

# **Elucidating the functional role of TDRD3 in stress granules**

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## **Abstract**

Tudor domain-containing protein 3, TDRD3, was first identified in a proteomic survey of the spliceosome machinery. Although its function remains elusive, elevated TDRD3 gene expression is associated with poor prognosis of estrogen receptor-negative breast cancer. The Tudor domain of TDRD3 is highly similar to the Tudor domain of the survival of motor neuron (SMN) and accordingly, it has been shown to bind dimethylated arginine residues. Our lab has previously demonstrated the association of TDRD3 with the translation machinery and most importantly, its localization to stress granules (SG) upon various cellular stresses. In this study, it was revealed that TDRD3 knockdown facilitates and accelerates SG assembly and consequently accelerates SG disassembly. Moreover, we showed that wildtype TDRD3 rescued this defect while a mutation in the Tudor domain of TDRD3, E691K, was not able to do so. Taken together, these findings allude to a prominent role for TDRD3, via its Tudor domain, in the proper formation of SGs.

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## List of Abbreviations

|         |                                                                   |
|---------|-------------------------------------------------------------------|
| aDMA    | asymmetrically dimethylated arginine(s)                           |
| ANOVA   | Analysis of variance                                              |
| Arg     | Arginine                                                          |
| ATCC    | American Type Culture Collection                                  |
| BSA     | Bovine serum albumin                                              |
| CARM1   | Coactivator-associated arginine methyltransferase 1 (a.k.a PRMT4) |
| CNV     | copy number variant                                               |
| CPEB    | cytoplasmic polyadenylation binding protein                       |
| DDX3    | DEAD/H box-3 protein                                              |
| DMA     | Dimethylated arginine                                             |
| DMEM    | Dulbecco's modified Eagle's medium                                |
| DSBs    | DNA double strand breaks                                          |
| DYRK3   | dual specificity tyrosine-phosphorylation-regulated kinase        |
| EEF1A1  | Eukaryotic translation Elongation Factor 1 alpha 1                |
| EJC     | Exon junction complex                                             |
| ER      | endoplasmic reticulum                                             |
| ER      | Estrogen receptor                                                 |
| EWS     | EWing Sarcoma breakpoint region 1                                 |
| FMRP    | Fragile X mental retardation protein                              |
| FRAP    | Fluorescence Recovery After Photobleaching (FRAP)                 |
| G3BP1   | ras-gap sH3 binding protein                                       |
| GAPDH   | Glyceraldehyde-3-phosphate dehydrogenase                          |
| GCN2    | general control nonderepressible 2                                |
| GTP     | Guanine triphosphate                                              |
| GW      | glycine-tryptophan                                                |
| HIF-1   | Hypoxia-inducible-factor -1                                       |
| HRR     | homologous recombination repair                                   |
| IB      | Immunoblotting                                                    |
| IF      | Immunofluorescence                                                |
| JMJD    | Jumonji domain-containing proteins                                |
| Lys     | Lysine                                                            |
| MLL     | mixed lineage leukemia                                            |
| MMA     | Monomethylated arginine                                           |
| MRN     |                                                                   |
| complex | Mre11/Rad50/NBS1 complex                                          |
| NOS     | nitric oxide synthase                                             |
| PABP-1  | Poly(A) binding protein, cytoplasmic 1                            |
| PAD4    | Peptidylarginine deiminase 4                                      |
| PARP    | Poly (ADP-ribose) polymerase                                      |
| PBS     | Phosphate buffered saline                                         |
| PBST    | Phosphate buffered saline with Tween                              |
| PERK    | PKR-like endoplasmic reticulum kinase                             |
| PKR     | protein kinase R                                                  |
| PRMT    | Protein arginine methyltransferase                                |

|          |                                                           |
|----------|-----------------------------------------------------------|
| PRMT     | Protein arginine methyltransferase                        |
| PTM      | post-translational modifications                          |
| PVDF     | Polyvinylidene difluoride                                 |
| RIPA     | Radioimmunoprecipitation assay                            |
| RISC     | RNA-induced silencing complex                             |
| RNA      | Ribonucleic acid                                          |
| Sam68    | Src-associated in mitosis 68K protein                     |
| sDMA     | symmetrically dimethylated arginine(s)                    |
| SDS      | Sodium dodecyl sulfate                                    |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| SERBP-1  | SERPINE mRNA binding protein 1                            |
| SG       | Stress granule                                            |
| shRNA    | small hairpin ribonucleic acid                            |
| siRNA    | small interfering ribonucleic acid                        |
| SMA      | splinal muscular atrophy                                  |
| SMN      | survival of motor neuron protein                          |
| SMN      | Survival of Motor Neuron protein                          |
| SN       | staphylococcal nuclease                                   |
| Stau1    | Staufen-1                                                 |
| Tdr      | Tudor domain                                              |
| TDRD3    | Tudor domain containing protein 3                         |
| TIA-1    | T-cell-restricted intracellular antigen-1                 |
| TRAF2    | tumor necrosis factor receptor-associated factor 2        |
| TTP      | tristetraprolin                                           |
| UTR      | Untranslated region                                       |
| WT       | Wildtype                                                  |

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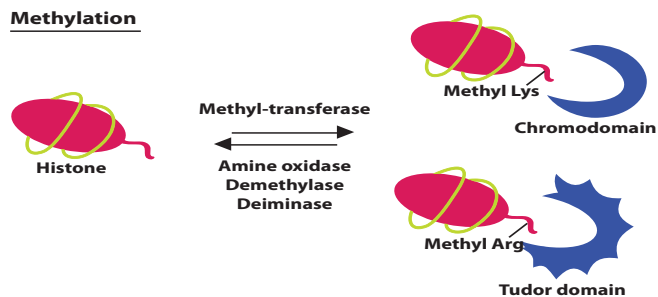
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## **Introduction**

## Post-translation modifications

Post-translational modifications (PTMs) offer a rapid and powerful way to modulate protein function, placing less overall demand on the available cellular resources (Deribe et al., 2010). These covalent modifications extend the diversity of protein function beyond that of the primary structure (McBride and Silver, 2001; Seo and Lee, 2004). PTMs include but are not limited to: phosphorylation, glycosylation, methylation, acetylation, and ubiquitination (Silva et al., 2013). Dysregulation of most of these PTMs are involved in various diseases (Lie et al., 2010; Thomas et al., 2004; Krueger and Srivastava, 2006). PTMs regulate protein stability, activity and localization as well as protein-protein interactions (Seo and Lee, 2004). Furthermore, PTMs exhibit a prominent role in regulating signal transduction pathways (Deribe et al., 2010). Phosphorylation was one of the first PTM, and since then has been the most extensively studied, affecting over 10,000 proteins (Beltran and Cutillas, 2012; Fischer and Krebs, 1955; Walsh et al., 1968). In recent years, methylation has gained significant research interest. Methylation can occur on both lysine and arginine residues (Lee et al., 2005a) and it is a significant epigenetic modification (Deribe et al., 2010). Methylation can result in mono-, di- or in the case of lysine, tri-methylated amino acids (**Figure 1**; Deribe et al., 2010; Lee et al., 2005a). Our lab is principally interested in arginine methylation.



