et al.*, 2002). DUSP19 serves as a scaffold for apoptosis signal-regulating kinase 1 (ASK1) that phosphorylates and activates MKK7, which in turn phosphorylates and activates JNK (Zama *et al.*, 2002). Experimentally, DUSP19 has been shown to bind both MKK7 and ASK1, but not JNK (Zama *et al.*, 2002; Zama *et al.*, 2002). The proposed model of JNK regulation by DUSP19 is that DUSP19 sequesters ASK1, preventing activation of MKK7 and JNK. At high levels of DUSP19 protein, it can bind ASK1 and MKK7 to facilitate the activation of JNK (Zama *et al.*, 2002; Zama *et al.*, 2002). Whether this is indeed the mechanism by which DUSP19 functions remains to be elucidated. Since DUSP19 levels affects the activation of JNK in cells, this can have important implication in cancer, as JNK is a critical regulator of the cellular stress response, hence, further investigations into whether DUSP19 has a role in cancer biology are needed.

DUSP21

Similarly to DUSP18, DUSP21 contains a highly conserved internal mitochondria localization signal (Rardin *et al.*, 2008). Detection of endogenous DUSP21 in cells reveals localization at the peripheral membrane of the inner membrane of the mitochondria, which is the opposing side to which DUSP18 is found (Rardin *et al.*, 2008). The expression of *dusp21* is restricted to the testis as indicated by northern, western, and qRT-PCR analyses (Rardin *et al.*, 2008; Hood *et al.*, 2002). DUSP21 exhibits activity against synthetic MAPK peptides *in vitro,* but cell based assays do not suggest that DUSP21 has activity against any MAPKs in the cell (Hood *et al.*, 2002), which is not surprising given its mitochondrial localization. Whether DUSP21 can be up-regulated and function in other tissues remains unknown.

DUSP27

DUSP27 has no described substrates, however, due to the solving of its 3D structure, it is thought to have substrates other than the MAPKs (Friedberg *et al.*, 2007). The catalytic site most likely can better fit dual phosphorylated substrates with residues separated by two amino acids, which differs from the catalytic site of DUSPs that can dephosphorylate MAPKs (Friedberg *et al.*, 2007). DUSP27 is mainly expressed in fat, skeletal muscle, and the liver in mice, with some expression also seen in the thymus, kidney, and heart (Friedberg *et al.*, 2007). Clearly, more work is needed to discover the function, substrates, and physiological role of this protein.

Conclusion

Analysis of the current literature on atypical DUSPs reveals that a large amount of research remains to be conducted to understand their various functions in both normal and cancer cell biology. Furthermore, aberrant expression of atypical DUSPs may have clinical importance. Using the cBio Cancer Genomics Portal (http://www.cbioportal.org/) and patient data deposited by the MSKCC Prostate Oncogenome Project (Taylor *et al.*, 2010), we found that patients harboring tumors with aberrant *dusp22* or *dusp23* expression (Table 2.1) had faster disease relapse than those harboring tumors with normal *dusp22* or *dusp23* expression (Figure 2.1).

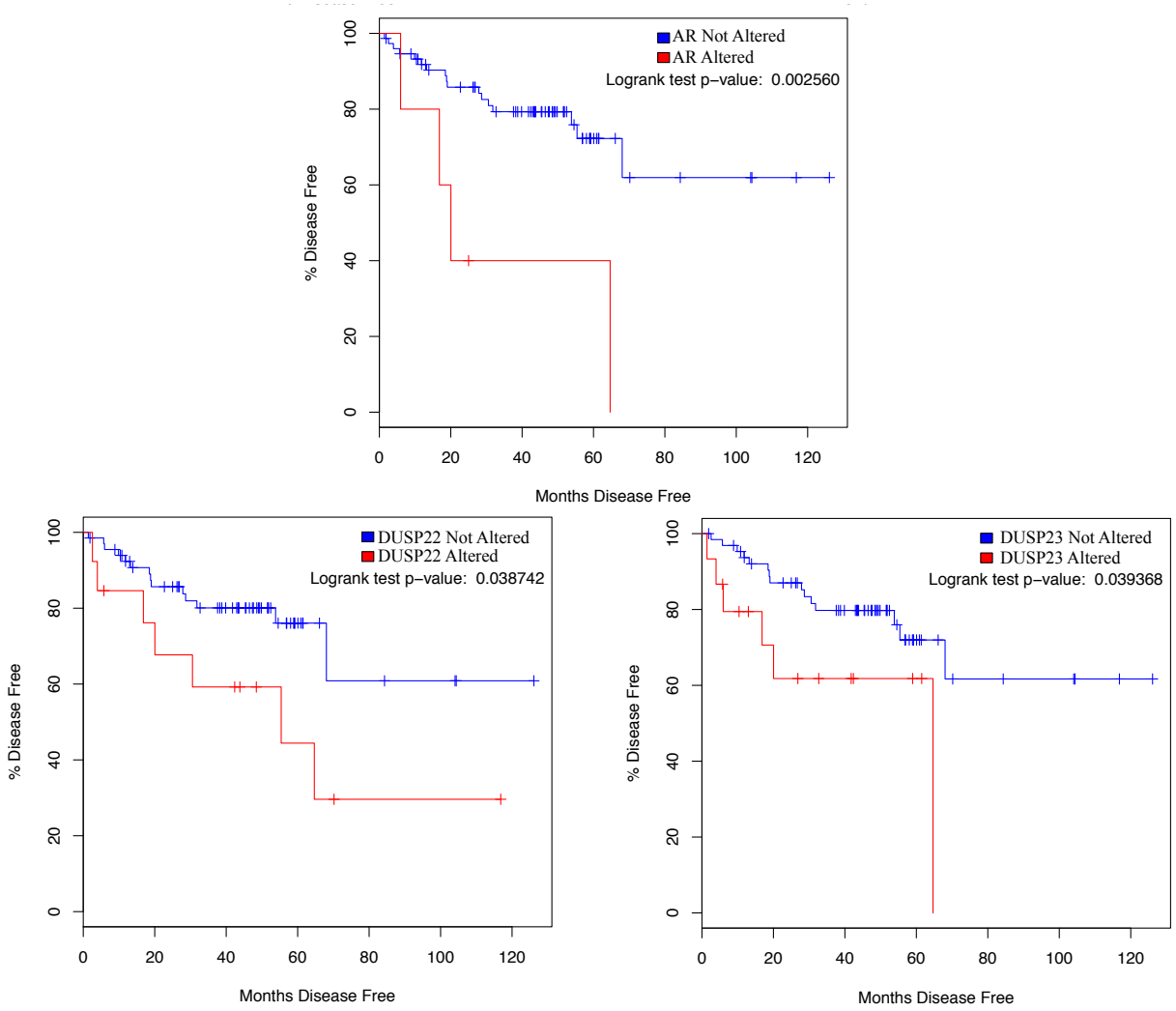

Figure 2.1 Kaplan-Meyer analysis of disease free survival for patients with altered expression of the androgen receptor or atypical DUSPs.

This graph was generated by the cBio Cancer Genomics Portal (http://www.cbioportal.org/) using data deposited by the MSKCC Prostate Oncogenome Project (Taylor *et al.,* 2010). Microarray expression data from tumors compared to normal prostate was used in this analysis with a threshold set at a Z score of +/-2. The androgen receptor was used as an example of a gene known to be differentially expressed in prostate cancer and as a gene that can impact patient survival and relapse (Taylor *et al.* 2010).

High throughput screening/genomic approaches have demonstrated that various atypical DUSPs are altered in cancer. Understanding the function of these proteins will provide valuable

insights into tumor cell biology and possibly, highlight new targets to be exploited therapeutically.

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Chapter 3 - Characterization of a human cell line stably over-expressing the candidate oncogene, dual specificity phosphatase 12

Erica L. Cain, Sterling E. Braun, and Alexander Beeser

Division of Biology, Kansas State University, Manhattan, Kansas, United States of America

Abstract

Background

Analysis of chromosomal rearrangements within primary tumors has been influential in the identification of novel oncogenes. Identification of the ‘‘driver’’ gene(s) within cancer-derived amplicons is, however, hampered by the fact that most amplicons contain many gene products. Amplification of 1q21–1q23 is strongly associated with liposarcomas and microarray-based comparative genomic hybridization narrowed down the likely candidate oncogenes to two: the activating transcription factor 6 (*atf6*) and the dual specificity phosphatase 12 (*dusp12*). While ATF6 is an established transcriptional regulator of the unfolded protein response, the potential role of DUSP12 in cancer remains uncharacterized.

Methodology/Principal Findings

To evaluate the oncogenic potential of *dusp12*, we established stable cell lines that ectopically over-express *dusp12* in isolation and determined whether this cell line acquired properties frequently associated with transformed cells. Here, we demonstrate that cells over-expressing *dusp12* display increased cell motility and resistance to apoptosis. Additionally, over-expression of *dusp12* promoted increased expression of the *c-met* proto-oncogene and the collagen and laminin receptor integrin alpha 1 (*itga1*) which is implicated in metastasis.

Significance

Collectively, these results suggest that *dusp12* is oncologically relevant and exposes a potential association between *dusp12* and established oncogenes that could be therapeutically targeted.

Introduction

Evaluation of the chromosomal region 1q21–1q23, frequently amplified in primary liposarcomas, by fluorescence in situ hybridization and comparative genomic hybridization reduced the list of candidate oncogenes contained by this amplicon to two genes: the activating transcription factor 6 (*atf6*) and the dual specificity phosphatase 12 (*dusp12*) (Kresse *et al.*, 2005). ATF6 is a transcription factor involved in the unfolded protein response (UPR), which responds to endoplasmic reticulum (ER) stress(Ma and Hendershot, 2004). While the UPR is indicated to be involved in tumorigenesis(Ma and Hendershot, 2004), the role, if any, of DUSP12 in tumorigenesis is not known. Interestingly, in four out of five liposarcomas examined, *dusp12* was expressed significantly higher than *atf6*, suggesting that *dusp12* may be the more relevant target of the 1q21–1q23 chromosomal amplification (Kresse *et al.*, 2005). In addition, *dusp12* is over-expressed in retinoblastomas, intracranial ependymomas, and the most common childhood malignant tumor, neuroblastoma (Gratias *et al.*, 2005; Mendrzyk *et al.*, 2006; Hirai *et al.*, 1999). As over-expression of *dusp12* is observed in multiple cancer types, it suggests that *dusp12* may play an important role in cancer biology.

The dual specificity phosphatases (DUSPs) are members of the protein tyrosine phosphatase (PTP) family that dephosphorylate serine, threonine, and tyrosine residues (Guan *et al.*, 1991) and are important regulators of multiple signaling pathways that modulate cell processes such as proliferation, apoptosis, and migration (Patterson *et al.*, 2009). Misregulation of DUSPs, and hence the pathways they regulate, play a major role in the development of many diseases, including cancer and diabetes (Pulido and Hooft van Huijsduijnen, 2008; Elchebly *et al.*, 1999). Members of the DUSP family can be subdivided into subgroups based on the presence of specific domains and sequence similarity. One poorly characterized subgroup, the atypical DUSPs, do not fit into any better characterized subgroups and often do not regulate known targets of DUSPs such as mitogen activated protein kinases (MAPKs) (Patterson *et al.*, 2009).

DUSP12 is an atypical DUSP whose function in human cells is poorly understood (Patterson *et al.*, 2009). DUSP12 was identified as a potential pro- survival phosphatase in an siRNA screen (MacKeigan *et al.*, 2005). The identification of DUSP12 as a pro-survival phosphatase has been supported by experiments where transient over-expression of DUSP12 in HeLa cells protects from apoptosis in response to a variety of apoptotic stimuli (Sharda *et al.*, 2009). Although DUSP12's function is poorly characterized in humans, DUSP12 is

evolutionarily conserved, and DUSP12 homologs exist in yeast (GeneID: 854844), flies (GeneID: 32963), fish (GeneID: 573998), nematodes (GeneID: 177903), and mice (GeneID: 80915). Of these organisms, the function of DUSP12 has been best characterized in the budding yeast, Saccharomyces cerevisiae, where the gene is designated *yvh1* (Guan *et al.*, 1992). Human DUSP12 and Yvh1p share 44% amino acid identity within their catalytic domain, this conservation extends to the essential C-terminal cysteine rich domain (59% identity) of unknown function that is only found in DUSP12 homologs. In S. cerevisiae, Yvh1p regulates cell growth and morphogenesis (Beeser and Cooper, 2000). Surprisingly, these abilities do not map to the phosphatase domain, but to the C-terminal rich domain, as catalytically inactive variants or variants that lack the entire N-terminal phosphatase domain suppress the phenotypes of *yvh1* deletion strains, suggesting a phosphatase independent role for Yvh1p function (Beeser and Cooper, 2000; Muda *et al.*, 1999). Importantly, ectopic expression of wild- type or catalytically inactive variants of the human *dusp12* gene in yeast also suppress the phenotypes of *yvh1* deletion strains, suggesting that the function(s) of DUSP12 and Yvh1p are evolutionarily conserved (Muda *et al.*, 1999). Recent work in yeast has also demonstrated that Yvh1p participates in 60S ribosome maturation in a phosphatase- independent manner (Kemmler *et al.*, 2009; Lo *et al.*, 2009). Although, it is clear that the human DUSP12 can functionally complement multiple phenotypes associated with yvh1 deletion in a phosphatase-independent manner (Muda *et al.*, 1999), whether DUSP12 functions similarly in human cells is currently unknown.

In this study, we have established for the first time, a stable cell line that selectively over-expresses *dusp12* in isolation and find that this cell line demonstrates increased cell motility, increased resistance to apoptotic stimuli, and has an increase in the transcript levels of two genes previously implicated in carcinogenesis, the proto-oncogene *c-met* and the collagen and laminin receptor *itga1*.

Results

Establishment of HEK293 cells stably over-expressing gfp-dusp12 in isolation

To evaluate the consequences of specific dusp12 over-expression, we established human embryonic kidney cells (HEK293), an immortalized, but non-tumorigenic cell line (Shen et al.,

2008) that stably over- expresses GFP or GFP-DUSP12 (Figure 3.1). Microscopic examination of the GFP-DUSP12 cell line revealed that they are morphologically similar in overall appearance to the GFP control cell line with the exception of more cortical actin present in the GFP-DUSP12 cell line (Figure 3.1A). In addition, there is no significant difference between the two cell lines under starvation conditions (Figure 3.1A). The GFP-DUSP12 cell line has close to a 60 fold increase in dusp12 expression compared to the control GFP cell line that only expresses endogenous *dusp12* as measured by qRT-PCR (Figure 3.1B). Expression of the full-length GFP-DUSP12 fusion protein was confirmed by immunoblotting (Figure 3.1C). All the data shown were generated with the use of one individual clone designated F78; however we observed similar results in other individual clones as well as in transient expression assays, suggesting that the phenotypes observed are not due to disruption of an unknown gene caused by the insertions of *gfp-dusp12* into the genome (data not shown-experiments performed by Cain, E.L. and Braun, S.E.).

Over-expression of dusp12 does not promote proliferation or anchorage independent growth

As *dusp12* has been described as a potential driver for the 1q21– 1q23 amplicon (Kresse *et al.*, 2005) and many oncogenes promote proliferation (Croce, 2008), we addressed whether over-expression of *dusp12* affected proliferation. Equivalent numbers of GFP and GFP-DUSP12 cells were seeded at day 0 and proliferation was assessed as a function of time by measurement of cellular ATP levels. We found no significant difference between cells over-expressing *gfp-dusp12* compared to the cell line over-expressing *gfp* alone (Figure 3.2A). As anchorage independent growth is another common property of some transformed cell lines, we asked whether *dusp12* over-expression allowed for growth in soft agar. Although we noted a slight increase in the number of colonies in the GFP-DUSP12 cell line after 21 days, there was no significant difference between the GFP-DUSP12 and GFP control cell line in either colony number or size (Figure 3.2B).