**Figure 1. Simplified representation of methylation of both lysine and arginine residues.** Both of these residues are recognized by their chromodomain and Tudor domain respectively (adapted from Deribe and Derick, 2010). Lys: lysine; Arg: arginine.

### Arginine methylation

Arginine is considered a distinctive amino acid due to its guanidino group possessing five hydrogen bond donors, positioned perfectly to interact with hydrogen bond acceptors (Gary and Clarke, 1998). Arginine methylation, although discovered over 40 years ago (Paik and Kim, 1967), has recently become a PTM that is almost as prominent as phosphorylation and acetylation; and in mammalian cells it has been noted as the most common protein methylation reaction (Najbauer et al., 1993; Yang and Bedford, 2013). Arginine methylation is the covalent modification that results in the addition of methyl groups to a guanidino nitrogen atom of the arginine side chains (Gary and Clarke, 1998). With the addition of each methyl group, a potential hydrogen bond donor is removed, rendering a change in the overall arginine shape (Yang and Bedford, 2013). Although the modification does not alter the cationic charge of arginine, it does however, disrupt the hydrogen bonding, thereby increasing the bulkiness and

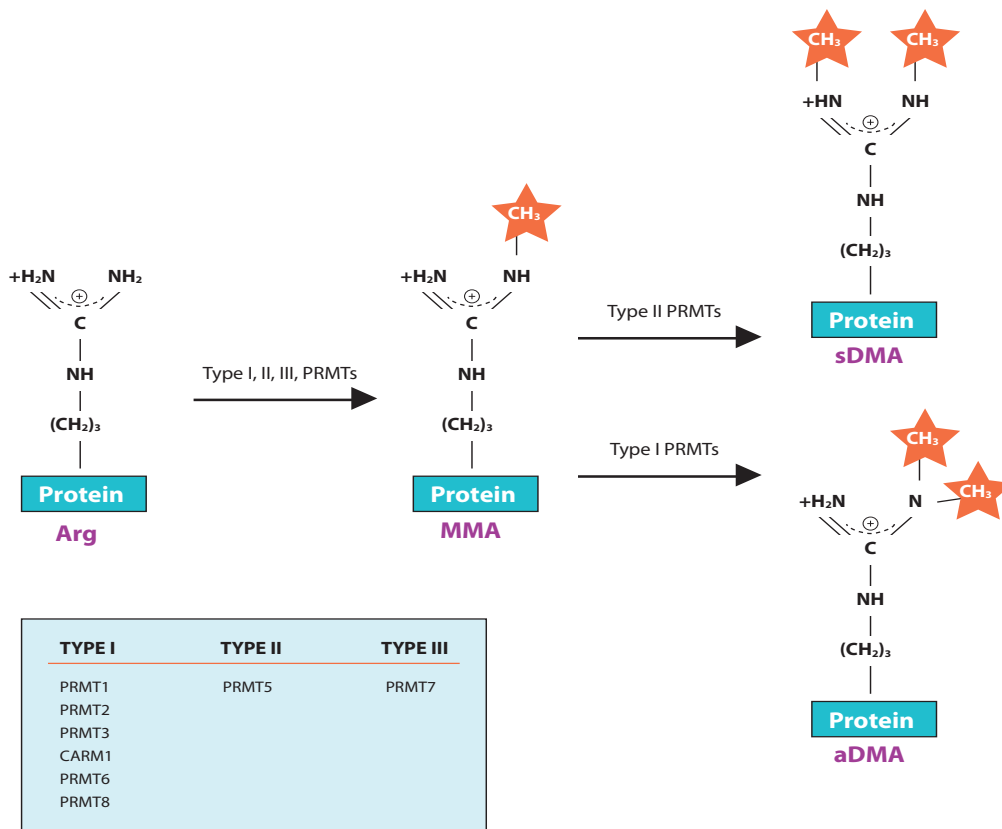


hydrophobicity of the arginine residue (Boisvert et al., 2005). The simplicity and efficiency of such a modification leads to a quick response to various stimuli. Recent evidence has demonstrated the reversibility of arginine methylation; however this still remains unclear and somewhat controversial (Zhang, 2004; Chang et al., 2007; Webby et al., 2009; Thompson and Fast, 2006; Wang et al., 2004; Cuthbert et al., 2004).

### **Protein Arginine Methyltransferase Family**

Arginine methylation is catalyzed by a specific class of enzymes known as protein arginine methyltransferases (PRMTs, **Figure 2**; Gary and Clarke, 1988). PRMTs are ubiquitously expressed enzymes (except for PRMT8) encompassing a family of nine related members that have been categorized into three distinct groups: Type I, II and III (Bedford, 2007). All three types catalyze the formation of  $\omega$ - $N^G$ -monomethylated arginine (MMA), placing a single methyl group on the terminal nitrogen atom of the arginine side chain (Bedford and Clarke, 2009). Type I PRMTs (PRMT1, PRMT2, PRMT3, PRMT4 (CARM1), PRMT6, PRMT8) lead to the subsequent production of the most prevalent type of methylated arginine, the asymmetrically  $\omega$ - $N^G$ -dimethylated arginine (aDMA) (Paik and Kim, 1980; Bedford, 2007). This entails the placement of two methyl groups on the same terminal nitrogen atom of the guanidino group (**Figure 2**; Bedford, 2007). Type II PRMTs catalyze the formation of symmetrically  $\omega$ - $N^G$ , $N'^G$ -dimethylated arginine (sDMA) by placing one methyl group on each of the guanidino nitrogens (Bedford and Richard, 2005). PRMT5 is the only clearly established type II PRMT (Bedford and Clarke, 2009). Type III PRMTs are thought to be responsible for generating predominantly  $\omega$ - $N^G$ -monomethylated arginines, and to date PRMT7 is

considered the only Type III PRMT in mammalian cells (Zurita-Lopez et al., 2012; Bedford and Clarke, 2009). Additionally, PRMT9 has been identified as a potential protein arginine methyltransferase, however its activity has not yet been determined (Cook et al., 2006). With increasing knowledge of PRMTs, the cellular processes attributed to arginine methylation continue to advance and expand.



**Figure 2. Arginine methylation and PRMTs.** All three types of PRMTs, Type I, II and III catalyze the formation of monomethylarginine (MMA), on one of the guanidine nitrogen atoms. Type I PRMTs catalyze the subsequent formation of asymmetric dimethylarginine (aDMA), Type II PRMTs catalyze the formation of symmetric dimethylarginine (sDMA), and Type III PRMTs generate only MMA.

## **Arginine methylation and cellular processes**

Arginine methylation is of great importance due to its implication in an array of cellular processes which include but are not limited to: signal transduction, pre-mRNA splicing, mRNA export, regulating mRNA stability, transcriptional co-activation and co-repression, nuclear/cytoplasmic shuttling, protein translocation and DNA repair (Lukong, 2004; Bedford and Clark, 2009; Bedford and Richard, 2005; Bedford, 2007; Lake and Bedford, 2007; Smith and Denu, 2009; McBride and Silver, 2001). Arginine methylation is further implicated in structural remodelling of chromatin gene transcription (Wolf, 2009).

## **Arginine methylation and protein-protein interaction**

Arginine methylation acts mainly by regulating protein-protein interaction both positively and negatively (Boisvert et al., 2009), although largely known for its negative regulation (Bedford and Clarke 2009). Negative regulation of protein-protein interaction is evident in the methylation of arginine residues close to proline rich motifs in Sam68, which in turn prevents binding of Sam68 to the SH3 domain but not to the WW domain (Bedford et al., 2000). Also, CARM1 methylates the C-terminal domain of GRIP1, blocking its interaction with p300 (Lee et al., 2005b). Moreover, methylation of H3R2 by PRMT6 inhibits the interaction of WDR5 with histone H3 (Couture et al., 2006). Negative regulation of arginine methylation is further evident in the interaction of SRC-3 with CARM1, where the methylation of SRC-3 promotes the dissociation of the SRC-3/CARM1 coactivator complex (Feng et al., 2006). In addition, RIP140 methylation by PRMT1 reduces the recruitment of histone deacetylases to RIP140, and thereby eases the

nuclear export of RIP140 (Mustaqul et al., 2006). PRMT1 has also been shown to methylate RUNX1 *in vivo* (Zhao et al., 2008). RUNX1, also known as acute myeloid leukemia 1 (*AML1*), is a transcription factor required for proper development of hematopoiesis (Zhao et al., 2008). RUNX1 methylation consequently abrogates the interaction of RUNX1 with SIN3A and ultimately promotes RUNX1 transcriptional activity (Zhao et al., 2008).

Positive regulation is evident in the requirement of symmetrical dimethylation of SmB by PRMT5, to bind with the Tudor domain of SMN, SPF30 and TDRD3 (Côté and Richard, 2005). Also, as mentioned above, CARM1 asymmetric dimethylation of CA150 provides a docking site for the SMN Tudor domain (Cheng et al., 2007).

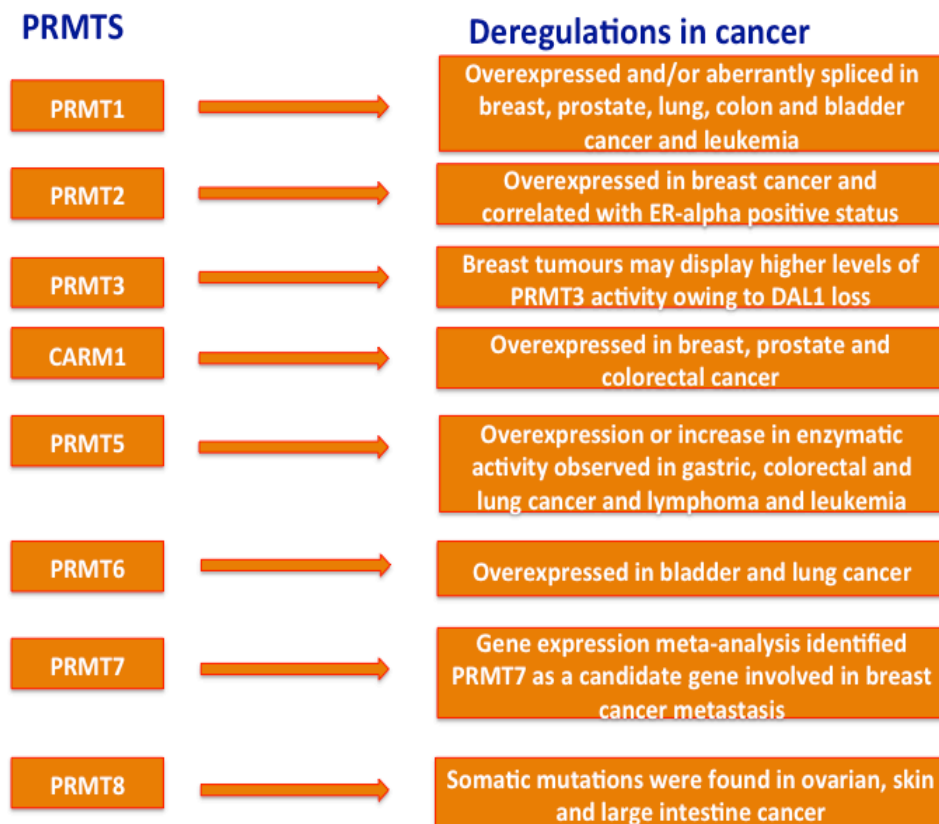
### **Arginine methylation and cancer**

Arginine methylation plays a vast role in an array of human pathologies and several studies have shown it has a potentially important role in cancer (Yang and Bedford, 2013). With the apparent deregulation of PRMTs in various cancers, the involvement of PRMTs in cancer has gained recent research interest (Bedford and Clarke, 2009; **Figure 3**). Many of the PRMTs are known to exhibit function in transcriptional regulation, a significant component of cancer metastasis (Ell and Kang, 2013). PRMT1 and CARM1 are the most well characterized transcriptional coactivators and alterations in their levels likely have an effect on the transcriptional pathways (Chen et al., 1999; Frietze et al., 2008; Bedford and Richard, 2005). For instance, overexpression or aberrant expression of PRMT1 has been linked to an array of human cancers including, but not limited to breast, prostate and bladder cancer, as well as leukemia (Goulet et al., 2007; Le Romancer et al., 2008; Cheung et al., 2007; Shia et al.,

2012; Yoshimatsu et al., 2011). PRMT1 is linked to breast cancer where *PRMT1* mRNA levels were shown to be higher in an array of breast cancer cell lines in comparison to normal cell lines (Goulet et al., 2007). Furthermore, estrogen receptor (ER) status is important for the prognosis of breast cancer, and ER $\alpha$  has been identified as a substrate for PRMT1 (Le Romancer et al., 2008; Le Romancer et al., 2010). PRMT1 is known to deposit an aDMA methylation mark, H4R3me2a, (Strahl et al., 2001; Wang et al., 2001) which has been positively correlated with the tumour grade and the risk of reoccurrence in prostate cancer (Seligson et al., 2005). Furthermore, PRMT1 has been linked to the maintenance of human telomeres (D  jardin and Kingston, 2009). Telomeric repeat-binding factor 2 (TRF2), a component known to protect telomeres, is a substrate of PRMT1 (Mitchell et al., 2009). Maintenance of telomeres exhibits a major role in carcinogenesis and accordingly PRMT1 knockout studies have associated PRMT1 with telomere maintenance, thereby demonstrating its role in carcinogenesis (Yang and Bedford 2013). Also, PRMT1 exhibits a prominent role in the regulation of leukemia, being a crucial component of the mixed lineage leukemia (MLL) oncogenic transcriptional complex (Cheung et al., 2007). PRMT1v2, an isoform of PRMT1, showed increased expression in various breast cancer cell lines and tumours (Goulet et al., 2007; Baldwin et al., 2012). Furthermore, its depletion leads to cancer cell apoptosis and decrease in cell invasion (Baldwin et al., 2012).