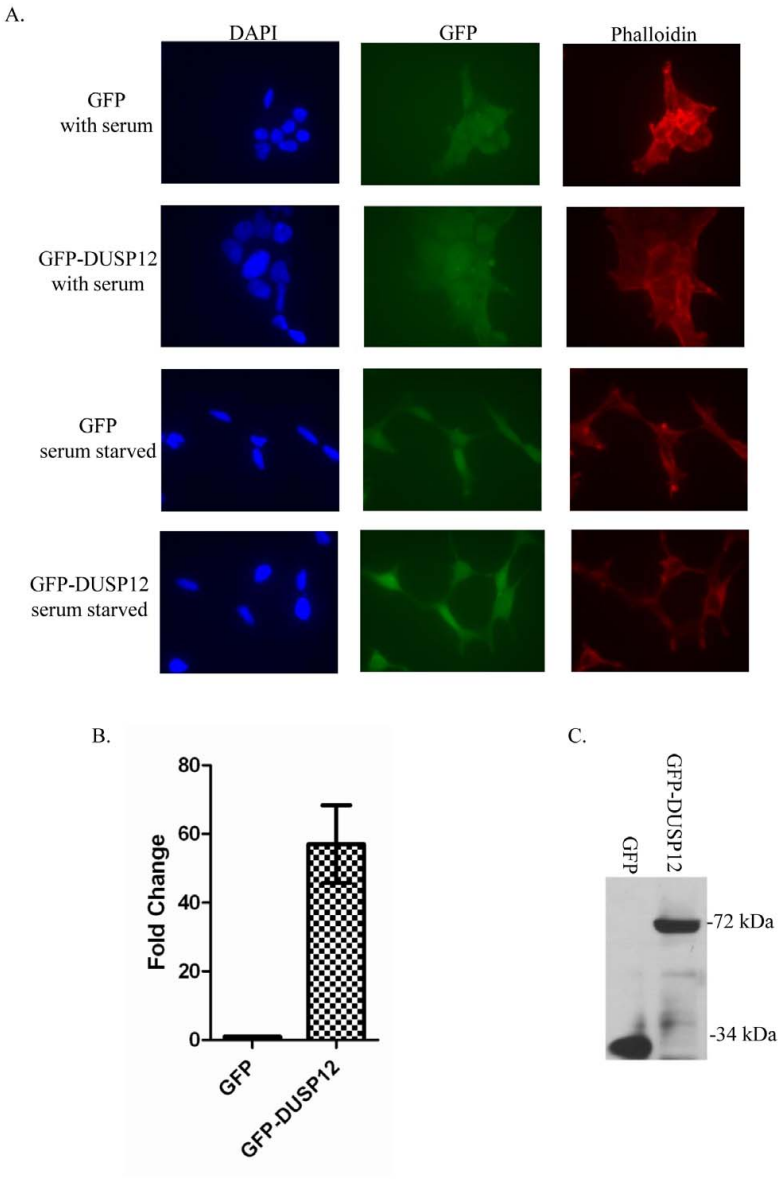

Figure 3.1 Establishment of HEK293 cells stably over-expressing either *gfp or gfp-dusp12* in isolation.

A. Confocal images of HEK293 cells stably expressing GFP or GFP-DUSP12. Cells were seeded on a fibronectin coated chamber slide. After attachment, cells were washed with PBS and complete or serum free media was added to the cells. After 18 hours, cells were fixed and stained with rhodamine-phalloidin and DAPI and viewed using confocal microscopy (magnification 100 X). B. Quantitative Real Time PCR was used to compare the expression level of *dusp12* in GFP-DUSP12 and GFP stable lines. *dusp12* specific primers were used and normalized to the average of the genes *b2m, actb*, and *gapd* genes. The fold change was calculated using the ΔΔCt method.

The mean of three independent experiments is graphed with error bars representing SEM. C. Immunoblot detecting GFP or GFP-DUSP12 in HEK293 stable lines. A single clone of GFP or GFP-DUSP12 cells was lysed in RIPA buffer. Equivalent amounts of lysates were fractionated by SDS-PAGE and GFP and GFP-DUSP12 were detected with a GFP specific antibody. The blot was also probed with a total ERK 1/2 specific antibody as a loading control. Numbers to the right of the blot indicate molecular weight

Over-expression of dusp12 promotes cell motility

Since *dusp12* over-expression is strongly associated with invasive liposarcomas (Kresse *et al.*, 2005), we tested the hypothesis that DUSP12 over-expression affects cell motility using a scratch wound assay. We observed that the GFP-DUSP12 cells were able to close the wound faster than the control cell line (Figure 3.3A). In order to better quantify the difference in cell motility, we conducted a transmigration assay using fetal bovine serum as the chemoattractant. The GFP-DUSP12 cell line had a statistically significant 1.2 fold increase in cell motility compared to the GFP control cell line (Figure 3.3B). Collectively, these results suggest that the selective over-expression of *dusp12* leads to increased cell motility.

Over-expression of dusp12 protects cells from apoptosis

Another common property of oncogene expression is the resistance to programmed cell death. In order to determine whether ectopic over-expression of *dusp12* affected apoptosis, we treated the GFP and GFP-DUSP12 cell lines with staurosporine (STS) a broad specificity kinase inhibitor that has been widely used to induce apoptosis in a variety of different cell types. Apoptosis was quantified by means of a luminescent Caspase 3/7 assay and by immunoblotting to detect cleaved PARP, a validated apoptosis marker (Koh *et al.*, 2005). Treatment of the control cell line with STS led to a significant increase in both Caspase 3/7 activity (Figure 3.4A, leftmost panel) and PARP cleavage (Figure 3.4A, right panel). The GFP-DUSP12 cells demonstrated both a reduced level of Caspase 3/7 activity and PARP cleavage after STS treatment. To ensure that these results were not specific to STS-induced apoptosis, we repeated these experiments using thapsigargin (TG), which induces the unfolded protein response (UPR) by perturbation of Ca^{2+} levels (He *et al.*, 2003). Similar to STS, over-expression of *gfp-dusp12* led to a decrease in both Caspase3/7 activity and PARP cleavage induced by TG (Figure 3.4B).

Collectively these results indicate that cells ectopically over-expressing *dusp12* have an increased resistance to apoptosis induced by different stimuli.

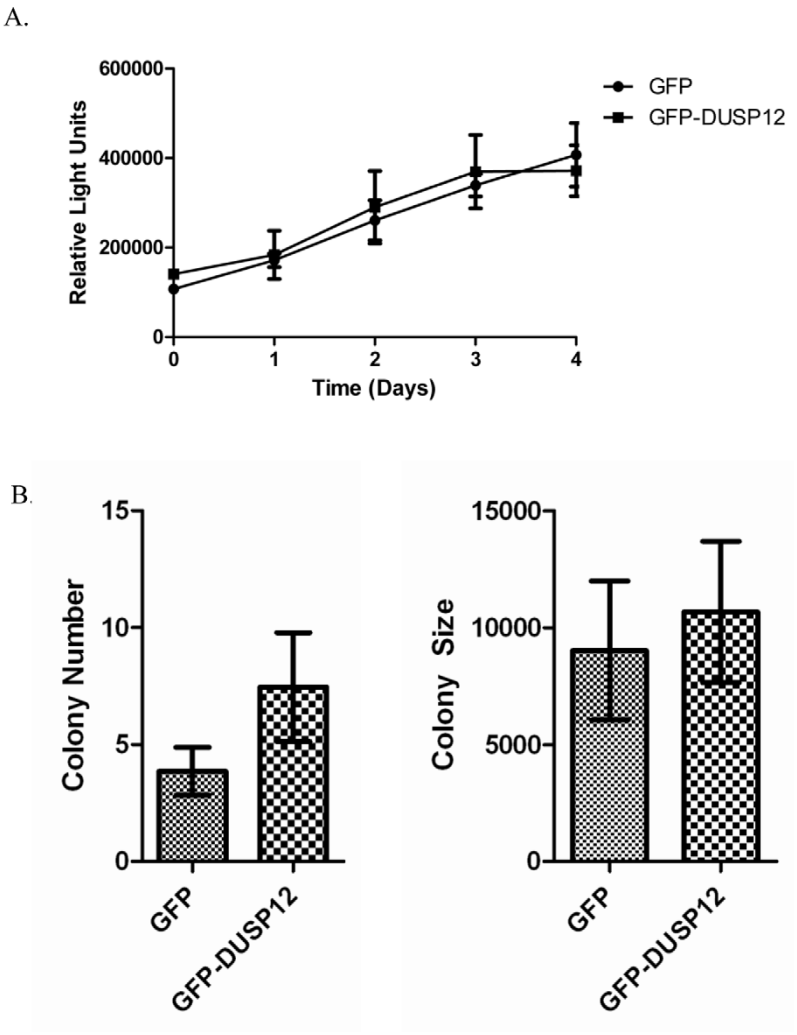

Figure 3.2 Selective over-expression of *dusp12* does not affect proliferation or anchorage independent growth.

A. Proliferation assay was performed by measuring viable cells over time with the Promega Cell Titer-Glo assay. Time zero was the measurement of GFP or GFP-DUSP12 cells immediately after seeding the wells. The means of three independent experiments are graphed with the error bars representing SEM. B. GFP or GFP-DUSP12 cells were suspended in soft agar for three weeks. Colony number and size were measured using ImageJ in five fields of vision. The mean of three independent experiments are shown and the error bars represent SEM.

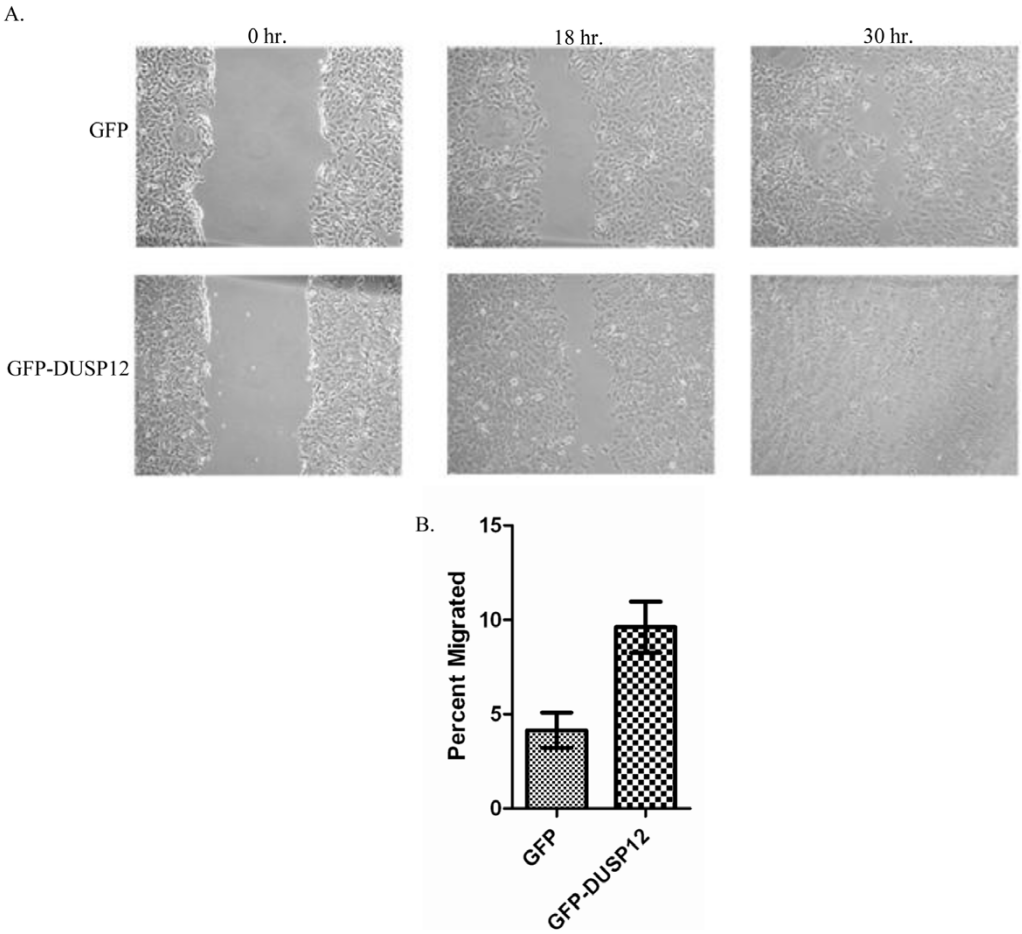

Figure 3.3 Over-expression of *dusp12* promotes cell motility.

A. A scratch wound assay was performed on confluent GFP and GFP-DUSP12 cells and the wound closure was monitored over time. Above is a representative experiment of three independent experiments. B. A transmigration assay using 0.8 mm HTS Fluoroblok transmigration chambers was performed with fetal bovine serum as the chemoattractant. Cells were pre-labeled with calcein AM and added to the wells in serum free media. After 22 hours the total number of live cells was measured and the percent of total cells that migrated to the lower chamber are graphed. The means of three independent experiments are graphed with the error bars representing SEM. Significance of a P value<0.05.

Over-expression of dusp12 up-regulates the proto-oncogene c-met and the metastasis factor itga1

To further characterize the consequences of ectopic *dusp12* expression, we used a Cancer Gene PCR array to compare the transcript levels of 84 genes previously implicated in transformation or tumorigenesis on RNA samples extracted from the GFP and GFP-DUSP12 cell lines. Candidate genes were identified as those demonstrating greater than a two-fold expression difference in the GFP-DUSP12 cell line compared to the GFP control cell line. This approach initially identified five candidate genes that were up-regulated by DUSP12: *bcl-2, cflar, itga1, vegfa,* and *c-met*. To confirm the initial PCR array results, qRT-primers for these genes were obtained and additional qRT-PCR analysis was performed using independent RNA samples extracted from the GFP and GFP-DUSP12 cell lines. This experiment confirmed that integrin alpha 1 (*itga1*) and the hepatocyte growth factor receptor tyrosine kinase (*c-met*) transcripts were significantly up-regulated in cells over-expressing *dusp12* (Figure 3.5A). The increased expression was also reflected at the protein level since western blots using c-MET and ITGA1 specific antibodies demonstrated increased amounts of these proteins in GFP-DUSP12 cells compared to the GFP control cell line (Figure 3.5B), suggesting that the increased transcript levels found in GFP-DUSP12 cells can have biological consequences.

Discussion

The GFP-DUSP12 cell line and the GFP control cell line were used to investigate whether over-expression of *dusp12* promotes oncogenic properties in a cell culture model. To our knowledge, this is the first time in which a stable cell line over-expressing *dusp12* in isolation has been described. This study demonstrates that over-expression of *dusp12,* may promote cancer development and progression by increasing migration and cell survival. Results obtained from the GFP-DUSP12 cell line can be replicated in transient assays where a Flag tagged DUSP12 is expressed in HEK293 cells (Figure 3.S1), showing that GFP is not altering DUSP12 function and that the phenotypes observed are not due to clonal variation.

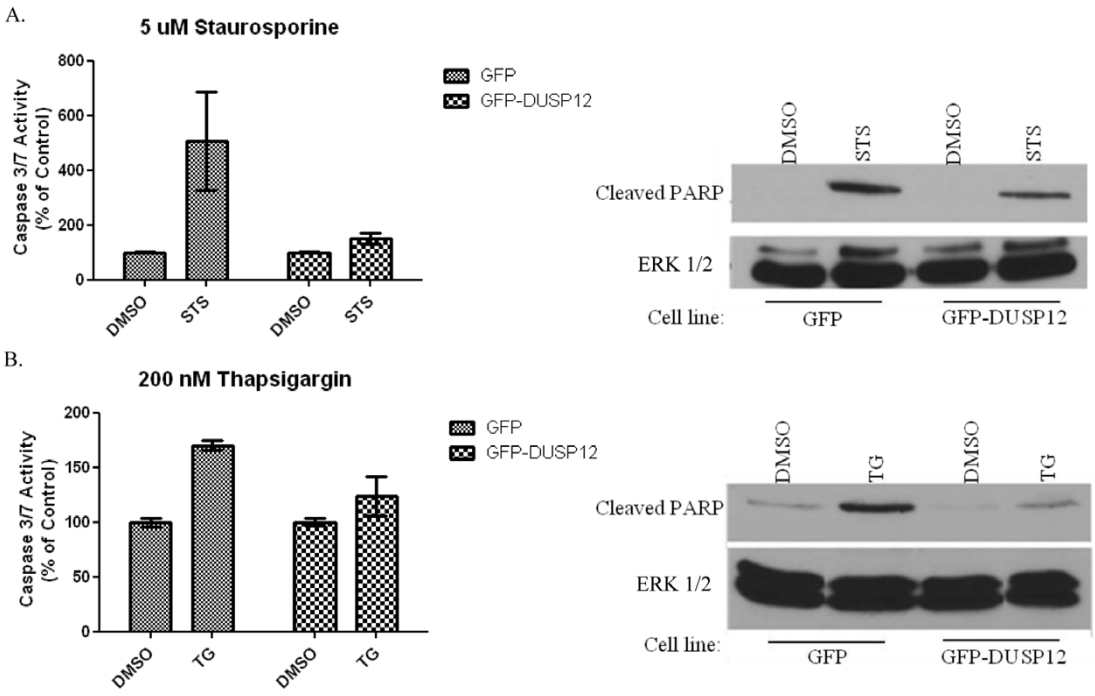

Figure 3.4 Over-expression of *dusp12* protects cells from apoptosis.