CARM1 is another PRMT prominently involved in cancer (Chen et al., 1999; Wang et al., 2001). Studies have shown that CARM1 is overexpressed in castration-resistant prostate cancer (Majumder et al., 2006; Hong et al., 2004), and is overexpressed in aggressive breast tumours along with cyclin E1, a protein that is transcriptionally

regulated (El Messaoudi et al., 2006). In addition, breast tumours that have elevated levels of CARM1 concurrently express high levels of the oncogenic co-activator, AIB1 (El Messaoudi et al., 2006). AIB1 has been associated with poor prognosis of breast cancer and, interestingly, is methylated by CARM1 (Lahusen et al., 2009; Feng et al., 2006; Naeem et al., 2007). Furthermore, CARM1 has been linked to colorectal cancer, where it has been found to be overexpressed in over 75% of clinical samples (Kim et al., 2010).



**Figure 3. Deregulation of PRMTs in various cancers (adapted from Yang and Bedford, 2013).**

### **Arginine methylation and other diseases**

Arginine methylation has been shown to play a significant role in cancer, but is also implicated in various other diseases, notably, cardiovascular diseases (Bedford and Richard, 2005). Nitric oxide is synthesized from L-arginine nitric oxide synthase (NOS) and plays numerous roles in the cardiovascular system. MMA and aDMA, but not sDMA, inhibit the activity of NOS, demonstrating a link between arginine methylation and cardiovascular diseases (Stuhlinger et al., 2001). Moreover, arginine methylation has been linked to viral pathogenesis, where different viral proteins are known PRMT substrates, and the consequent methylation of these viral proteins can lead to disruption in cellular processes (Boulanger et al., 2005). Lastly, the SMN protein implicated in spinal muscular atrophy, SMA, contains a Tudor domain and point mutations within this domain were identified in SMA patients (Tadesse et al., 2008; Cheng et al., 2007). This alludes to a role for arginine methylation in SMA (Tadesse et al., 2008; Cheng et al., 2007; Friessen et al., 2001).

### **Arginine methylation and stress granules**

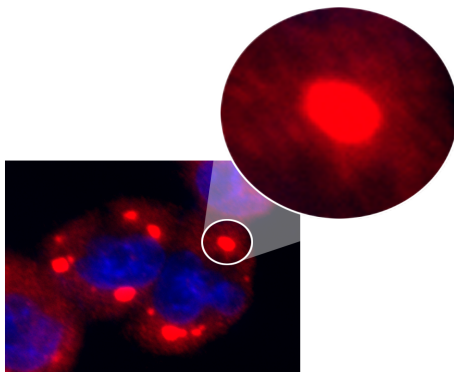
Arginine methylation has been associated with stress granules. Stress granules (SG) are cytoplasmic RNA structures harboring mRNAs stalled in translation initiation that accumulate in the face of environmental stress (Kedersha and Anderson, 2007). Many proteins sequestered into stress granules are known substrates of PRMTs (Anderson and Kedersha, 2008). The process of arginine methylation has been implicated in SGs because arginine methylated proteins are recruited to SGs (De Leeuw et al., 2007). Furthermore, methylarginine effector molecules are also recruited to stress



granules in periods of stress (Hua and Zhou, 2004). These effector molecules include SMN and TDRD3, proteins that contain Tudor modules known to bind methylated arginines (Hua and Zhou, 2004; Goulet et al., 2008).

### **Stress Granules**

With ever-fluctuating environmental conditions, cells need to evolve strategies to deal with various cellular stresses. A prominent stress response involves the inhibition of translation initiation and the consequent formation of cytoplasmic ribonucleoprotein (RNP) foci, the SGs (**Figure 4**; Buchan and Parker, 2009). These SGs range in size from 0.1 to 2.0 $\mu$ m, and have been observed in a variety of organisms, including yeast, protozoa, metazoa and plants (Kedersha and Anderson, 2007). SG assembly is triggered in cells upon exposure to various environmental stresses including heat shock, oxidative stress, osmotic stress, viral infection, hypoxia, and UV irradiation (Anderson and Kedersha, 2002a).



**Figure 4. A representative image of a cell containing stress granules.** An individual stress granule is magnified (Red: DDX3; Blue: DAPI)

In metazoans, there are five eIF2 $\alpha$  kinases that monitor stress response and are activated following specific stress exposures (Kedersha and Anderson, 2007). These eIF2 $\alpha$  kinases include: (1) protein kinase R, (PKR): activated by viral infection, heat and UV irradiation (Srivastava et al., 1998); (2) PKR-like endoplasmic reticulum kinase, (PERK): an endoplasmic reticulum resident protein activated upon accumulation of unfolded proteins in the endoplasmic reticulum lumen (Harding et al., 2000a; Harding et al., 2000b); (3) general control nonderepressible 2, (GCN2) which senses amino acid starvation (Wek et al., 1995); (4) heme-regulated initiation factor 2 $\alpha$  kinase, (HRI): senses oxidative stress by arsenite, heat shock and osmotic stress, and ensures a balanced synthesis of globin chains and heme during erythrocyte maturation (McEwen et al., 2005); and (5) Z-DNA kinase: an enzyme involved in the global host antiviral response (Wek et al., 1995; Kedersha and Anderson, 2007; Anderson and Kedersha, 2002a; **Figure 5**). All of the abovementioned enzymes, when activated by their respective stressors, will phosphorylate eIF2 $\alpha$  on Ser51, reducing the availability of eIF2-GTP-tRNA<sup>Met</sup>, thereby preventing the assembly of the 48S pre-initiation complex (Anderson and Kedersha, 2002a). This stalled pre-initiation complex promotes the disassembly of polysomes as the translating ribosomes “run off” resulting in an inhibition of global protein translation. This is due to the reduced availability of eIF2-GTP-tRNA<sub>i</sub><sup>Met</sup> ternary complex needed for cap-dependent translation initiation (Anderson and Kedersha, 2009; Kedersha et al., 2008). It is also important to note that eIF2 $\alpha$ -independent induction of SGs is possible and involves proteasome inhibition or inactivation of certain components of the translation initiation machinery (Dang et al., 2006; Mazroui et al., 2006).