A. Left: GFP or GFP-DUSP12 cells were treated with DMSO or 5 mM staurosporine (STS) overnight and the Promega Caspase 3/7 Glo assay was used to measure apoptosis. Means of three independent experiments are graphed with error bars representing SEM. Significance of a P value<0.05. Right: Immunoblot detecting cleaved PARP in lysates of GFP and GFP- DUSP12 cells treated overnight with 5 mM STS. Lysates were collected with RIPA buffer and equalized by total protein using a BCA assay. A representative blot from three independent experiments is shown. B. Left: GFP or GFP-DUSP12 cells were treated with DMSO or 200 nM thapsigargin (TG) for 48 hours and the Promega Caspase 3/7 Glo assay was used to measure Caspase 3/7 activation as an indication of apoptosis. Means of three independent experiments are graphed with error bars representing SEM. Significance of a P value<0.05. Right: Immunoblot detecting cleaved PARP in lysates of GFP and GFP-DUSP 12 cells treated with 200 nM TG for 48 hours. Lysates were collected with RIPA buffer and equalized by total protein using a BCA assay. Results depicted are representative of at least three independent experiments.

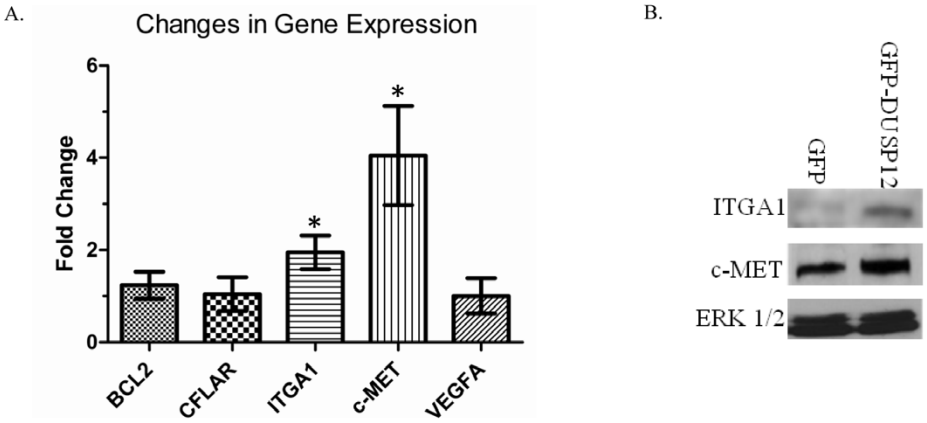

Figure 3.5 Over-expression of *dusp12* up-regulates the proto-oncogene c-MET and the metastasis factor ITGA1.

A. The expression levels of five proto-oncogenes in GFP-DUSP12 cells were compared to the expression levels in GFP cells and normalized to the average of the housekeeping genes *b2m*, *actb*, and *gapd*. The fold change was calculated using the ΔΔCt method. The mean of three independent experiments is graphed with error bars representing SEM. * indicates P value<0.05. B. Immunoblot of GFP and GFP-DUSP12 cells probed with c-MET, ITGA1, and ERK 1/2 specific antibodies. Lysates were collected in RIPA buffer and equalized by total protein as measured by a BCA assay. The blot shown is a representative blot of three independent experiments.

The up-regulation of *c-met* and *itga1* in cells over-expressing *dusp12* further indicates that *dusp12* may function as a novel oncogene, since ITGA1 is known to promote proliferation, invasion, angiogenesis, and metastasis (Lochter *et al.*, 1999; Pozzi *et al.*, 2000; Pozzi *et al.*, 1998; Schadendorf *et al.*, 1996; Senger *et al.*, 2002), while c-MET can affect proliferation, survival, and migration (Zhang and Vande Woude, 2003; Birchmeier *et al.*, 2003; Martin and Jiang, 2010). The ability of DUSP12 to up-regulate c-MET and ITGA1 may explain the increased cell motility observed in the GFP-DUSP12 cell line since both of these proteins are known to increase cell migration (Senger *et al.*, 2002; Birchmeier *et al.*, 2003). As DUSP12 is known to be over-expressed in invasive sarcomas (Kresse *et al.*, 2005), and we have demonstrated that selective up- regulation of DUSP12 leads to increased *c-met* expression, it

would be interesting to examine whether primary sarcomas containing the 1q21–1q23 amplicon also demonstrate increased expression of the *c-met* proto-oncogene.

In addition to regulating the expression of two genes previously implicated in various aspects of tumorgenesis, we found that *dusp12* over-expression increased resistance to apoptosis. Initially, *dusp12* was identified as a pro-survival gene in an siRNA screen using HeLa cells (MacKeigan *et al.*, 2005). This was supported by other experiments where transient over-expression of *dusp12* protected HeLa cells from apoptosis induced by a variety of stimuli (Sharda *et al.*, 2009). Our results with the immortal, but non-tumorigenic, HEK293 cell line suggest that DUSP12 over-expression can promote apoptosis resistance is the third instance of *dusp12* being described as a pro-survival gene, and suggests that DUSP12 can protect from apoptosis in a variety of cellular contexts. The ability of DUSP12 to promote apoptosis resistance may be due to the up-regulation of c-MET which has been described to promote cell survival (Martin and Jiang, 2010). However, we were unable to detect c-MET activation by immunoblotting with an antibody specific to c-MET phosphorylated at Tyr 1234/1235 (data not shown), suggesting that DUSP12 may protect from apoptosis in a manner independent of c-MET activation. Investigations into mechanisms by which DUSP12 can protect from apoptosis are ongoing.

c-met is over-expressed in many different neoplastic diseases (Christensen *et al.*, 2005). Studies have found that over-expression of *c-met* imbues cells with properties of cellular transformation, however, properties such as anchorage independent growth require the c-MET ligand, hepatocyte growth factor (HGF), supplied either in an autocrine or paracrine manner (Zhang and Vande Woude, 2003; Martin and Jiang, 2010; Fukuda *et al.*, 1998; Kuhnen *et al.*, 1998; Di Renzo *et al.*, 1995; Rahimi *et al.*, 1998; Tomida and Saito, 2004). Our inability to observe c- MET activation, growth in soft agar, or increases in proliferation in the GFP-DUSP12 cells may be due in part to the low expression of HGF by HEK293 cells (Rahimi *et al.*, 1998). Although growth in soft agar is often used as a metric for cellular transformation, not all oncogenes are capable of promoting anchorage-independent growth. Notably, over-expression of the *bcl-2* oncogene in normal rat epithelial cells (WBF443) and JB6 mouse epidermal cells, does not affect growth rate nor does it allow for anchorage independent growth (DeoCampo *et al.*, 2000; Amstad *et al.*, 1997). We speculate that in the correct cellular context and microenvironment DUSP12's ability to up-regulate c-MET could result in cellular

transformation. For reasons that are not well understood despite repeated efforts we were able to recover cells that only stably over-express GFP, but not the GFP-DUSP12 fusion, from a human fibrosarcoma (HT1080), cervical adenocarcinoma (HELA), breast adenocarcinoma (MCF-7) or a fibroblast (NIH3T3) cell line (data not shown).

At this time, investigations are ongoing that are examining how DUSP12 can cause the phenotypes described here. Of interest is whether the phosphatase or the unique cysteine rich domain of DUSP12 is required for the up-regulation of genes and increases in survival and cell motility. Previous work in HeLa cells suggests that the pro-survival function of DUSP12 is dependent on phosphatase activity (Sharda *et al.*, 2009). However, Yvh1p affects yeast growth (Beeser and Cooper, 2000; Muda *et al.*, 1999) and promotes 60S ribosome maturation (Kemmler *et al.*, 2009; Lo *et al.*, 2009) in a phosphatase independent manner. Both protein translation and ribosome biogenesis are coordinated with cell proliferation, and interfering with these processes can retard cell growth and animal development (Dai *et al.*, 2007). A role for ribosome biogenesis in cancer progression comes from the recent observation that c-MYC localizes to nucleoli where it functions as a regulator of ribosome biogenesis (Grandori *et al.*, 2005). Whether DUSP12 contributes to ribosome biogenesis in mammalian cells and if so, to what extent, awaits further investigation.

In summary, we describe for the first time the establishment of a cell line that over-expresses the *dusp12* gene in isolation and demonstrate that these cell lines are endowed with several cancer relevant properties: increased motility, resistance to apoptosis, and up-regulation of two genes (*c-met* and *itga1*) which are implicated in transformation and/or metastasis. As *dusp12* is present within the 1q21–1q23 amplicon present in primary liposarcomas and other tumor types, this study suggests a possible role for DUSP12 in cancer progression.

Materials and Methods

Plasmids and plasmid construction

The human *dusp12* cDNA in plasmid pOTB7 was obtained from OpenBiosystems. This plasmid was used as a template for polymerase chain reaction using Pfusion polymerase (NEB) with the following oligonucleotides incorporating 59 BamHI and 39 EcoRI restrictions sites 59-GCCCGGATCCATGTTGGAG- GCTCCG-39 and 59-GCGAATTGTCATATTTTTCCTGTT-39. The resulting PCR fragment was digested with BamHI and EcoRI and ligated into the

baculoviral transfer vector pFast- BacHTB (Invitrogen) similarly digested. The clone was then confirmed by sequencing the entire length of the insert. The DUSP12 fragment was digested with BamHI/EcoRI and ligated in to pEGFP-C1 (Clontech) digested with BglII/EcoRI to create plasmid pEGFP-DUSP12.

Immunoblotting

Cells were lysed in radioimmunoprecipitation assay buffer (RIPA) containing 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, and 1 mM sodium orthovanadate (Fisher). Cleared cell lysates were obtained by centrifugation (210006 g for 10 min at 4 C) and standardized by total protein as measured by a BCA assay (Pierce). Equivalent amounts of cleared lysates were fractionated by SDS/PAGE and transferred to PVDF membranes (Millipore). The membranes were blocked in 5% fat free milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for at least 1 hour at room temperature. All primary antibodies were diluted in blocking buffer at 1:1000 and incubated rolling at 4 C overnight. Primary antibodies used in this study are GFP (Cell Signaling #2555), Cleaved PARP (Cell Signaling #9541), p44/ p42 MAPK (ERK 1/2) (Cell Signaling # 4695), ITGA1 (Abcam #ab78479), and MET (Cell Signaling #3127). HRP- linked secondary antibodies were from Cell Signaling (#7074 and #7076) and used at a dilution of 1:30000 in blocking buffer and incubated at room temperature for one hour. For signal detection the Immobilon Chemiluminescent HRP substrate was used as recommended by the manufacturer (Millipore),and signals were obtained by exposing blots to X-ray film (MIDSCI).

Cell culture

HEK293 cells were obtained from ATCC (CRL-1573). All growth media, serum and supplements were purchased from Hyclone. HEK293 cells were grown in 16 Eagle's Minimal Essential Medium (EMEM) supplemented with 10% fetal bovine serum and penicillin/streptomycin. The GFP and GFP-DUSP12 HEK293 stable lines were cultured in the complete HEK293 media with the addition of 800 mg/ml G418 (Fisher). All experiments using HEK293 or derivative cell lines were conducted in the absence of G418 except the soft agar assays. Cells were grown at 37 C, 5% CO2 in a humidified chamber.

Establishment of GFP and GFP-DUSP12 stable cell lines

pEGFP-C1 or pEGFP-DUSP12 were transfected into HEK293 cells using the TransIT-293 transfection reagent (Mirus) in a 6 well plate following the manufacturer's recommendations. 24 hours later, cells were washed in phosphate buffered saline (PBS), trypsinized, and placed in one 100 mm tissue culture dish, and allowed to attach. Complete media was removed and replaced with complete media containing 800 mg/ml G418. Every two days the G418 containing media was replenished. After approximately 16–20 days of selection, individual clones were isolated using cloning discs, and individually expanded followed by screening for the recombinant GFP-DUSP12 or GFP expression by immunoblotting with a GFP specific antibody.

Immunofluorescence

GFP or GFP-DUSP12 cells were seeded at 40–50% confluence in Millipore 8 chambered slides coated with 200 ml of 50 mg/ml fibronectin (Sigma). After over-night attachment, cells were washed in PBS, and complete media or serum free media was added to the cells and incubated. After 18 hours the cells were washed in PBS, fixed in 3.7% formaldehyde in PBS for 15 minutes at room temperature, washed with PBS, and permeabilized using 0.2% Triton X in PBS for 10 minutes at room temperature. Cells were labeled with rhodamine-phalloidin as described by the manufacturer (Invitrogen). Cells were washed three times in PBS and 100 ng/ml DAPI (Roche) was added for five minutes prior to addition of ProLong antifade reagent (Invitrogen). Images were taken at 100X using a Zeiss confocal microscope.

Quantitative real time PCR

Total RNA was isolated from GFP and GFP-DUSP12 stable cell lines cultured in complete media, using the RNAeasy kit (Qiagen) including the optional on the column DNAse treatment. The quality of total RNA for each sample was verified by the Agilient 2100 Bioanalyzer. cDNA was produced using the RT2 First Strand kit as described by manufacturer (SABiosciences). Quantitative real time PCR was performed using the RT2 SYBR Green Master Mix following the manufacturer's protocol (SABiosciences) using an iCylcer iQ Real Time PCR Detection System (BioRad). Fold change was calculated using the ΔΔCt method. Expression levels of *dusp12* were monitored using *dusp12* specific primers described in (Caunt *et al.*, 2008). qRT-PCR primers for housekeeping genes (*actb, b2m*, and *gapd*) were obtained from

RealTimePrimers.com. To screen for cancer relevant genes that are up or down regulated by DUSP12 over-expression, we used a cancer relevant PCR array from SABiosciences (PAHS-033) as described by the manufacturer. The array contains 84 genes that are known to be involved in tumorigenesis. Genes identified from the arrays with at least a two-fold change were then verified by qRT-PCR done in triplicate with primers for the *c-met, itga1, vegfa, cflar*, and *bcl2* genes from SABiosciences.

Proliferation assay

GFP or GFP-DUSP12 cells were counted using trypan blue exclusion and a hemacytometer. 250 cells in 75 ml were seeded per well of a 96 well plate. Viable cell number was monitored over time using the Cell Titer Glo kit as described by the manufacturer (Promega).

Soft agar assay

GFP or GFP-DUSP12 stable cells were suspended in 1 ml of 0.3% Difco Noble Agar (BD) in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, penicillin/streptomycin, and 800 mg/ml G418, and then added to a six-well plate with a foundation layer of 0.5% agar in triplicate. 24 hours later, the cells received 1 ml of complete medium containing G418 before incubation for 21 days. Complete media was replenished at least once a week to prevent drying. Colonies were stained with .005% crystal violet (Fisher) dissolved in phosphate buffer saline solution for one hour at 37 C. Five fields of vision at 46 magnification were taken for each well and analyzed for colony number and size by ImageJ (NIH).

Caspase activity

Viable cells were counted using trypan blue exclusion and a hemacytometer. Approximately 2.6 x 10^4 cells were added per well to a 96 white walled clear bottom plate. The next day, either staruospoine (STS) or thapsigargin (TG) (Acros Organics) were added to the cells at a final concentration of 5 mM STS or 200 nM TG, and incubated for an additional 16 hours or 48 hours respectively. For both treatments addition of DMSO (Fisher) serves as a vehicle control. Following incubation with STS or TG, the Promega Caspase 3/7 Glo assay was performed as described by the manufacturer. Luciferase activity was measured using a Perkin-

Elmer Victor 3V. For immunoblots examining cleaved PARP, cells were seeded in a 100 mm plate and treated with a final concentration of 5 mM STS, 200 nM TG, or DMSO and lysates were collected in RIPA buffer at the indicated times.