## **SG constituents**

Despite extensive efforts, purification of SGs has yet to be achieved, so their content most currently are defined by immunostaining (Kedersha and Anderson, 2007). Their constituents can be categorized into four different groups. The first group is composed of stalled initiation complexes and translation-related factors. These include but are not limited to, mRNA transcripts, eIF4F, eIF4B and PABP-1 (Anderson and Kedersha, 2008; Anderson and Kedersha, 2002a). The second group is composed of mRNA binding proteins linked to translational silencing or to mRNA stability and include TIA-1, TIAR (Kedersha et al., 1999), FMRP, FXR1 (Mazroui et al., 2007), Argonaute (Leung et al., 2006) and CPEB (Wilczynska et al., 2005; Anderson and Kedersha, 2008). The third group includes RNA-binding proteins that regulate RNA metabolism, other than mRNA translation or decay (Anderson and Kedersha, 2002a). This class includes G3BP (Ras-GTPase-activating protein SH3-domain-binding protein) (Tourriere et al., 2003), FAST (Kedersha et al., 2005), SMN (Hua and Zhou, 2004), and Staufen (Thomas et al., 2005; Anderson and Kedersha, 2008). The last group encompasses signalling proteins that demonstrate no known direct link to RNA metabolism such as tumor necrosis factor receptor-associated factor 2 (TRAF2 ;Anderson and Kedersha, 2002a), but may have a role in cell signalling and survival (Anderson and Kedersha, 2008). The composition of SGs however may vary slightly, depending on the type of stress and during the course of the response (Thomas et al., 2011). Overexpression of various RNA-binding proteins is sufficient to induce SG assembly without stress (Kedersha et al., 1999). These proteins include TIA-1/TIAR (Gilks et al., 2004), FMRP/FXR-1 (Mazroui et al., 2006), ras-gap SH3 binding protein (G3BP1;

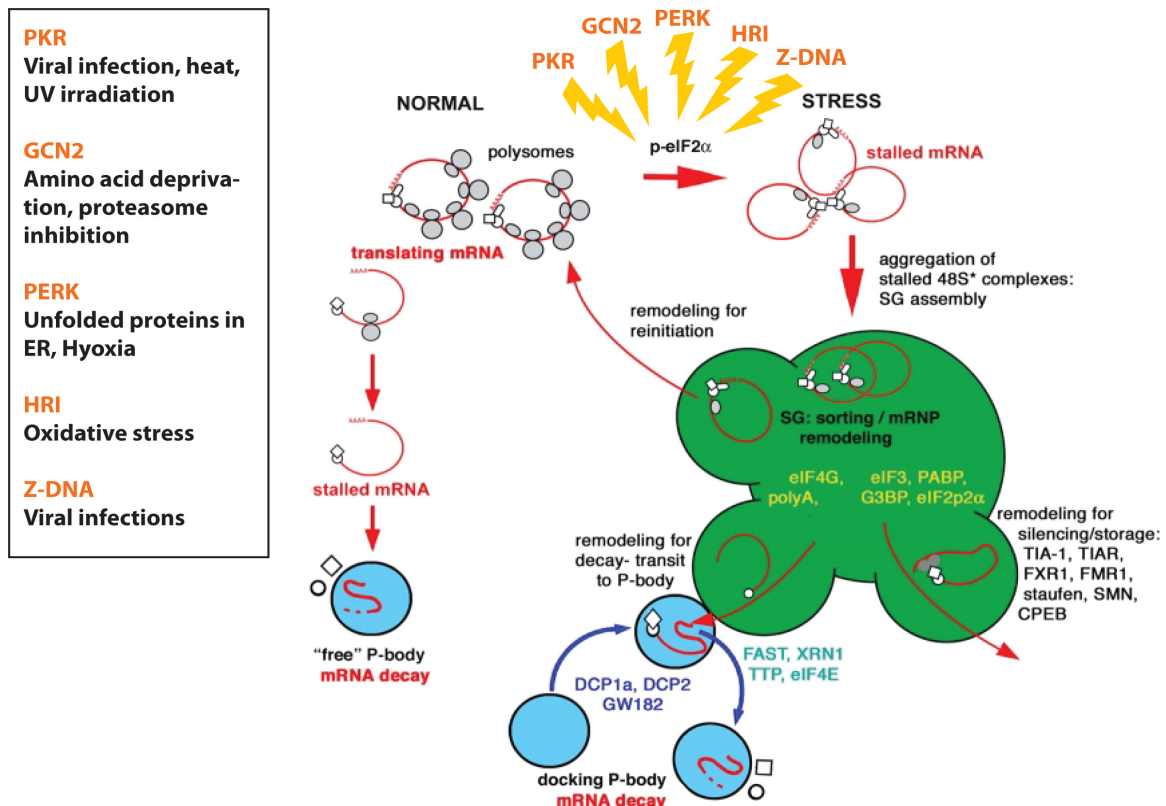
Tourriere et al., 2003), SMN (Hua and Zhou, 2004), cytoplasmic polyadenylation binding protein (CPEB; Wilczynska et al., 2005), p54/RCK (Wilczynska et al., 2005), tristetraprolin (TTP; Stoecklin et al., 2004) and smaug (Baez and Boccaccio, 2005; Kedersha and Anderson, 2007)

Stress granules are believed to serve as a site of mRNA triage where sequestered mRNAs are sorted and routed to sites of translation re-initiation, degradation or storage (Kedersha et al., 2002). Furthermore, some transcripts are sent from SGs to processing bodies (PBs) for facilitated decay (Kedersha et al., 2005). PBs, also known as decapping bodies, or glycine-tryptophan (GW) bodies, are SG-related mRNA-silencing foci (Thomas et al., 2009).

### **Processing bodies and mRNA cycle**

PBs consist of the 5' to 3' decay machinery which includes the 5' to 3' exonuclease Xrn1, decapping enzymes DCP1/2, Lsm proteins 1-7, scaffolding proteins heds/GE-1, nonsense mediated decay pathway proteins (SMG5, SMG7, UPF1), RNA induced silencing machinery (GW182, microRNA, argonaute) and activators of mRNA decay pathways (4ET, p54/RCK, CBEP) (Anderson and Kedersha, 2006; Kedersha and Anderson, 2007). Much less is known about the stimuli causing the induction of PBs. Unstressed cells display PBs, however their assembly is increased upon treatment with arsenite in conjunction with the formation of SGs, but this assembly is eIF2 $\alpha$  phosphorylation-independent (Cougot et al., 2004; Ferraiuolo et al., 2005; Wilczynska et al., 2005; Anderson and Kedersha, 2008). SGs have been observed juxtaposed to PBs, allowing for interaction, and the aforementioned exchange/transfer of messenger RNPs (Kedersha et al., 2005; Wilczynska et al., 2005). This analysis of SGs and PBs supports

the notion of an mRNA cycle and the existence of mRNAs in potentially different environments, polysomes, PBs, and stress granules (**Figure 5**; Balagopal and Parker, 2009).



**Figure 5. mRNA cycles between stress granules, P-bodies, and polysomes.** Different kinases are activated upon various stresses, ultimately phosphorylating eIF2 $\alpha$  (adapted from Kedersha et al., 2005).