Cell motility

For the scratch wound assay, GFP or GFP-DUSP12 cells were placed on a coverslip in a six well plate. Once cells reached confluence, a wound was created by using a micropipette tip. Pictures of the same area of the scratch were taken at 10X magnification at the indicated times. Each assay was performed in triplicate. For the transmigration assay, GFP or GFP-DUSP12 cells were pre-labeled with Calcein AM as described by the manufacturer (BD). Following labeling, cells were suspended in serum free media and placed in a HTS Fluorblok transwell in a 24 well format (BD). Complete medium was used as a chemoattractactant. Cells were also placed in a well of the 24 well plate without a transwell to be used to measure total fluorescent signal of the cells. Fluorescence was read at 0 and 22 hours using a Perkin Elmer Victor 3V. The signal at 0 hours of the transwell was subtracted from the 22 hour read. Percent migration was calculated by dividing the background subtracted 22 hour transwell signal by the 22 hour signal from the control wells that contained no transwell. Each assay was done in triplicate.

Statistics

P values were obtained using either a standard two-tailed t-test or a two- way ANOVA with Bonferroni Post-Test using GraphPad Prism.

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Author Contributions

Conceived and designed the experiments: AEB ELC. Performed the experiments: ELC SEB. Analyzed the data: AEB ELC. Wrote the paper: ELC AEB.

Supporting Information

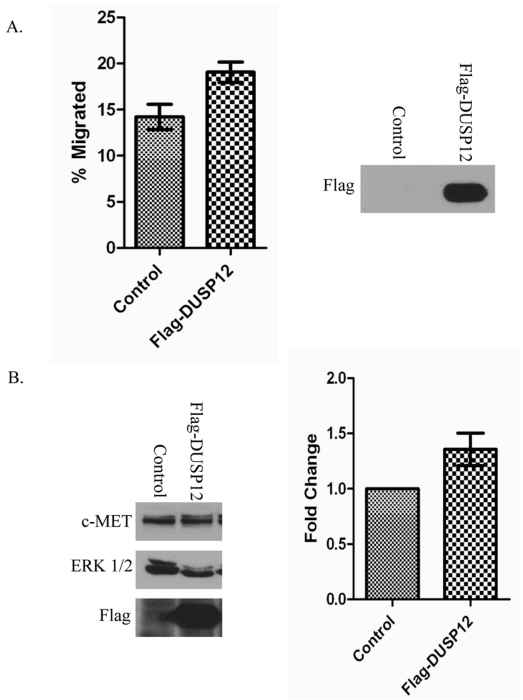

Figure 3.S1 Transient expression of Flag tagged DUSP12 in HEK293 cells promotes cell motility and up-regulation of c-MET.

A. Left: A transmigration assay using 0.8 mm HTS Fluoroblok transmigration chambers was performed with fetal bovine serum as the chemoattractant. At 24 hours post transfection, cells were pre-labeled with calcein AM and added to the wells in serum free media. After 22 hours the total number of live cells was measured and the percent of total cells that migrated to the lower chamber are graphed. The means of three independent experiments are graphed with the error bars representing SEM. Right: Immunoblot of lysates from HEK293 cells transiently expressing Flag tagged DUSP12 or the empty vector. Blot was probed with an anti-Flag antibody (Sigma #F3165). Immunoblot shown is representative of three independent experiments. B. Left: Immunoblot of lysates from HEK293 cells transiently expressing a Flag tagged DUSP12 or the empty vector. Blot was probed with antibodies specific to c-MET, ERK 1/2 (loading control), and Flag. Immunoblot shown is representative of three independent experiments. Right: Densitometry was performed using ImageJ. The fold change compared to the empty vector control is graphed after normalization with the loading control (ERK 1/2). Graphed are the results of three independent experiments with the error bars representing SEM.

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Chapter 4 - The Dual Specificity Phosphatase 12 interacts with the DDX3 DEAD Box RNA Helicase and Affects Stress-dependent Pathways

Erica L. Cain[1], Yasuaki Hiromasa[2], John M. Tomich[2], and Alexander Beeser[1]

From [1]Division of Biology and [2]Biotech/Proteomic Core Facilities, Kansas State University, Manhattan, Kansas, United States of America

Abstract

The dual specificity phosphatase 12 (*dusp12*) is a candidate oncogene from a 1q21-1q23 amplicon observed in liposarcomas and other cancers. Ectopic over-expression of *dusp12* promotes cancer-relevant properties in cell lines, but how DUSP12 affects these processes is unknown. Using tandem affinity purification, we identified the DEAD box helicase DDX3 as a novel, and evolutionarily conserved, DUSP12 interacting protein. DEAD box helicases affect multiple aspects of mRNA metabolism including splicing, mRNP shuttling and sequestration, ribosome maturation, and protein translation. DDX3 function is of specific importance under conditions of cellular stress where cells employ multiple regulatory mechanisms to ensure or restore proteostasis. Supporting a functional role for DUSP12 in these pathways, we found that loss of the budding yeast DUSP12 ortholog, Yvh1p, ameliorated the slow growth phenotype associated with transcriptional repression of the DDX3 ortholog (Ded1p). DUSP12 over-expression in mammalian cells additionally inhibited the DDX3-dependant translation of transcripts driven by a hepatitis C virus IRES. Cells over-expressing *dusp12* additionally have altered stress-induced DDX3-dependent responses, including stress granule formation and repression of general translation. Collectively, we demonstrate that *dusp12* interacts with and negatively regulates DDX3, and affects multiple stress-related mRNA-dependent signaling pathways.

Introduction

The atypical dual specificity phosphatase, *dusp12*, is one of only two candidate genes for the target of a genetic amplification associated with human invasive liposarcomas (Kresse *et al.*, 2005) and other cancers (Gratias *et al.*, 2005; Hirai *et al.*, 1999; Mendrzyk *et al.*, 2006)

suggesting that DUSP12 may play a role in carcinogenesis. Consistent with this hypothesis, selective over-expression of *dusp12* promotes cancer-relevant properties in cells (Cain *et al.*, 2011; Sharda *et al.*, 2009), but how DUSP12 affects these diverse cellular processes remains largely unknown.

Dual specificity phosphatases (DUSPs) are members of the protein tyrosine phosphatase superfamily that dephosphorylate serine, threonine, and tyrosine residues (Guan *et al.*, 1991) and contribute to multiple signaling pathways regulating processes such as proliferation, apoptosis, and migration (Patterson *et al.*, 2009). Misregulation of DUSPs plays a major role in the pathogenesis of numerous diseases, including cancer and diabetes (Pulido and Hooft van Huijsduijnen, 2008). The cellular function(s) of any specific phosphatase is often informed by identification of the proteins they dephosphorylate (Jeffrey *et al.*, 2007), however validated substrates for DUSP12 remain largely unknown.

DUSP12 is strongly evolutionarily conserved; all DUSP12 isoforms contain an N-terminal phosphatase domain followed by a C-terminal zinc binding cysteine rich domain (CRD) (Muda *et al.*, 1999). Insights into DUSP12 function come largely from the budding yeast ortholog, Yvh1p (Guan *et al.*, 1992). *YVH1* transcription is induced by a variety of environmental stresses including nitrogen starvation and low temperature (Guan *et al.*, 1992; Sakumoto *et al.*, 1999). Deletion of *YVH1* results in several phenotypes including; slow growth, sporulation defects, improper glycogen accumulation (Beeser and Cooper, 2000), and cold sensitive growth (Sakumoto *et al.*, 1999). Ectopic expression of human *dusp12* is able to suppress all *yvh1Δ* phenotypes in yeast, suggesting that DUSP12 is a functional ortholog (Muda *et al.*, 1999). Yvh1p was also recently characterized as a ribosome assembly factor required for cytoplasmic maturation of 60S ribosomal particles (Liu and Chang, 2009), and *yvh1* phenotypes are suppressed by specific mutations in *MRT4*, a gene previously implicated in mRNA turnover and ribosome maturation (Kemmler *et al.*, 2009). Intriguingly, phosphatase-deficient variants of Yvh1p and DUSP12 suppress all *yvh1* phenotypes, and the zinc-binding CRD (which completely excludes the N-terminal phosphatase domain) of either protein is required and sufficient to complement *yvh1Δ* strains (Beeser and Cooper, 2000; Muda *et al.*, 1999). This suggests that although both proteins have protein phosphatase activity, their cellular function(s), in yeast are phosphatase independent (Beeser and Cooper, 2000; Muda *et al.*, 1999).

Several studies have implicated DUSP12 as a pro-survival protein (MacKeigan *et al.*, 2005; Sharda *et al.*, 2009; Cain *et al.*, 2011). In order to gain a better understanding of DUSP12 function, we sought to identify protein(s) that interact with DUSP12. Since DUSP12 is an ortholog of Yvh1p, we first used tandem affinity purification (yeast experiments conducted by Beeser, A. and mass spectrometry performed by Hiromasa, Y, among others) to identify potential Yvh1p interactants. These approaches identified the DEAD box RNA helicase (Ded1p), as a binding partner for Yvh1p. Genetic experiments employing transcriptional repression of *DED1* suggested that Yvh1p negatively regulated Ded1p function. Further validating the use of Yvh1p as a model for DUSP12 function, the human Ded1p ortholog, DDX3, specifically interacts with DUSP12. Ded1p/DDX3 regulate multiple aspects of mRNA metabolism including splicing, ribosome maturation, translation, nucleocytoplasmic shuttling and the formation of mRNP particles (Tarn and Chang, 2009) including stress granules, a transient mRNP complex formed under stress where the decision to store, translate, or destroy specific mRNA's is made (Hilliker *et al.*, 2011; Kedersha *et al.*, 2005; Shih *et al.*, 2011). Here, we demonstrate that *dusp12* over-expression promotes stress granule formation and reduces both cap dependent and independent translation. Consistent with a role for DUSP12 in stress responsive pathways affecting translation, over-expression of *dusp12* repressed the unfolded protein response (UPR). Collectively, these findings identify DUSP12 as a novel DDX3-interacting protein and implicate it in several important stress-dependent signaling pathways.

Results

Yvh1p functionally interacts with the DEAD box RNA helicase Ded1p.

To gain insights into DUSP12 function, we sought to identify DUSP12 interacting proteins. Since DUSP12 functionally complements *yvh1Δ* strains (Muda *et al.*, 1999), we used the genetic tools available in yeast to examine the function of Yvh1p as a surrogate for DUSP12. To identify Yvh1p interacting proteins we used endogenous TAP-tagged yeast strains and mass spectrometry. These approaches identified several proteins including the RNA polymerase II degradation factor, Def1p, the transcription regulator, Spt5p, the translation termination factor, Sup35p, and, Ded1p, a DEAD box RNA helicase (Figure 4.1A) (Woudstra *et al.*, 2002; Lindstrom *et al.*, 2003; Funakoshi *et al.*, 2007; Iost *et al.*, 1999).

As Yvh1p and human DUSP12 both affect RNA metabolism and ribosome maturation (Lo *et al.*, 2009; Lo *et al.*, 2010; Liu and Chang, 2009; Sugiyama *et al.*, 2011), we focused our attention on Ded1p. *DED1* is an essential gene in *S. cerevisiae* (Struhl, 1985), so we used conditional transcriptional repression of *DED1* by validated Tet-Off yeast strains (Mnaimneh *et al.*, 2004), which display a slow-growth phenotype in the presence of doxycycline (Beckham *et al.*, 2008). Consistent with previous reports, disruption of *YVH1* leads to a slow growth phenotype in this Tet-*DED1* genetic background in the absence of doxycycline (Figure 4.1B). With increasing transcriptional silencing of *DED1*, the differential growth advantage of Tet-*DED1* strains over Tet-*DED1 yvh1*::HIS3 strains is diminished to where at 10 µg/ml doxycycline, the Tet-*DED1 yvh1*::HIS3 double mutant grows better than the Tet-*DED1* strain (Figure 4.1B). As loss of *YVH1* promotes growth of strains transcriptionally repressing

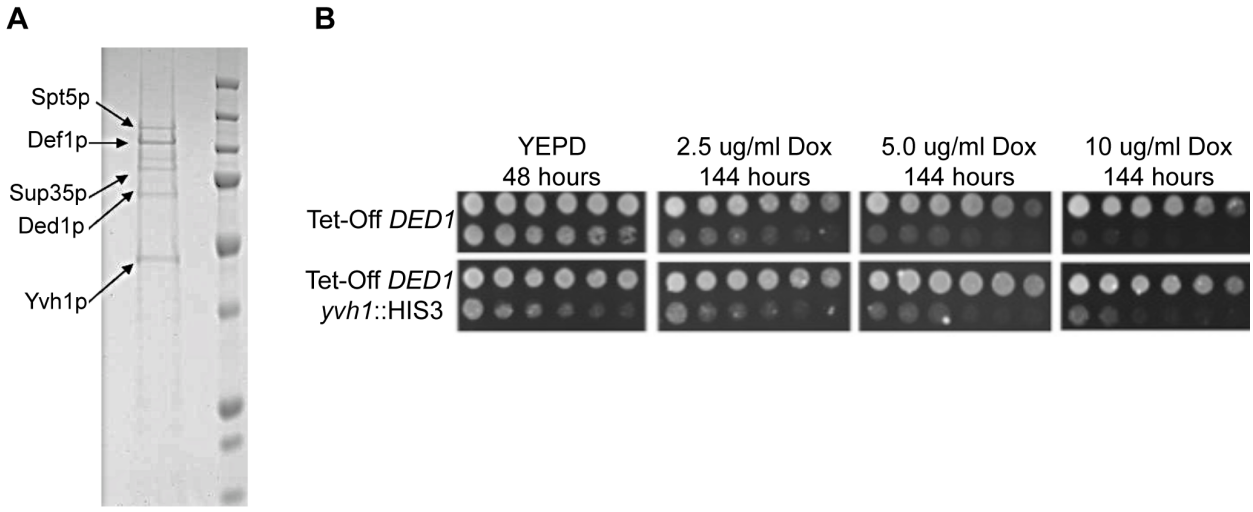

Figure 4.1 Yvh1p interacts with the DEAD box RNA helicase, Ded1p.

A. Coomasie stain of Yvh1p associated proteins isolated by tandem affinity purification using TAP tagged Yvh1p as bait. Bands were excised and proteins identified by tandem mass spectrometry. B. Five fold serial dilutions of Tet-off *DED1* or Tet-off *DED1 yvh1::*HIS3 strains were spotted onto YEPD with increasing amounts of doxycycline (Dox) to transcriptionally suppress *DED1* expression and pictures of the growth were taken at the indicated times. Experiments in yeast and Coomasie stain performed by Beeser, A. Tandem mass spectrometry performed by Hiromasa, Y.

endogenous *DED1*, it suggests a genetic interaction between *YVH1* and *DED1* with Yvh1p functioning as a negative regulator of *DED1*.

The Yvh1p-Ded1p interaction is evolutionarily conserved.

We next asked whether the interaction between Yvh1p and Ded1p is retained in humans. In order to determine whether DUSP12 interacts with DDX3, the human Ded1p homolog, and to map which domains of DUSP12 are necessary for the binding with DDX3, we tested if GFP tagged variants of DUSP12 (Figure 4.2A) could be immunoprecipitated with endogenous DDX3 from HEK293 cells (Figure 4.2B). This approach demonstrated that DDX3 efficiently co-immunoprecipitated GFP-DUSP12, but not GFP alone. Analysis of GFP-DUSP12 variants indicated that this interaction was dependent on the N-terminal phosphatase domain, as the N-terminal domain co-precipitated with DDX3 whereas the DUSP12 CRD was poorly recovered in DDX3 immunoprecipitates, despite roughly equivalent levels of expression (Figure 4.2B). These results indicate that the interaction between DUSP12 and DDX3 is conserved from yeast to humans and that the DUSP12 N-terminal phosphatase domain mediates this interaction.

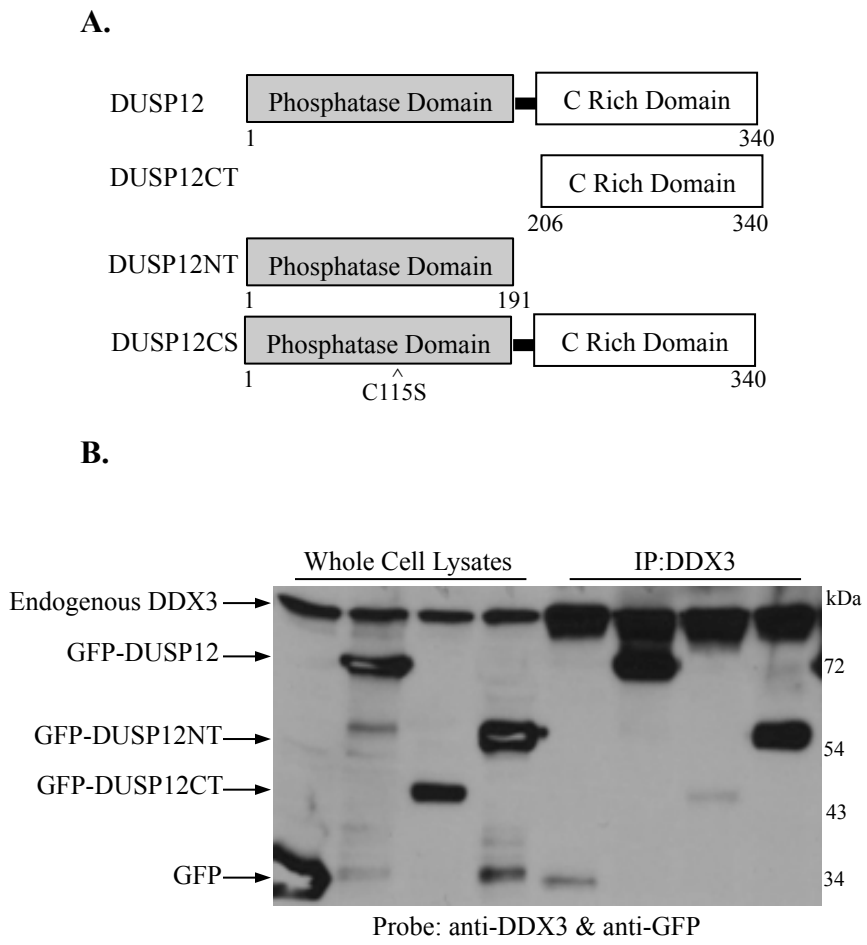

Figure 4.2 DUSP12 interacts with DEAD box RNA helicase 3, (DDX3).

A. Diagram of the different DUSP12 constructs used in this study. All constructs contain a N-terminal GFP tag (not shown). **B.** HEK293 cells were transiently transfected with indicated vectors, lysates were collected and immunoprecipitation of DDX3 performed. A representative immunoblot probed with DDX3 and GFP specific antibodies is shown.

Over-expression of dusp12 increases stress granule formation.

dusp12 over-expression protects cells from a variety of apoptotic stimuli (Cain *et al.*, 2011; Sharda *et al.*, 2009). As DUSP12 interacts with DDX3, we asked whether DUSP12 participates in DDX3-dependent processes. Although the specific role of DDX3 in many RNA-dependent functions remains unresolved (Tarn and Chang, 2009), it is an important stress granule-nucleating factor, and when down regulated, viability in response to a variety of stresses is significantly reduced (Shih *et al.*, 2011). Accordingly, we asked whether *dusp12* over-expression affected stress granule formation. HEK293 cells stably over-expressing *gfp* or *gfp-*

dusp12 (Cain *et al.*, 2011) were treated with sodium arsenite and cells subjected to immunofluorescence with the stress granule-specific marker, eIF3a (Kedersha *et al.*, 2005). In the absence of sodium arsenite, GFP-DUSP12 cells failed to promote stress granule formation (Figure 4.3). However, after sodium arsenite treatment the GFP-DUSP12 cells had significantly more stress granules per cell than the GFP control cell line (Figure 4.3). In addition to promoting and resolving stress granule formation, DDX3 itself is a component of stress granules (Lai *et al.*, 2008). To independently confirm the eIF3a results we determined the number of stress granules per cell by counting DDX3 foci. This approach revealed the same increase as observed with eIF3a after sodium arsenite treatment (Figure 4.S1). Collectively, these results implicate DUSP12 as a positive regulator of stress granule formation in human cells.

The phosphatase activity of DUSP12 is required to promote stress granule formation.

Since we found that the DUSP12 phosphatase domain is required for binding with DDX3 (Figure 4.2B), we next asked whether this domain was required to promote stress granule formation. At this time we also switched to the NIH3T3 mouse fibroblast cell line to examine stress granule formation, as stress granules are more easily observed in this cell line compared to HEK293 cells. NIH3T3 cells were transfected with the following constructs: GFP, GFP-DUSP12, GFP-DUSP12NT, GFP-DUSP12CT and GFP-DUSP12CS, a full-length variant in which the phosphatase activity is abrogated by mutation of the catalytic cysteine (Zhang *et al.*, 1994) (Figure 4.2A). Stress granule formation was monitored by the formation of DDX3 foci by immunofluorescence. DUSP12 was originally reported to be predominantly a nuclear protein, but also present in the cytoplasm in a mesh-like pattern in HeLA cells (Muda *et al.*, 1999). In NIH3T3 cells, under non-stress conditions, GFP-DUSP12 was localized in both the nucleus and cytoplasm, and in the absence of sodium arsenite DDX3 foci were absent (Figure 4.4). We noted that cells transfected with GFP-DUSP12CT, formed puncta (Figure 4.4) that did not colocalize with DDX3 or EIF3a (data not shown) suggesting that the puncta were not stress granules. The puncta may be aggregated DUSP12 C-termini due to instability as others have reported expression problems when expressing the DUSP12 C-terminus alone (Sharda *et al.,* 2009). Treatment with sodium arsenite failed to promote GFP-DUSP12 foci, but there was a shift of DUSP12 from the nucleus to the cytoplasm (Figure 4.5). As observed previously (Figure 4.3), GFP-DUSP12 expression increased the number of stress granules in response to sodium arsenite,

as did the DUSP12 N-terminal phosphatase domain. However, phosphatase-deficient variants of DUSP12 abolished the ability of DUSP12 to increase the number of DDX3 foci under stress conditions (Figure 4.5). These results were recapitulated when immunofluorescence was conducted using the stress granule specific marker eIF3a (Kedersha *et al.,* 2005) (Figure 4.S2), demonstrating that the phosphatase activity of DUSP12 was required for the increased formation of stress granules.

Over-expression of dusp12 promotes general translation repression during stress.

DDX3 affects protein translation at several levels (Tarn and Chang, 2009). In yeast, Ded1p modulates translation by the formation and resolution of mRNP particles that contain the translation initiation factor eIF4A in an ATP-dependent process (Hilliker *et al.*, 2011). As DUSP12 interacts with DDX3 (Figure 4.2B), and *YVH1* functions as a negative regulator of Ded1p function (Figure 4.1B), we next determined whether *dusp12* over-expression affected protein translation in mammalian cells. Based on our previous stress granule results (Figures 4.3 & 4.5), we investigated whether DUSP12 affected translation in cells pretreated with sodium arsenite by puromycin incorporation assays. Puromycin, a structural analog of tyrosyl-tRNA incorporates into nascent peptide chains (Nathans, 1964) and functions as a metric for general translation when monitored by a puromycin specific antibody (Schmidt *et al.*, 2009). While cells over-expressing *dusp12* did not show significant changes in puromycin incorporation under non-stress conditions, in the presence of sodium arsenite a greater translational repression compared to the GFP stable cell lines (Figure 4.6) was observed. This reduction does not derive from clonal selection of our stable cell lines as the effect could be reproduced by transiently over-expressing *dusp12* in HEK293 cells (Figure 4.S3). This data suggests that *dusp12* over-expression synergizes with arsenite treatment to promote translational attenuation in cells.

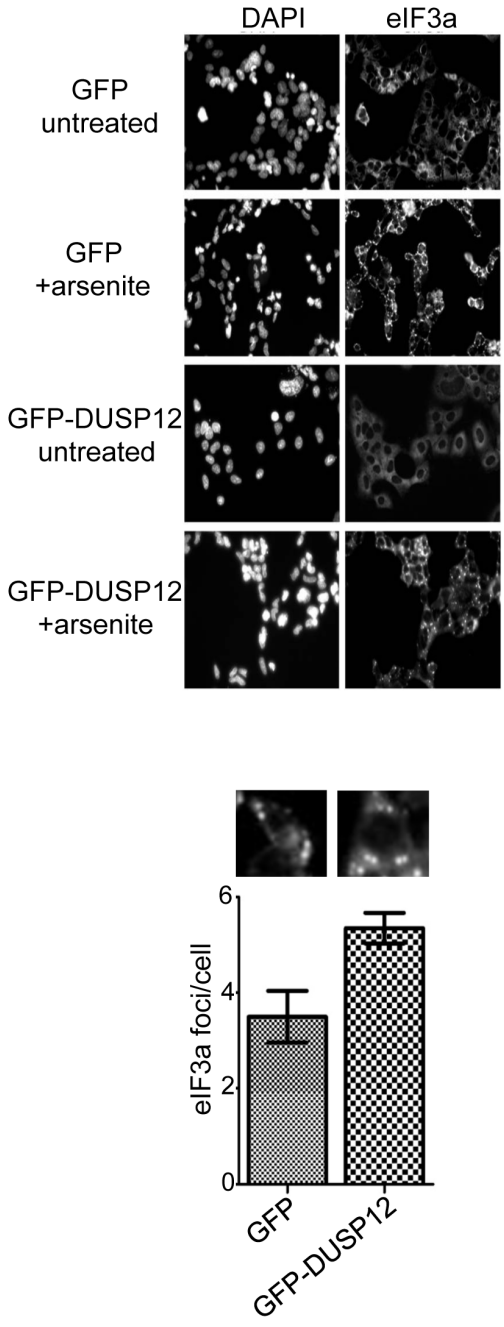

Figure 4.3 Over-expression of *dusp12* increases the number of stress granules per cell.

GFP and GFP-DUSP12 stable cells were treated with 1mM sodium arsenite for 30 minutes, fixed, and immunofluorescence using an eIF3a (stress granule marker) specific antibody was performed followed by DAPI staining. Pictures are representative of three independent experiments. The number of cytoplasmic foci and DAPI stained nuclei were counted in three

different 20x fields of view to calculate the number of foci per cell. The means of three independent experiments are graphed with error bars representing SEM. Significance of $P<0.05$.

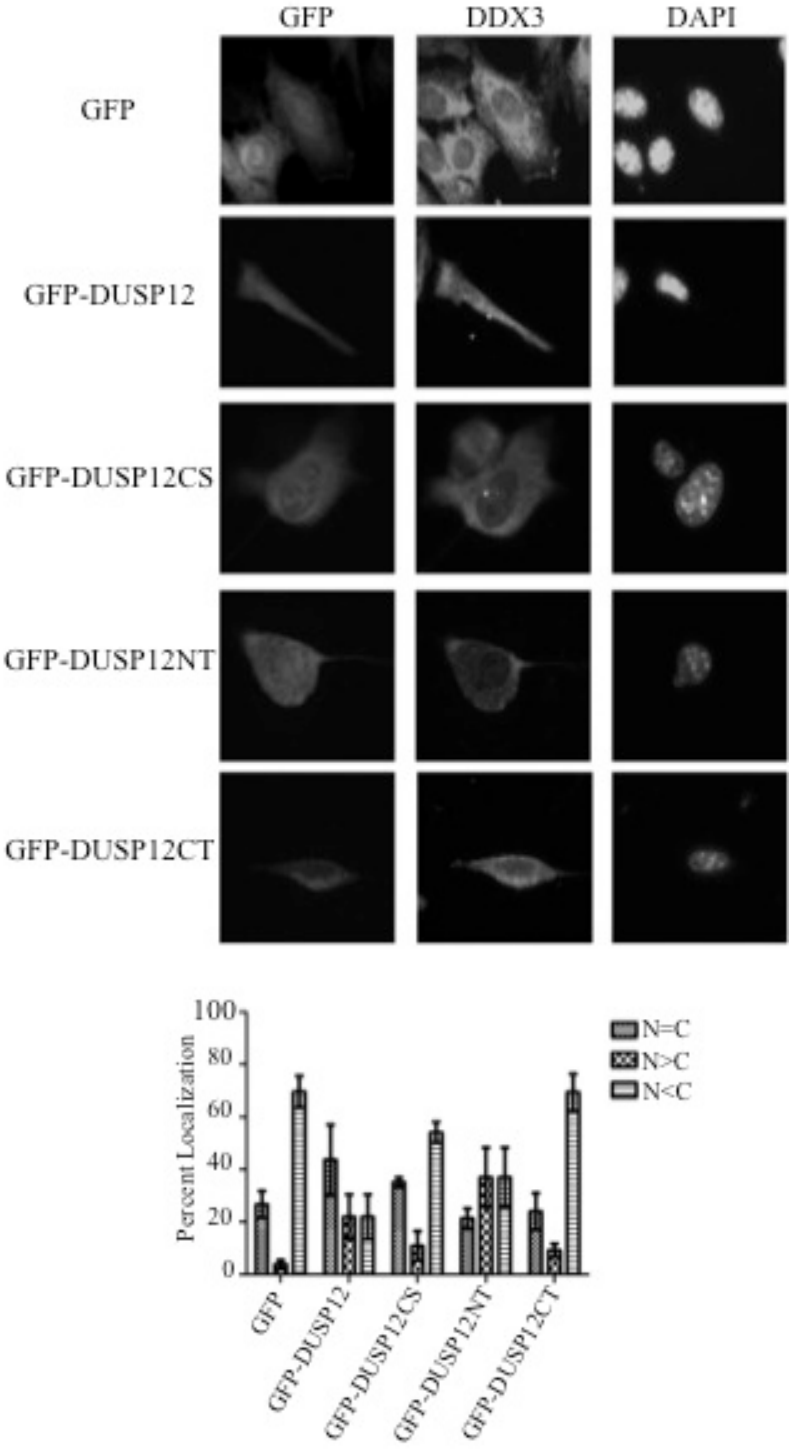

Figure 4.4 Over-expression of *dusp12* or *dusp12* variants does not promote stress granule formation under non-stress conditions.

NIH3T3 cells were transfected with the indicated constructs and the localization of DDX3 (stress granule marker) by immunofluorescence using a DDX3 specific antibody and of the GFP tagged DUSP12 variants observed. At least 10 images at 40X magnification were taken and localization was scored blind. The mean of three independent experiments is shown with error bars representing SEM.

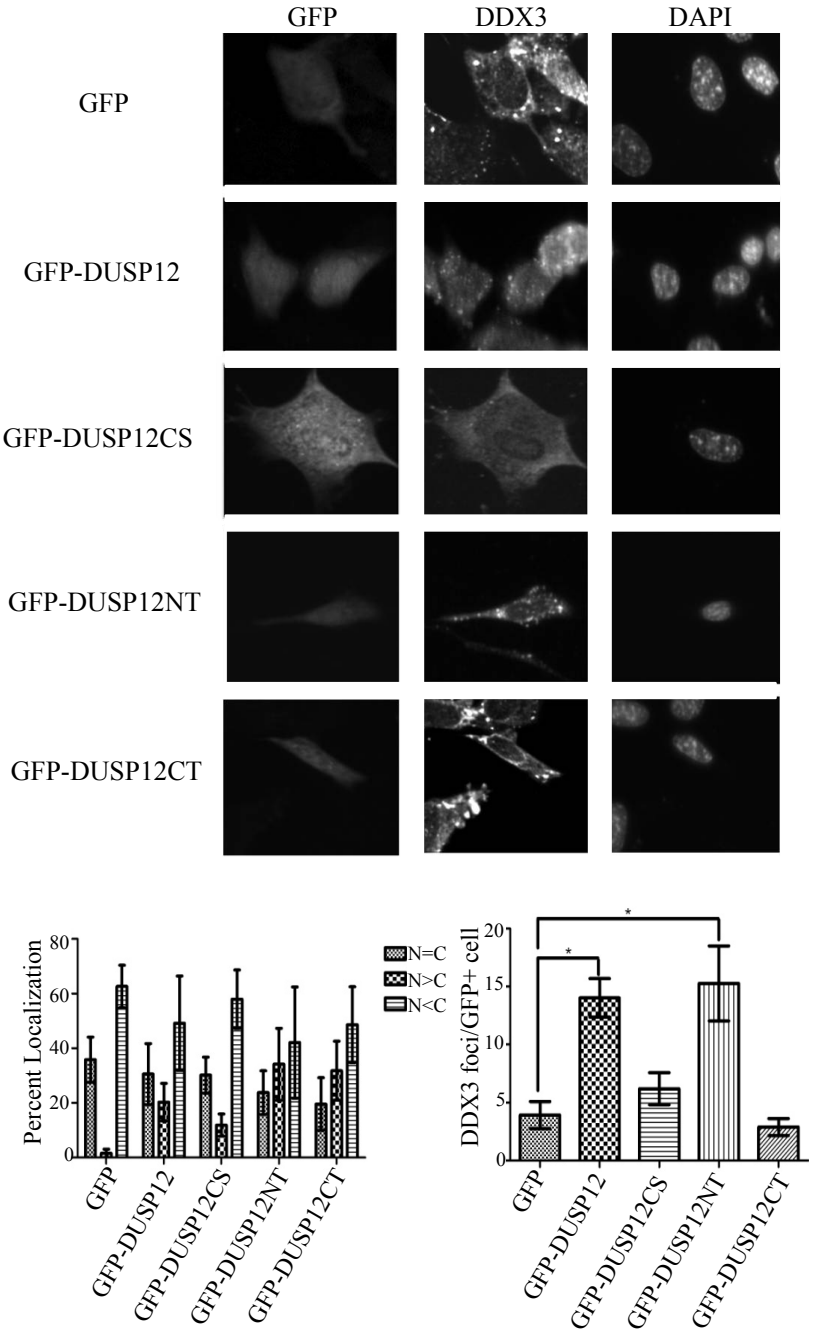

Figure 4.5 The DUSP12-dependent stress granule formation requires phosphatase activity.