Under normal conditions and through translation, mRNA produces polypeptides (Decker and Parker, 2013). However, upon translational defects, mRNA will interact with translation initiation repressors such as Dhh1/Rck which could result in a possible run-off of translating ribosomes and subsequently, recruitment of the decapping

machinery and transcript degradation (Anderson and Kedersha, 2006; Decker and Parker, 2013; Balagopal and Parker, 2009). At this stage, mRNAs may then accumulate in PBs. (Balagopal and Parker, 2009). mRNAs that do not accumulate in PBs, in the face of these translational defects, can aggregate into SGs (Balagopal and Parker, 2009; Decker and Parker, 2013). It is speculated that the mRNA will preferentially aggregate in SGs, PBs or as polysomes depending on the overall “biochemical state” of the cell (Balagopal and Parker, 2009). The mRNA that exists within SGs is not degraded and upon stress recovery quickly moves back to the polysomes (Kedersha et al., 2000).

Despite the similarities between SGs and PBs, they remain distinct entities (Kedersha et al., 2000; Kedersha et al., 2008). SGs are defined by translation initiation factors, whereas PBs are primarily defined by the decay machinery (Balagopal and Parker, 2009). Proteins that are exclusive to SGs include eIF3, PABP-1, eIF4G and FXR1 (Kedersha and Anderson, 2007). In addition to these proteins, DDX3 has been identified as a SG-nucleating factor and a SG marker (Shih et al., 2012). DDX3 was recently shown to also be part of PBs (Chahar et al., 2013). PB-exclusive proteins include DCP1a, DCP2, hedls/GE-1, 4E-T, and GW182 (Kedersha and Anderson, 2007). There are however multiple SG markers, including TIA-1, TIAR and HuR, which are not entirely SG specific, as they associate with PBs in unstressed cells (Kedersha and Anderson, 2007)

SGs are dynamic entities that, upon stress, are assembled hastily and ultimately disassemble upon the removal of stress to reinstate homeostasis (Buchan and Parker, 2009). SG disassembly associates with the translational recovery of each mRNA (Lian and Gallouzi, 2009), although full SG disassembly is not required for the recovery of translation (Loschi et al., 2009). Upon removal of stress, SGs increase in size due to

fusion of smaller SGs, and eventually disperse (Kedersha et al., 2000). The rate of disassembly is potentially accelerated upon treatment with drugs that stabilize polysomes, such as emetine (Kedersha et al., 2000). Conversely, drugs that dismantle polysomes, such as puromycin, promote SG assembly (Kedersha et al., 2000, Mazroui et al., 2002).

### **Stress granule function and implication in disease**

SGs seem to primarily function as a protective mechanism, promoting survival under stressful conditions (Kim et al., 2005; Arimoto et al., 2008; Eisinger-Mathason et al., 2008). Their protective role is evident by poor cell survival upon the impairment of stress granule assembly (Baguet et al., 2007; Eisinger-Mathason et al., 2008). SGs play a prominent role in the decision of whether or not a stressed cell should enter apoptosis (Kim et al., 2005; Arimoto et al., 2008; Eisinger-Mathason et al., 2008).

SGs sequester a variety of apoptotic factors and key signalling proteins, preventing their interactions with other factors and thereby ultimately promoting survival (Kim et al., 2005; Arimoto et al., 2008; Eisinger-Mathason et al., 2008). This is evident in cancer cells, where SGs formation under hypoxic conditions (type 1 stress) inhibits apoptosis induced by etoposide (type 2 stress) (Arimoto et al., 2008). This is due to the sequestration of RACK-1 into SGs, which inhibits the stress-responsive MAP kinase apoptotic pathways (MTK1-SAPK signalling) (Arimoto et al., 2008). This is also relevant to hypoxia-induced cancer cell resistance to chemotherapy, which is associated with the presence of the SGs observed inside the hypoxic core of breast tumours (Moeller et al., 2004).

Similarly, sequestration of mitogen-activated kinase ribosomal S6 kinase 2 (RSK2) in breast cancer promotes survival and proliferation in response to stress (Eisinger-Mathason et al., 2008). Furthermore, the nucleo-cytoplasmic shuttling protein, MLN51, a core component of the exon junction complex (EJC) was shown to be overexpressed in breast carcinomas and promote SG assembly (Baguet et al., 2007). In breast cancer patient samples, MLN51 was shown to localize to discrete SG-like foci (Baguet et al., 2007).

Consistently, sequestering tumour necrosis factor (TNF)- $\alpha$  receptor associated factor 2 (TRAF2) into SGs upon heat shock, blocks TNF signalling, leading to an impairment of NF- $\kappa$ B, a prominent transcriptional regulator of both apoptosis and inflammatory responses (Kim et al., 2005). Therefore, SGs appear to play a role in apoptosis via sequestering and inhibiting apoptosis promoting factors.

SGs have been associated with tumour radioresistance (Moeller et al., 2004). Hypoxia-inducible Factor-1 (HIF-1) is a major angiogenesis regulating transcription factor which is activated by hypoxia (Dewhirst et al., 2008). In response to radiation, HIF-1's pro-angiogenic activity is increased, partly due to the accumulation/sequestration of HIF-1 mRNA in SGs inside hypoxic tumours (Moeller et al., 2004). Following radiotherapy, and subsequent re-oxygenation, SGs dissociate and release sequestered HIF-1 target gene mRNAs (Moeller et al., 2004). This results in the translation of HIF-1 dependent transcripts, ultimately promoting tumour progression (Moeller et al., 2004).

Interestingly, bortezomib, a proteasome inhibitor and chemotherapeutic agent, has been shown to efficiently induce SG formation in cancer cells (Fournier et al., 2010). The study hypothesized that formation of SGs by bortezomib might provide mechanistic



insights by which the cancer cells resist bortezomib-mediated apoptosis (Fournier et al., 2010). A recent study has shown the contribution of the oncogenic mTORC1-eukaryotic translation initiation factor 4E pathway to the formation of SGs, thus inhibiting apoptosis (Fournier et al., 2013). Inactivation of this pathway results in an impairment of SG formation and thus hinders the growth of bortezomib chemoresistant tumours (Fournier et al., 2013). All of the abovementioned results further support the existence of SGs in the tumour microenvironment, which influence tumour cell survival and resistance to cancer therapy (Fournier et al., 2013, Moeller et al., 2004, Dewhirst et al., 2008).

SGs have been further implicated in an array of diseases, other than cancer (reviewed in Anderson and Kedersha, 2007). SGs are thought to contribute to the pathogenesis of spinal muscular atrophy, ischemia-reperfusion injury, and immunity (Rossoll et al., 2003; Hua et al., 2004; Kayali et al., 2005; De Gracia et al., 2006; Scheu et al., 2006). In addition, SGs have been associated with fragile X syndrome, where fragile X mental retardation protein (FMRP) was shown to be a component of SGs (Khandijian et al., 2004; Antar et al., 2005). Interestingly, FMRP was shown to interact with a Tudor domain protein known as Tudor domain containing-protein 3 (TDRD3; Linder et al., 2008).