NIH3T3 cells were transfected with the indicated constructs and treated with 1 mM sodium arsenite for 30 minutes and stress granules monitored by immunofluorescence with a DDX3 specific antibody. Representative images of the cells are shown (top). The cellular localization of the different GFP constructs during sodium arsenite treatment (bottom left) and the number of stress granules as indicated by DDX3 foci per GFP positive cells (bottom right) were scored blind in three independent experiments with at least 10 pictures each using the 40X objective. The means of three independent experiments are shown with the error bars representing SEM. * indicates significance of $P<0.05$.

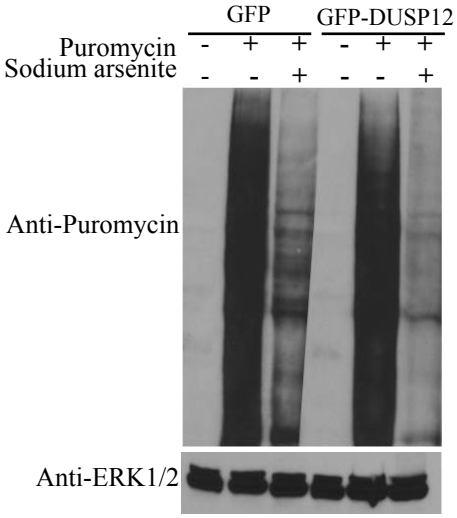

Figure 4.6 Over-expression of *dusp12* promotes general translation repression during stress.

Immunoblot showing puromycin incorporation in GFP and GFP-DUSP12 stable cell lines in the presence and absence of 1 mM sodium arsenite. An ERK 1/2 specific antibody was used as a loading control. A representative blot from three independent experiments is shown.

Over-expression of dusp12 inhibits cap-independent translation.

The specific role(s) of DDX3 in multifactorial processes such as protein translation remains to be elucidated (Tarn and Chang, 2009). DDX3 is, however, specifically implicated in cap-independent translation from an HCV-dependent IRES (Hilliker *et al.*, 2011; Shih *et al.*, 2008). Accordingly, we asked whether *dusp12* over-expression affected DDX3-dependent cap-independent translation from a biscistronic reporter with Renilla luciferase (RL) driven by cap-

dependent translation and firefly luciferase (FL) translated via the HCV IRES. GFP and GFP-DUSP12 cells were co-transfected with this reporter and with Flag-tagged full-length DDX3 or the Flag empty vector and cap (FL) and IRES (RL)-dependent translation was determined by dual luciferase assays. Over-expression of *ddx3* in GFP-DUSP12 cells resulted in significantly less IRES-mediated translation compared to *ddx3* over-expression in the GFP control cell line, while no significant changes were seen for cap-dependant firefly luciferase signal (Figure 4.7). These results suggest that DUSP12 inhibits the DDX3-dependent increase of cap-independent translation and further support our previous results in yeast, implicating DUSP12 as a negative regulator of DDX3.

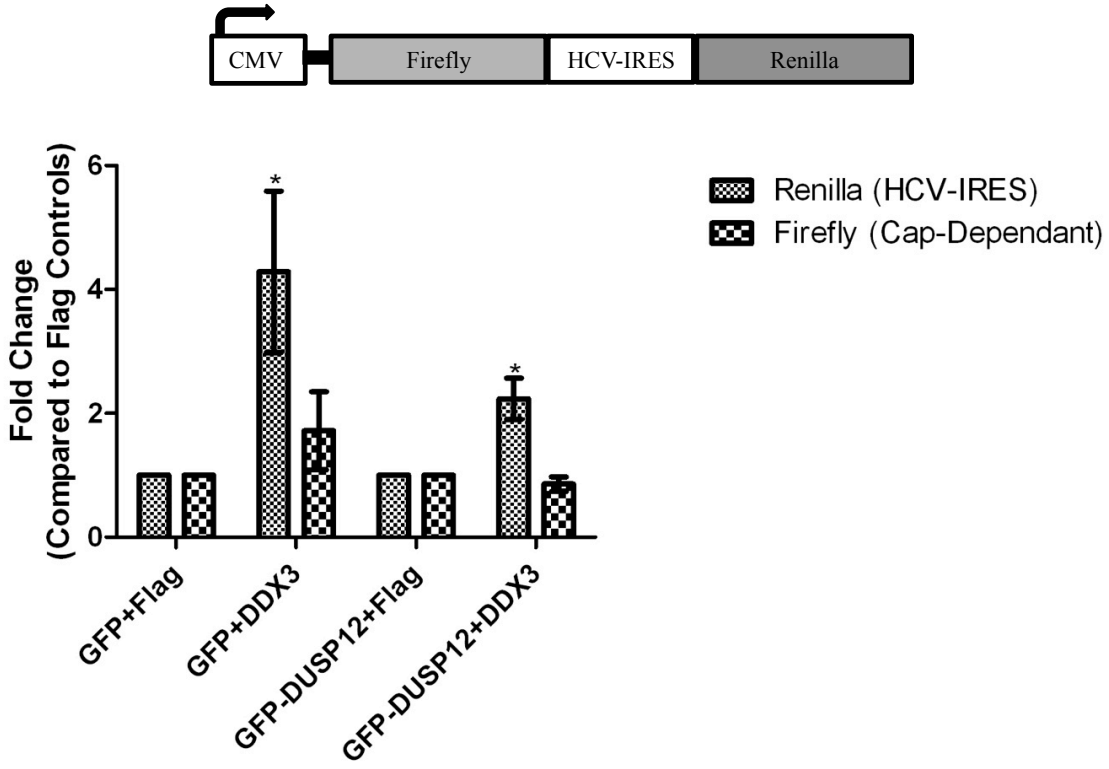

Figure 4.7 Over-expression of *dusp12* reduces the DDX3 dependent translation of a HCV IRES driven reporter.

GFP and GFP-DUSP12 cells were transfected with a biscistronic luciferase reporter that had firefly luciferase (FL) under cap-dependent translation and Renilla luciferase (RL) driven by HCV IRES mediated translation. GFP and GFP-DUSP12 cells were also transfected with a

plasmid expressing a Flag tagged DDX3 or the Flag vector. The means of four independent experiments is shown with error bars representing SEM. * indicates significance of $P<0.05$.

The unfolded protein response is attenuated in cells over-expressing dusp12

Previously, we observed that cells over-expressing *dusp12* resistant to apoptosis induced by endoplasmic reticulum (ER) stress (Cain *et al.*, 2011). ER stress induces the unfolded protein response (UPR), a homeostatic signaling pathway that responds to the accumulation of unfolded or misfolded proteins (Ron and Walter, 2007). The UPR induces a variety of changes in the cell such as increased chaperone production, translational attenuation, and remodeling of the ER (Ron and Walter, 2007). Persistent UPR activity also drives both type 1 programmed cell death and autophagy (Ron and Walter, 2007). One of the first consequences of UPR activation is reduced global translation and stress granule formation to presumably reduce the protein load at the ER in order to alleviate ER stress (Harding *et al.*, 1999). Since over-expression of *dusp12* protected cells from ER stress (Cain *et al.*, 2011) and promoted stress granule formation and reduction of general translation (Figures 4.3, 4.5, & 4.6), we hypothesized that UPR activity would be attenuated in cells over-expressing *dusp12*. To test this, we examined different aspects of the UPR in the GFP and GFP-DUSP12 stable cell lines treated with thapsigargin (TG).

One of the three arms of the UPR is mediated by PERK, a kinase that reduces general protein translation by phosphorylation of eIF2α on serine 51 (Harding *et al.*, 1999). S51 phosphorylation of eIF2α reduces general translation through the formation of a stalled 43S ternary complex (Hershey and Merrick, 2000; Hinnebusch, 1994). eIF2α S51 phosphorylation also promotes stress granules formation (Kedersha *et al.*, 1999). Accordingly, we examined the level of eIF2α S51 phosphorylation in cells expressing *gfp* or *gfp-dusp12*. GFP-DUSP12 cells had a slight, but reproducible, relative increase in basal levels of eIF2α S51 phosphorylation, and a more robust increase after TG treatment (Figure 4.8A). This increased eIF2α S51 phosphorylation is consistent with both the observed decrease in stress-induced general translation (Figure 4.6) and increase in stress granule numbers (Figures 4.4 & 4.6). In addition to promoting general translation repression, eIF2α S51 phosphorylation promotes the selective translation of specific mRNA's (Lu *et al.*, 2004)), so we asked whether *dusp12* over-expression affected the expression of ATF4. ATF4 is a transcription factor induced by the UPR and is

regulated by several upstream open reading frames uORFs (Lu *et al.*, 2004). Under conditions of eIF2α S51 phosphorylation, uORFs are translationally bypassed and translation initiation is resumed at the downstream cognate ATF4 ORF (Lu *et al.*, 2004). ATF4 expression in response to TG was characterized by dual luciferase assays in GFP and GFP-DUSP12 cells transfected with a plasmid constitutively expressing firefly luciferase reporter regulated by ATF4 uORFs. Surprisingly, despite elevated eIF2α S51 phosphorylation (Figure 4.8A), cells over-expressing *dusp12* did not significantly induce ATF4 (Figure 4.8B). Since ATF4 functions upstream of the pro-apoptotic transcription factor, CHOP (Harding *et al.*, 2000; Zinszner *et al.*, 1998), we examined whether CHOP expression was affected by *dusp12* over-expression. Consistent with the lack of ATF4-luciferase expression, GFP-DUSP12 cells failed to up-regulate CHOP during ER stress (Figure 4.8C). Collectively, these experiments demonstrate that *dusp12* over-expression affects UPR components downstream of PERK, and importantly, results in the lack of a significant induction of the pro-apoptotic factor CHOP.

Since we observed that DUSP12 reduced general translation during stress and that the activity of the PERK branch of the UPR was attenuated, we expected that the activity of the other two branches of the UPR would also be affected. To test this, we used an UPRE-luciferase reporter plasmid that responds to two prominent UPR transcription factors, ATF6 and XBP1 (Yamamoto *et al.*, 2004). Although TG treatment of GFP-DUSP12 cells was able to induce expression of the UPRE-dependent reporter transcription, it did so significantly less than the GFP control cell line (Figure 4.8D), demonstrating that cells over-expressing *dusp12* retain TG-induced UPR responsiveness, but to a lesser extent than the GFP control cells.

Discussion

In this paper we demonstrate that the candidate oncogene, *dusp12,* interacts with the DEAD box RNA helicase, DDX3. This interaction, which requires the N-terminal phosphatase domain, is evolutionarily conserved as the yeast ortholog of DUSP12, Yvh1p, interacts with the Ded1p DEAD box helicase. Both DUSP12 and DDX3 are capable of respectively suppressing the phenotypes of *yvh1Δ* and *ded1Δ* yeast strains arguing for an evolutionarily conserved role for both of these proteins (Muda *et al.*, 1999; Tarn and Chang, 2009). The observations that *DED1* is essential whereas deletion of *YVH1* only results in a slow-growth phenotype (Iost *et al.*, 1999; Beeser and Cooper, 2000), and over-expression of *DED1* (Tarn and Chang, 2009), but not *YVH1*

(Beeser and Cooper, 2000), leads to a slow growth phenotype, argues against the two proteins functioning equivalently in promoting growth in yeast. As Ded1p functions in multiple aspects

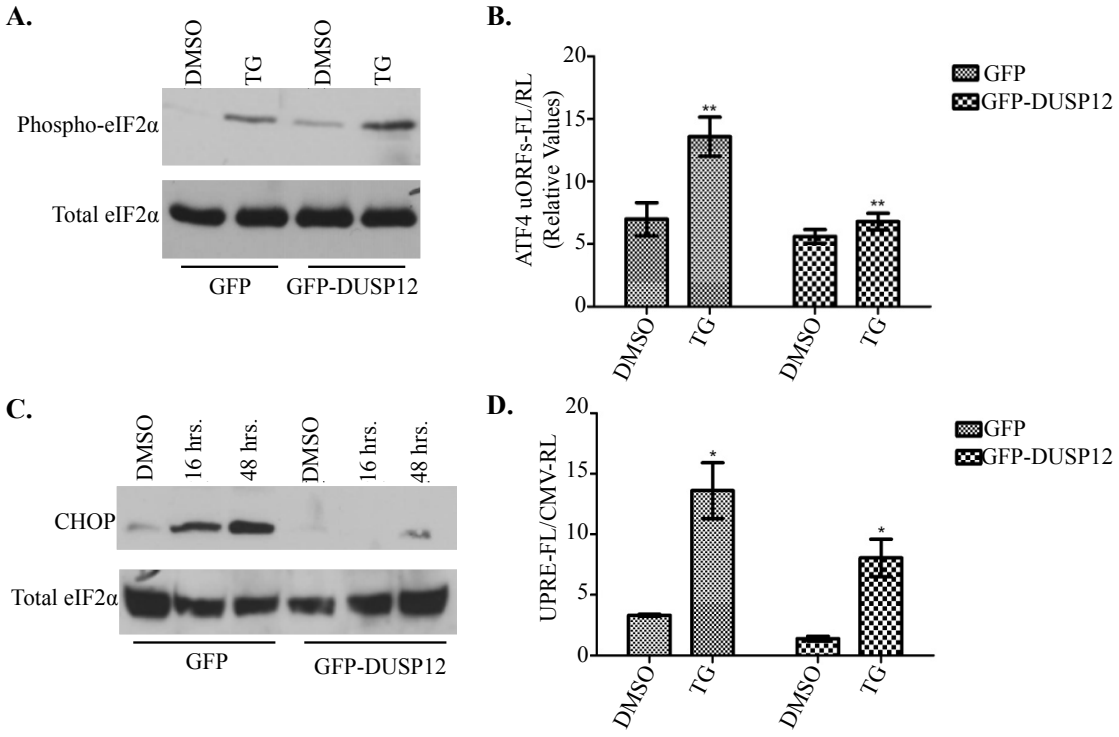

Figure 4.8 The activity of the unfolded protein response (UPR) is attenuated in cells over-expressing *dusp12*.

A. Immunoblot detecting eIF2α S51 phosphorylation after treating GFP and GFP-DUSP12 cells with DMSO or 200 nM thapsigargin (TG) for four hours. A representative blot from three independent experiments is shown. B. GFP and GFP-DUSP12 cells were transfected with the firefly luciferase (FL) gene regulated by ATF4 uORFs and treated with DMSO or 200 nM TG for four hours. A plasmid constitutively expressing Renilla luciferase (RL) was used as a transfection control. The means of four independent experiments are shown with the error bars representing SEM. ** indicates a significance of $P<0.01$. C. Immunoblot detecting CHOP and total eIF2α (loading control) in GFP and GFP-DUSP12 cells treated with DMSO or 200 nM TG for the indicated times. A representative blot from three independent experiments is shown. D. GFP and GFP-DUSP12 cells were transfected with FL driven by the UPR responsive promoter, UPRE, and treated with DMSO or 200 nM TG overnight. pRL-CMV was used as a transfection

control. The means of three independent experiments are shown with the error bars representing SEM. * indicates a significance of $P<0.05$.

of mRNA metabolism, the discrepancy between the observed phenotypes may result from Ded1p having additional important Yvh1p-independent function(s). Characterization of these functions is complicated by the fact the specific role(s) of Ded1p in yeast are currently poorly understood (Tarn and Chang, 2009), but our observed genetic interaction between *YVH1* and *DED1* under conditions of transcriptional repression suggests that *YVH1* genetically functions as a negative regulator of Ded1p function in yeast.

For our studies of human DUSP12, we rely solely on a *dusp12* over-expression model because despite the use of various siRNAs and shRNAs and cell lines, we were unable to sufficiently knock down *dusp12* (data not shown). The inability to study cells with *dusp12* knocked down may be due to the fact that reduced *dusp12* levels is detrimental to cells as knock down of *dusp12* in HELA cells results in spontaneous apoptosis (MacKiegan *et al.,* 2005) and disrupts the cell cycle in HEK293 cells (Kozarova *et al.,* 2011). However, we believe that our over-expression model is valid in that it mimics the pathological state, as *dusp12* is over-expressed in many cancers (Hirai *et al.,* 1999; Kresse *et al.,* 2005; Gratias *et al.,* 2005; Mendrzyk *et al.,* 2006).

Using *dusp12* over-expression in cell culture and immunoprecipitation we found that DUSP12 interacts with the Ded1p homolog, DDX3. Although DDX3 has pleotropic roles in humans, its role in cap-independent translation is well established (Tarn and Chang, 2009; Shih *et al.*, 2011; Mamiya and Worman, 1999). Here, we show that *dusp12* over-expression reduced cap-independent translation; further supporting our hypothesis that Yvh1p/DUSP12 functionally represses Ded1p/DDX3 function. Whether DUSP12 accomplishes this by regulating the RNA helicase activity of DDX3 in a manner similar to that as the recently characterized GLE1 (Bolger and Wente, 2011), or whether DDX3 acts as a physiological substrate for DUSP12 in human cells is currently being evaluated in our laboratory. Additionally, we so no significant difference in cap-dependent translation in cells over-expressing both *dusp12* and *ddx3*. However, in regards to cap-dependent translation, no conclusion can be declared as DDX3's affect on cap-dependent translation is debated and may be dependent on the cellular levels of DDX3 (Geissler *et al.,* 2012).

Although DUSP12 function is largely enigmatic, one of the more surprising results is that many of the reported function(s) of DUSP12 homologs are independent of phosphatase activity (Kozarova *et al.*, 2011; Beeser and Cooper, 2000). This likely does not result from the creation of "substrate-trapping" variants (Flint *et al.*, 1997), as expression of the CRD in isolation is capable of suppressing all of the phenotypes associated with *yvh1Δ* (Beeser and Cooper, 2000). Analysis of the CRD primary sequence suggests that it contains a domain that has been alternatively described as RING-variant, FYVE, or a zinc finger domain (Muda *et al.*, 1999; Alonso *et al.*, 2004; Liu and Chang, 2009), The DUSP12 CRD is more strongly evolutionarily conserved than the phosphatase domain between yeast and humans. There are at least 16 different atypical DUSPs in humans and phylogenetic analysis indicates that DUSP12 is most closely related to DUSP3 (Patterson *et al.*, 2009), but this similarity does not extend to the CRD. Biochemically, the CRD binds zinc and mutation of the evolutionarily conserved cysteines dramatically reduces protein stability (Muda *et al.*, 1999). Despite the fact that Yvh1p was one of the first eukaryotic DUSP's identified (Guan *et al.*, 1992), the biological function(s) of its CRD is currently unknown. However, our observations that the DUSP12/DDX3 interaction requires the phosphatase domain and stress granule formation is dependent on the phosphatase activity suggest that, unlike in yeast, phosphatase activity of DUSP12 is required for some functions in humans.

Yvh1p was recently identified to function in ribosome biogenesis (Liu and Chang, 2009; Lo *et al.*, 2009; Lo *et al.*, 2010; Sugiyama *et al.*, 2011). As Ded1p/DDX3 affects multiple steps of mRNA metabolism (Tarn and Chang, 2009), and DUSP12 interacts with DDX3, we further show that *dusp12* over-expression also affects proteostatic pathways in mammalian cells. Proteostatic defects can promote a variety of different diseases, and drugs that prevent protein turnover or induce perturbations of the integrated stress response are currently being used or evaluated as targeted cancer therapies (Ruggero and Pandolfi, 2003). Our finding that *dusp12* over-expression leads to increased stress-induced translational repression may explain our previous observation that *dusp12* over-expression protects cells from thapsigargin-induced apoptosis (Cain *et al.*, 2011). Once again, whether DDX3 also contributes to this resistance is difficult to establish as the specific role for DDX3 in general translation remains controversial (Tarn and Chang, 2009). Nevertheless, the observation that *dusp12* over-expression leads to

increased stress granule formation and general translational repression in response to cellular stress implicates DUSP12 as a component of stress-induced pathways to maintain proteostasis.

Our findings that cells over-expressing *dusp12* have increased eIF2α S51 phosphorylation, increased stress granule numbers, and translation attenuation in response to stress, may explain why DUSP12 can protect cells from apoptosis induced by stresses such as heat shock and thapsigargin, but not by DNA damaging agents (Sharda *et al.*, 2009), as cisplatin does not induce eIF2α phosphorylation or stress granule formation (Martins *et al.*, 2011). A stressed cell is able to conserve energy and regain homeostasis by producing stress granules, where the decision to store, degrade, or to translate existing transcripts is made (Anderson and Kedersha, 2008). The ability to promote stress granules may also provide insights into why DUSP12 can protect from FAS induced apoptosis (Sharda *et al.*, 2009), since stress granules have been found to also be protective from apoptosis induced by extrinsic means by disruption of cell signaling such as NF-kB (Li *et al.*, 2004).

The ability of DUSP12 to function as a pro-survival protein (MacKeigan *et al.*, 2005; Sharda *et al.*, 2009; Cain *et al.*, 2011), may be due in part, to the ability of DUSP12 to reduce translation in times of stress, possibly via its interaction with DDX3. As proteostasis results from the integration of multiple interdependent regulatory pathways, it was not surprising that *dusp12* over-expressing cells would ultimately affect the unfolded protein response (UPR). Since one of the first actions of the UPR is to shut down general translation (Harding *et al.*, 1999), the ability of DUSP12 to inhibit translation could reduce ER stress, leading to reduced UPR activation. Surprisingly, although *dusp12* over-expression increased eIF2α S51 phosphorylation, we did not see a corresponding increase in ATF4 expression. Failure to induce ATF4 has important consequences, including the failure to induce the pro-apoptotic factor, CHOP (Harding *et al.*, 2000; Zinszner *et al.*, 1998) possibly explaining why cells over-expressing *dusp12* are highly resistant to ER-stress mediated apoptosis induced by thapsigargin. Why ATF4 expression fails to respond to increased eIF2α phosphorylation when *dusp12* is over-expressed, remains unknown, but this observation has been previously reported (Woo *et al.*, 2009). *Woo et al.* suggest that TRIF-mediated signaling can result in attenuated translational activation of ATF4 and CHOP under conditions where eIF2α remains phosphorylated (Woo *et al.*, 2009). This is an interesting possibility since it has been demonstrated that DDX3 can regulate TBK1/IKK-mediated signaling, which are downstream of TRIF (Schroder *et al.*, 2008). In addition, we show that

basal levels of eIF2α S51 phosphorylation were increased in GFP-DUSP12 cells, and it is possible that the suppression of the UPR we observed may be similar to what has been described as adaptive resistance to ER stress in cells that undergo chronic ER stress (Rutkowski *et al.*, 2006). Alternatively, since DDX3 has been proposed to selectively affect the translation of specific mRNAs (Marsden *et al.*, 2006) it is possible that DDX3/DUSP12 could directly affect the translation of ATF4. In fact, studies in yeast have revealed that translation of a reporter regulated by the GCN4 (ATF4 yeast homolog) uORFs is reduced in yeast with reduced Ded1p levels (Berthelot *et al.*, 2004), leading to the suggestion that Ded1p is necessary for the derepression of GCN4 (Watanabe *et al.*, 2010). It will be informative to determine whether DDX3 also affects selective translation of mRNAs regulated by uORFs in mammals as many proto-oncogenes, receptor proteins, signal transduction components, and immune response genes contain uORFs (Kozak, 1991).

Because DUSP12 is thought to play a role in oncogenesis, our findings regarding the proteostatic effects of DUSP12 and the identification of protein interactants provides new insights into DUSP12 function that may have implications for both cell biology and disease. Protein translation is exquisitely coordinated with cell growth and proliferation, and proteins affecting the rate limiting translational initiation events are sufficient to transform cells in *vitro* and *in vivo* (Ruggero and Pandolfi, 2003). This is of specific importance to liposarcomas, where *dusp12* is one of only two candidate genes for the target of a genetic amplification (Kresse *et al.*, 2005). If amplification of 1q21-1q23 results in neoplastic diseases by affecting proteostasis through DUSP12, it may represent a novel vulnerability that could be exploited therapeutically.

Materials and Methods

Cell culture

All growth media, serum and supplements were purchased from Hyclone. The GFP and GFP-DUSP12 HEK293 stable lines were grown as described in (Cain *et al.*, 2011). All experiments were conducted in the absence of G418. NIH3T3 cells were obtained from ATCC and cultured as recommended.

Plasmids

The pEGFP-C1 was obtained from Clontech and pEGFP-DUSP12 construction is described in (Cain *et al.*, 2011). The DUSP12 C-terminus (amino acids 206-340) was amplified from pEGFP-DUSP12 using the following oligonucleotides: 5’GAAGATCTGCTGTTGACCCAACTACC3’ and 5’CGAATTCTCATATTTTTCCTGTTTGTGA3’. The PCR product was digested with BglII/EcoRI and ligated into pEGFP-C1 similarly cut. The N-terminus of DUSP12 containing the phosphatase domain (amino acids 1-191) was amplified from pEGFP-DUSP12 using the oligonucleotides 5’GGAATTCTAGATCTATGTTGGAGGCTCCGGGC3’ and 5’CCCAAGCTTTCAGGATCCATACTTCTCTGTAACC3’. The PCR product was digested with BglII and HindIII and ligated into pEGFP-C1 similarly digested. The plasmid containing DUSP12 harboring a C/S mutation was constructed by using the Pfusion site directed mutagenesis kit and the 5' phosphorylated primers 5'ACGGCCCTCAGCGCGGGCCTG3' and 5'GCGGTGTTGGTGCACAGTCATGCAGGAGTCAGT3' with the pOTB7-DUSP12 (Open Biosystems) as template. The mutated DUSP12 was then amplified from this construct using the oligonucleotides 5'GAAGATCTATGTTGGAGGCTCCGGC3' and 5'CGAATTCTCATATTTTTCCTGTTTGTGA3', digested with BglII and EcoRI and inserted into the similarly digested pEGFP-DUSP12CT. All constructs generated by PCR were confirmed by DNA sequencing. The pFR_HCV_xb plasmid was obtained from Addgene (plasmid 11510) (Petersen *et al.*, 2006). In this plasmid, the promoter driving the biscistronic message is thymidine kinase. The Firefly-IRES-Renilla portion was removed by digestion with HindIII and XbaI and cloned into the CMV based vector pCS2+ (A gift from Dr. Chris Thorpe, Kansas State University) to allow for more robust expression. Human DDX3X (Gene ID 1654) (Gene was amplified from clone ID 3617040, catalog #MHS1011-75670 (Open Biosystems), using the oligonucleotides 5'GGAATTCGATGAGTCATGTGGCAG3' and 5'CGGGATCCGTTACCCCACCAGTCAAC3' which also added 5' EcoRI and 3' BamHI restriction sites. The amplified product was cloned into the pGEM-T Easy vector (Promega) and verified by sequencing. The pGEM-T-DDX3 clone was digested with EcoRI and BamHI and ligated into pCMV-FLAG-MAT-1 vector (Sigma) similarly cut. Plasmid p5xATF6-GL3 was obtained from Addgene (Plasmid 11976) (Wang *et al.*, 2000), and responds to both ATF6 and XBP1 (Yamamoto *et al.*, 2004), pRL-CMV, (Promega) was used as an internal control reporter.

The pGL3-Basic Vector lacks a eukaryotic promoter upstream of the luciferase gene (Promega E1751). For the assay to examine ATF4 expression by measuring luciferase, we used Addgene (Plasmid 21850) (Harding *et al.*, 2000), which contains the luciferase gene with the ATF4 uORF sequence upstream of the firefly luciferase gene.

Immunoblotting

Immunoblotting was performed as described in (Cain *et al.*, 2011). All primary antibodies were diluted in blocking buffer at 1:1000 and incubated rolling at 4°C overnight. Primary antibodies used in this study are DDX3 (Cell signaling #2635), GFP (Cell signaling #2555), p44/p42 MAPK (ERK 1/2) (Cell signaling # 4695), eIF2α (L57A5) (Cell signaling #2103), Phospho-eIF2α (Ser51) (D9G8) XP (Cell signaling #3398), and CHOP (L63F7) (Cell signaling #2895). The puromycin specific antibody was diluted at 1:5000 and was a generous gift provided by Dr. Peter Walter (University of California, San Francisco). HRP- linked secondary antibodies were from Cell Signaling (#7074 and #7076).

Dual luciferase assays

For all dual luciferase assays, the Dual-Glo Luciferase Assay System was used as described by the manufacturer (Promega) and plates were read using a Perkin-Elmer Victor 3V. To monitor up-regulation of ATF4 translation and transcription driven by the UPRE promoter, transfections of GFP and GFP-DUSP12 cells were performed using the TransIT-293 transfection reagent in a six well plate as described by the manufacturer (Mirus). pRL-CMV plasmid was used as a transfection control for both assays. The ratio of RL to FL was 1:40 (μg/μg). For the ATF4 translation assay, cells were co-transfected with pRL-CMV and plasmid 21850 or pRL-CMV and pGL3-Basic vector plasmids and for the UPRE transcription assay pRL-CMV and p5xATF6-GL3 or pRL-CMV and pGL3-Basic Vector plasmids were used. 24 hours post transfection, each well of cells was split into six wells of a 96 well white walled, clear bottom, plate (Nunc) and allowed to incubate for 24 hours at 37 C. The media was removed from the wells and 75μl of complete media with DMSO or 200 nM thapsigargin (TG) was added to three wells of each transfection and allowed to incubate for 4 hrs. (ATF4 assay) or over-night (UPRE assay) and the FL and RL activity was measured. For each well, the ratio of FL: RL was calculated. Wells transfected with a pGL3-Basic and pRL-CMV were used to calculate background signals, which were subtracted from the experimental wells, and the relative values

calculated. The means of four (ATF4 assay) or three (UPRE assay) independent experiments were then graphed with error bars representing SEM. To monitor translation driven by the HCV-IRES, equal amounts of GFP and GFP-DUSP12 cells were co-transfected with pCMV-FLAG-MAT or pFlag-DDX3 with the HCV IRES plasmid using the ProFection transfection reagent (Promega) in a six well plate following the manufacturer's recommendations. 24 hours post transfection; FL and RL activity was measured. The means of four independent experiments were graphed as described previously.

Immunofluorescence

GFP and GFP-DUSP12 HEK293 stable lines were seeded onto a fibronectin coated eight-chambered slide (Millipore). After attachment, the media was replaced with media containing PBS or 1 mM sodium arsenite. Cells were incubated for one hour at 37^{o} C, washed with PBS and fixed with -20° C methanol for 3 minutes. After fixation, cells were washed three times with PBS and blocked in 5% bovine serum albumin in PBS for one hour at room temperature. The primary antibody, eIF3a (#3411 Cell Signaling) was diluted in blocking buffer at 1:1000 dilution. Cells were then incubated overnight at 4° C., washed three times in PBS and the Alexa Fluor 555 linked goat anti-rabbit antibody (Invitrogen), diluted 1:1000 in blocking buffer, was added to the wells and incubated at room temperature for one hour. Wells were washed three times in PBS, stained with 100 ng/ml DAPI (Roche), and mounted using ProLong AntiFade reagent (Invitrogen). Images of ten fields of view at 20X were taken of each well and the number of stress granules and DAPI stained nuclei were counted to determine the number of stress granules per cell. For transient assays, NIH3T3 cells in a six well plate were transfected with pEGFP-C1, pEGFP-DUSP12, pEGFP-DUSP12CS, pEGFP-DUSP12NT, or pEGFP-DUSP12CT using the Arrest-In Transfection Reagent as described by the manufacturer (Open-Biosystems). 24 hours post transfection; cells were split onto an eight-chambered slide (Millipore). At 48 hours post transfection, the media was removed from the cells and complete media containing PBS or 1 mM sodium arsenite was added and cells incubated for 30 minutes at 37^{o} C. Cells were then fixed and immunofluorescence was performed as described above using the primary antibody DDX3 (cat# 98711Santa Cruz) diluted 1:200 in blocking buffer. For each experiment, at least ten images were taken at 40X magnification and the number of foci counted

blind per GFP positive cell. Subcellular localization of GFP-DUSP12 and its variants was also scored blind.