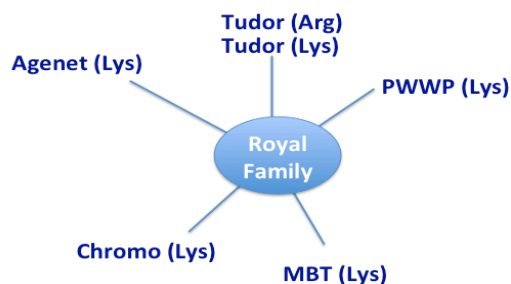
## **Tudor domain-methyl binding domain**

### **'Royal Family'**

The Tudor protein was initially discovered in *Drosophila Melanogaster* in 1985 through a genetic screen for maternally expressed genes involved in embryonic development and fertility (Boswell and Mahowald, 1985). Later, in 1997, the conserved Tudor protein itself was subsequently identified as 11 repeats found in a variety of

proteins functioning in RNA metabolism (Ponting, 1997; Callebaut and Mornon, 1997). Since then, the Tudor domain has been found in a wide range of eukaryotic organisms with over 200 Tudor-containing proteins, and approximately 30 from the mammalian genome (Lasko, 2010; Chen et al., 2011). The conserved protein structure of the Tudor domain consists of approximately 60 amino acids which make up a core structure that forms a hydrophobic barrel-like structure composed of anti-parallel  $\beta$ -sheets, surrounded by negatively charged and aromatic residues, together forming a possible protein-protein interaction surface (Selenko et al., 2001; Sprangers et al., 2003). Based on structural fold similarities, the Tudor domain was proposed to be closely related to chromo (chromatin binding), Malignant Brain Tumor (MBT), the conserved proline-tryptophan-tryptophan-proline' domain (PWWP) and plantAgenet domains, which together form the so-called “Royal Family” that are known to recognize methylated arginine and lysine residues (**Figure 6**; Maurer-Stroh et al., 2003).

In humans, there are over a hundred proteins containing a domain from this “Royal Family” (Mallette and Richard, 2012). Tudor domains are the founding member of the “Royal Family” (Maurer-Stroh et al., 2003).



**Figure 6. Representation of the purported “Royal Family”.** This family recognizes both methylated arginines (Arg) and lysines (Lys).

All members of the “Royal Family” with the exception of Tudor domains are known to solely recognize methylated lysine (Maurer-Stroh et al., 2003). Different Tudor domains bind both methylated arginines and lysines, however Tudor domains are better known for binding methylated arginines (Liu et al., 2012; Zhang et al., 2012). Indeed, many Tudor proteins have demonstrated prominent roles in RNA metabolism including RNA processing, translocation, stability and translation, as well as in germ cell development, transposon silencing, histone modification and chromatin remodelling (Chen et al., 2011). Moreover, Tudor domain proteins have also exhibited roles in DNA damage response pathways, and can interact with methylated histones (Pek et al., 2012).

### **Subtypes of Tudor domains**

Four subtypes of Tudor domains can be distinguished, based solely on their flanking sequences and/or their linkage to other conserved motifs; these allow the different Tudor domain-containing proteins to possess distinct biological function and properties (Jin et al., 2009). The first group is the original germ-line type Tudor domain proteins (TDRD1, 2, 4, 5, 6, 7, 8, 9). This group is characterized by multiple Tudor repeats, some of which are flanked on their amino-terminal side by an  $\alpha$  helix and  $\beta$  strands, and bind to proteins with dimethylated arginine or lysine residues (Jin et al., 2009). These Tudor domain-containing proteins accumulate in nuage/polar/germinal granules (germ-line specific ribonucleoprotein complexes), and are necessary for recruiting other methylated Piwi-type proteins, involved in retro-transposon silencing during gametogenesis (Siomi et al., 2010; Lasko, 2010).

The second group is called the Tudor domain of SND1 (p100, Tudor-SN), which like the first group associates with arginine methylated PIWIL1/Miwi in germ cells and harbours five tandem staphylococcal nuclease (SN)-like domains, with its Tudor domain inserted into the fifth one (Shaw et al., 2007; Liu et al., 2010). SND1 was shown to participate in several pathways and was identified as a prominent component of the RISC complex (RNA-induced silencing complex; Caudy et al., 2003; Gau et al., 2010). A third group of Tudor domains is referred to as “tandem Tudor” or “JMJD” and recognizes methylated lysines on histone tails (Charier et al., 2004; Kim et al. 2006). This group of Tudor domains includes 53BP1, which participates in the DNA repair pathway, (Botuyan et al., 2006) and JMJD2A, involved in transcriptional repression (Klose and Zhang, 2007).

The final group contains the Tudor domain of the SMN protein which plays a substantial role in the assembly of the core spliceosomal machinery, U small nuclear ribonucleoproteins (Brahms et al., 2001; Meister et al., 2001; Pu et al., 1999). The Tudor domain of SMN is the best characterized of all the Tudor domains (Goulet et al., 2008). The SMN Tudor domain has also recently been shown to be involved in RNA-related processes, including but not limited to, mRNA stability and co-transcriptional pre-mRNA splicing (Tadesse et al., 2008; Hubers et al., 2011; Chen et al., 2010). This final group of Tudor domains recognizes methylated arginines, most prominently in the context of RG-rich repeats in proteins (Cheng et al., 2007; Friesen et al., 2001a; Friesen et al., 2001b; Brahms et al., 2001; Côté and Richard, 2005). In addition to SMN, this group also includes splicing factor 30 (SPF30/SMNrp; Rappsilber et al., 2001; Meister et al., 2001) and TDRD3 (Côté and Richard, 2005).

### Tudor domain containing protein-3

Tudor-domain containing protein 3 (TDRD3) is a novel modular protein whose function remains elusive to date. It was first identified in a proteomic survey of the complete mammalian pre-spliceosome machinery (Hartmuth et al., 2002). TDRD3 is an 83 kDa protein consisting of 744 amino acids (Linder et al., 2008). It harbours a Tudor domain highly similar to that of SMN, and accordingly, has been shown to bind dimethylated arginines in polypeptides (Côté and Richard, 2005). In addition to its C-terminal Tudor domain, TDRD3 possesses a putative sugar and/or nucleic acid recognition motif (DUF/OB-fold motif) (Theobald et al., 2003), an ubiquitin-binding domain (UBA) (Hurley et al., 2006), and an exon junction complex binding motif (EBM) (Kashima et al., 2010; **Figure 7**).



**Figure 7. TDRD3 structure.** TDRD3 consists of 744 amino acids which include the Tudor domain, the putative DUF/OB fold motif, the ubiquitin-binding domain (UBA domains), and the exon junction complex binding motif (EBM) (adapted from Goulet et al., 2008).

TDRD3 has been shown to bind arginine methylated peptides (Côté and Richard, 2005; Kim et al., 2006). Its Tudor domain has the ability to bind a variety of histone methyl marks, suggesting a function for TDRD3 as a versatile reader of various methyl

marks (Yang et al., 2010). It binds strongly to H3R17me2a and H4R3me2a, which are histone marks deposited by the transcriptional co-activators CARM1 and PRMT1, respectively (Yang et al., 2010). Furthermore, the Tudor domain of TDRD3 preferentially binds to the aDMA modification on histone 4's arginine residue 3 (H4R3), a PRMT1 generated methyl mark associated with transcription activation, over its alternative sDMA PRMT5 generated methyl mark, H4R3me2s (Liu et al., 2012; Yang et al., 2010).