Puromycin Incorporation

An equal number of GFP and GFP-DUSP12 cells were seeded into a 6- well plate. After attachment, media was removed and replaced with media with or without 1 mM sodium arsenite. Cells were incubated for 30 minutes, washed once with PBS and then media containing 1 uM puromycin was added and cells incubated for an additional 30 minutes. One well did not receive puromycin as a control to detect non-specific binding of the puromycin specific antibody. Lysates were collected and immunoblotting performed as described previously.

Tandem affinity purification

Tandem affinity purification was conducted on TAP-tagged yeast strains (Open Biosystems, YSC1178-750118) from 6 liters of YPD media. Cell pellets were collected and lysed by sonication and processed as described in (Rigaut *et al.*, 1999).

YVH1 and DED1 genetic interaction assay

The Tet-off *DED1* strain (Clone ID TH_2831) was obtained from Open Biosystems. To construct the Tet-*DED1*/*yvh1*::HIS3 strain, genomic DNA from the diploid strain HPY120(Beeser and Cooper, 2000) was prepared by Phenol/chloroform/isoamyl alcohol extraction and glass beads. The genomic DNA was used as template for polymerase chain reaction (PCR) using oligonucleotides annealing 200 bp upstream and downstream of the *yvh1*::HIS3 gene. The PCR product was used to transform the Tet-off *DED1* strain by standard methods and transformants were selected on YNB His- plates. Genomic DNA was extracted and PCR using DNA oligonucleotides distinct from those used to make the PCR KO cassette were used to validate candidate transformants. For transcriptional repression, overnight cultures grown in YEPD were diluted into YEPD and grown to A600 approximately 0.5. Cell numbers were determined by hemocytometer and 5 fold serial dilutions of both strains were spotted onto YEPD plates containing the indicated concentration of doxycycline hydrochloride (Fisher). Cell spots were allowed to adsorb into the plate at room temperature and then the plates were incubated inverted at 30° C and photographed as a function of time (Alpha Imager). This work was conducted by Beeser, A.

Immunoprecipitation

HEK293 cells were transfected with pEGFP-C1, pEGFP-DUSP12, pEGFP-DUSP12NT, or pEGFP-DUSP12CT using the Mirus TransIT-293 transfection reagent following the manufacturer's protocol in a 100 mm plate. 48 hours post transfection; cells were lysed in radioimmune precipitation assay buffer (RIPA) containing 1mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, and 1mM sodium vanadate (Fisher). Cleared lysates were equalized by total protein as measured by the BCA assay (Pierce). Immunoprecipitation using 1µg of DDX3 (2253C5a) antibody (Santa Cruz Biotechnology, #sc-81247) of the equalized lysates was conducted using the PureProteome Protein G Magnetic Beads following the manufacturer's indirect immunoprecipitation protocol (Millipore). Immunoblotting was performed of the input and eluate using the DDX3 and GFP (Cell Signaling) antibodies.

Statistics

P values were obtained using either a standard two-tailed t-test or a two-way ANOVA with Bonferroni Post-Test using GraphPad Prism.

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Supporting Information

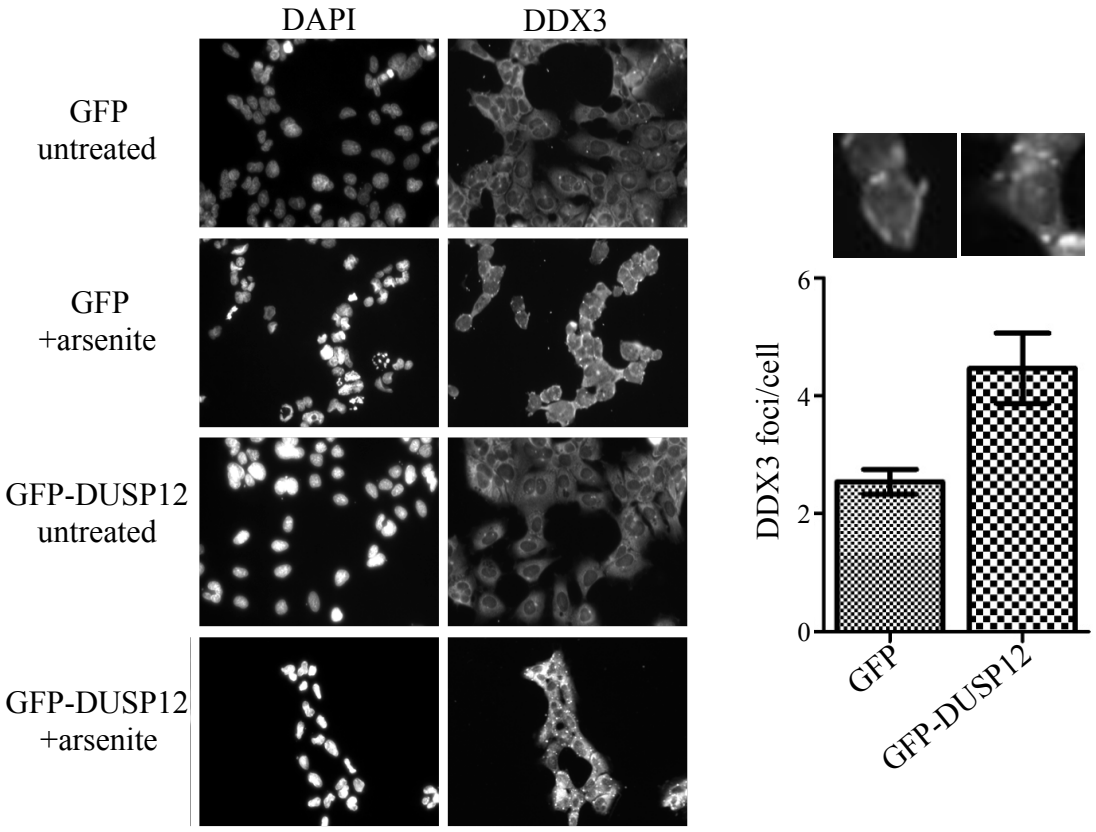

Figure 4.S1 Over-expression of *dusp12* increases the number of stress granules per cell as indicated by the formation of DDX3 foci.

GFP and GFP-DUSP12 cells were treated with 1mM sodium arsenite for 30 minutes, fixed, and immunofluorescence using a DDX3 specific antibody was performed followed by DAPI staining. Pictures are representative of three independent experiments. The number of cytoplasmic foci and DAPI stained nuclei were counted in three different 20x fields of view to calculate the number of foci per cell. The means of three independent experiments are graphed with error bars representing SEM. Significance of $P<0.05$.

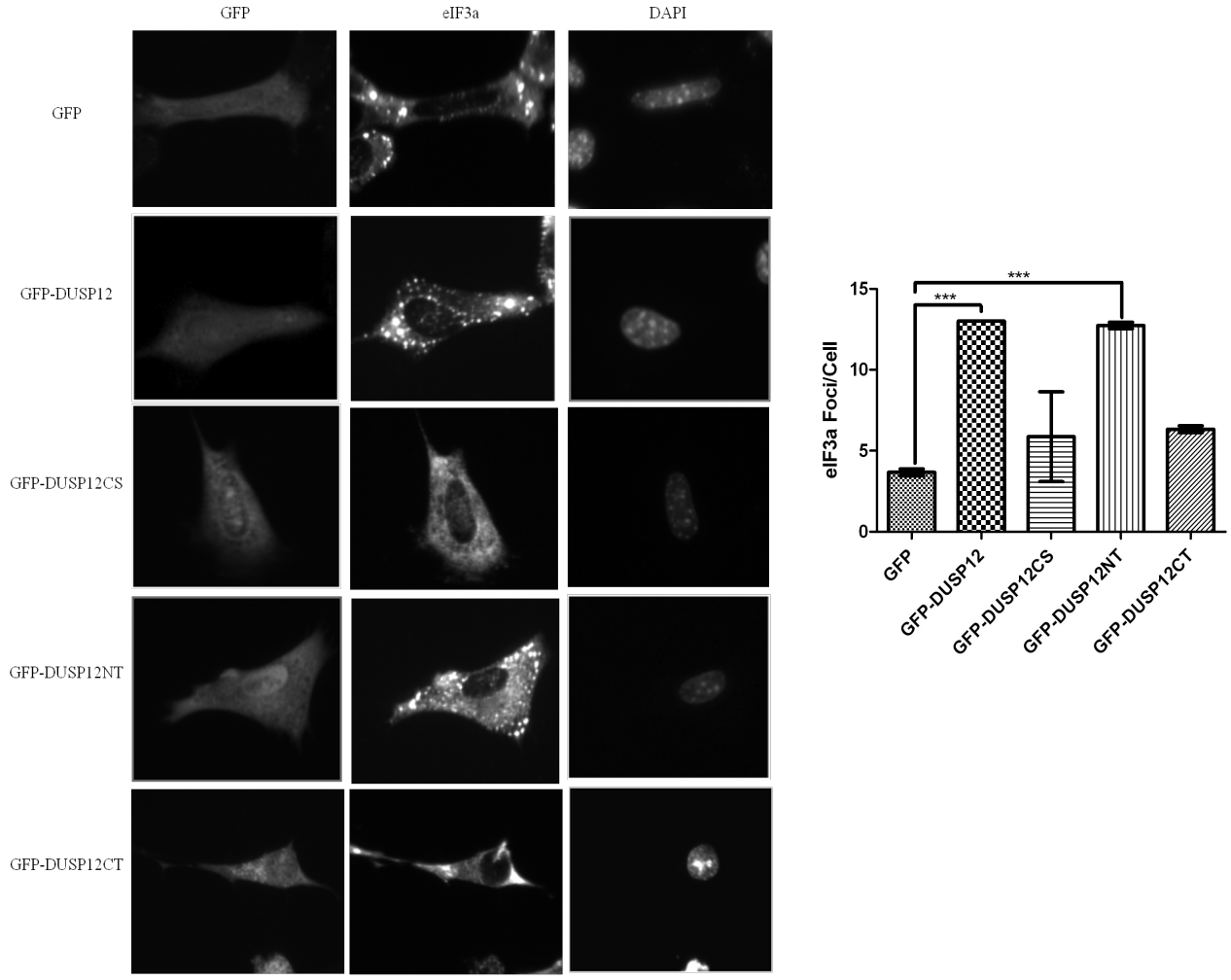

Figure 4.S2 The DUSP12-dependent stress granule formation requires phosphatase activity.

NIH3T3 cells were transfected with the indicated constructs and treated with 1 mM sodium arsenite for 30 minutes and stress granules monitored by immunofluorescence with a eIF3a specific antibody. Representative images of the cells are shown (top). The cellular localization of the different GFP constructs during sodium arsenite treatment (bottom left) and the number of stress granules as indicated by eIF3a foci per GFP positive cells (bottom right) were scored blind in three independent experiments with at least 10 pictures each using the 40X objective. The means of three independent experiments are shown with the error bars representing SEM. *** indicates significance of $P<0.001$.

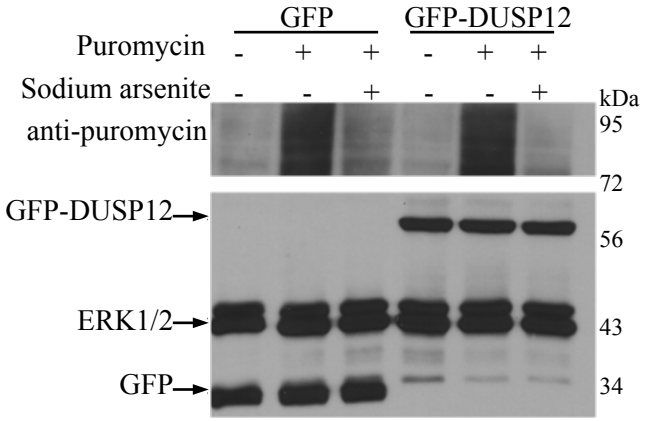

Figure 4.S3 Transient over-expression of *dusp12* promotes general translation reduction during stress.

HEK293 cells were transfected with GFP or GFP-DUSP12 and translation was monitored 48 hours post transfection plus or minus a 30 minute 1 mM sodium arsenite treatment, followed by the addition of puromycin. Puromycin incorporation was monitored by immunoblotting with a puromycin specific antibody (top blot). The lower portion of the blot was probed with GFP and ERK 1/2 (loading control) specific antibodies. A representative blot from three independent experiments is shown.

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Chapter 5 - Conclusion

The research accomplished as part of this thesis work supports a role for the dual specificity phosphatase 12 (DUSP12) in cancer development and/or progression as over-expression of *dusp12* promotes cancer attributes in cells, such as resistance to apoptosis and increased cell motility (Cain *et al.*, 2011). Work in this thesis is the first to demonstrate the ability of DUSP12 to promote cell motility and the expression of the cancer relevant genes, integrin alpha 1 (*itga1*) and the hepatocyte growth factor receptor (*c-met*) (Cain *et al.*, 2011). At this time, it is unknown how DUSP12 promotes cell motility and oncogene expression.

As DUSP12 interacts with DDX3, it is necessary to examine whether regulation of DDX3 by DUSP12 is responsible for the changes in cell motility and oncogene up-regulation. Besides regulating translation, DDX3 is a positive and negative regulator of transcription in a cell type dependent manner (Chao *et al.,* 2006; Botlagunta *et al.,* 2008). GST-purified DDX3 and purified SP1 transcription factor directly interact (Chao *et al.,* 2006). In the HuH7 hepatocarcinoma cell line, over-expression of *ddx3*, in a SP1-dependent manner, promoted the transcription of the cyclin dependent kinase inhibitor, p21, which inhibits progression of the cell cycle (Chao *et al.,* 2006). Additionally, over-expression of *ddx3* in MCF-10A mammary epithelial cells repressed the transcription of the cell adhesion molecule, E-cadherin, and caused increased cell motility (Botlagunta *et al.,* 2008). In contrast to research using the HuH7 cell line, in the MCF-10A cell line, over-expression of *ddx3* did not alter p21 transcript levels, and DDX3 bound directly to the E-cadherin promoter as demonstrated by chromatin immunoprecipitation assays (Botlagunta *et al.,* 2008). As over-expression of *dusp12* in HEK293 cells promoted cell motility (Cain *et al.,* 2011), it would be interesting to determine whether E-cadherin levels are reduced in these cells. Additionally, as the transcriptome regulated by DDX3 is mostly unknown, it would be important to examine whether DDX3 regulates the expression of the cancer relevant genes, *c-met* and *itga1*.

While this thesis work was being conducted, others demonstrated that transient over-expression of *dusp12* in HeLa cells protected cells from apoptosis (Sharda *et al.,* 2009). Consistent with these findings, I demonstrated that stable over-expression of *dusp12* in HEK293 cells also protected cells from apoptosis (Cain *et al.,* 2011). This suggests that DUSP12 can protect from apoptosis in different cell types. The DDX3-DUSP12 interaction may also help to

protect cells from apoptosis as *dusp12* over-expression promotes two processes known to involve DDX3, stress granule formation (Shih *et al.*, 2011) and stress-induced translation repression (Hilliker *et al.*, 2011). Additionally, further work is required to examine whether DUSP12 and DDX3 directly interact. Since the phosphatase domain of DUSP12 was required for the interaction with DDX3 and DUSP12's phosphatase activity was required to increase stress granules formation, studies investigating whether DDX3 is a DUSP12 substrate are also warranted. If DUSP12 functions in cancer biology to protect cancer cells from apoptosis, DUSP12 may be a potential target for cancer therapies. In order to further explore this possibility further research to unravel the function of DUSP12 in both normal and cancer cell biology are warranted.

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