The Tudor domain of TDRD3 is unique in its ability to bind methyl marks distinctively associated with transcriptional activation (Yang et al., 2010). TDRD3 is recruited to the pS2 promoter, where it is believed to function as a transcriptional co-activator (Yang et al., 2010). Moreover, unbiased chromatin immunoprecipitation (ChIP) sequence analysis has also revealed that TDRD3 is enriched at transcriptional start sites (TSS) of highly transcribed genes, such as pS2 and *MYC*, genes known to be regulated by estrogen receptor (ER) (Yang et al., 2010).

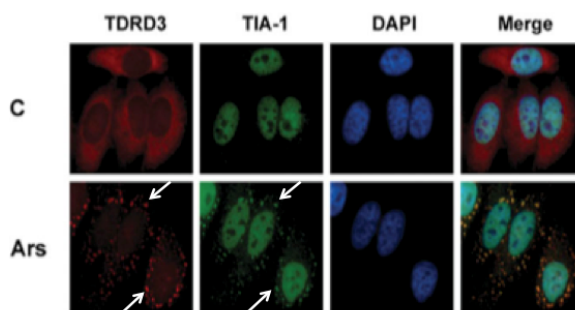
In light of TDRD3's association with translation, the carboxyl terminal domain (CTD) of RNA polymerase II has recently been shown to be methylated by CARM1, and this methylation in turn provides a docking site for TDRD3 (Sims et al., 2011). This thereby signifies a potential role for TDRD3 in regulating the activity of RNAPII, and in turn, in transcription. Consistent with its potential role in translation and in linking RNA processing, TDRD3's EBM domain has been demonstrated to interact with RNA binding protein 8A (Y14), Mago nashi homolog (Magoh) and Poly(A)-Binding Protein 1 (PABP-1; Kashima et al., 2010).

TDRD3's different protein domains come together to enhance the binding of the Tudor domain of TDRD3 to methyl marks associated with transcriptional co-activation

(Yang et al., 2010). Furthermore, it has been proposed that TDRD3 might function as a scaffolding molecule, bringing protein complexes together (Linder et al., 2008; Yang et al., 2010).

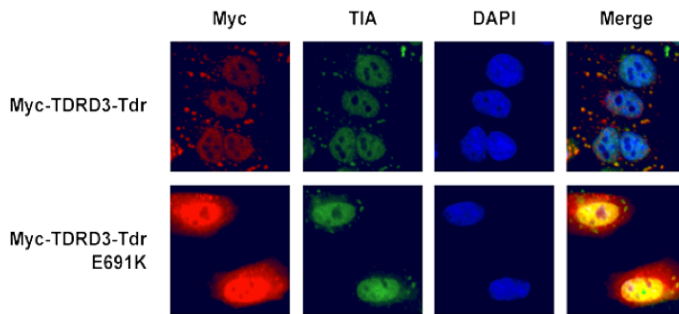
TDRD3 has been shown to localize to both the nucleus and cytoplasm, but predominantly to the cytoplasm (Goulet et al., 2008). TDRD3 demonstrates its effector role in the nucleus, where it binds methyl marks generated by transcriptional co-activators, CARM1 and PRMT1, and promotes the transcription of regions, such as estrogen-responsive elements (Yang et al., 2010).

*In vitro*, TDRD3 was proposed to repress translation (Linder et al., 2008). Concurrently, with its proposed role in translational repression, our lab was the first to demonstrate the localization of TDRD3 to cytoplasmic stress granules (SG) in response to various cellular stresses (**Figure 8**; Goulet et al., 2008). The Tudor domain of TDRD3 was demonstrated to be both necessary and sufficient for recruiting TDRD3 to SGs upon stress (Goulet et al., 2008).



**Figure 8. Localization of TDRD3 to SGs upon exposure to arsenic stress.** Following treatment with sodium arsenite (Ars) SGs became detectable, using TIA-1 as a SG marker (Goulet et al., 2008).

Furthermore, a mutation in the Tudor domain of TDRD3, E691K, which disrupts its interaction with methylated arginines, demonstrates the importance of the methyl binding Tudor domain for TDRD3 interactions (Goulet et al., 2008). The E691K mutation also abolishes TDRD3's recruitment to SGs upon stress (**Figure 9**; Goulet et al., 2008).



**Figure 9. The E691K mutant abolishes the recruitment of TDRD3 to SGs following arsenic treatment.** E691K mutant contains a point mutation in the Tudor domain of TDRD3 and also prevents TDRD3 binding to methylated arginines found in proteins (Goulet et al., 2008).

Our lab has identified a number of interacting proteins for the Tudor domain of TDRD3 through a glutathione-S-transferase (GST) pull-down. These proteins are potentially and concomitantly methylated RNA binding proteins (**Table 1**; Goulet et al., 2008). These include the EWS (EWing Sarcoma breakpoint region 1), FUS, DDX3 (DEAD/H box- 3), EEF1A1 (Eukaryotic translation Elongation Factor 1 alpha 1) and SERPINE mRNA binding protein 1 (SERBP1; Goulet et al., 2008). All of these proteins have prominent roles in RNA handling, and most of them are arginine methylated, further advocating a potential role for TDRD3 in translation (Goulet et al., 2008). Intriguingly, of these interacting proteins, EWS, FUS (Anderson et al., 2008; Gal et al., 2011; Bosco et



al., 2010), DDX3 (Shih et al., 2012) and SERBP1 (Goulet et al., 2008) were shown to also localize to SGs upon stress.

| Protein name<br>(GenPept acc. no.)                                | Short name | Arginine methylation | RG-motifs                                                                                                                                                                        | Known or putative function(s)                                            |
|-------------------------------------------------------------------|------------|----------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------|
| Ewing sarcoma breakpoint region 1<br>(NP_005234)                  | EWS        | PRMT1                | 19 x RGG<br>300RGRGRGG <sup>308</sup><br>314RGGRGGGRGG <sup>323</sup>                                                                                                            | RNA binding protein<br>(transcription and pre-mRNA splicing machineries) |
| DEAD/H box-3<br>(NP_001347)                                       | DDX3       | PRMT1*               | 10 x RG<br>121RGG <sup>123</sup>                                                                                                                                                 | Translation/<br>mRNA transport                                           |
| Fus<br>(NP_004951)                                                | Fus        | PRMT1                | 2 x RGG<br>216RGGGRGG <sup>222</sup><br>245RGRGGGRGGGRGG <sup>256</sup><br>360RGGGNRGGRG<br>RGGPMGRGG <sup>369</sup><br>475RGGGRGGYDRGGYRGRGG<br>DRGGFRGGRGGGDRGG <sup>508</sup> | RNA splicing and<br>nuclear/cytoplasmic shuttling                        |
| Eukaryotic translation elongation factor 1 alpha 1<br>(NP_001393) | eEF1A1     | nd                   |                                                                                                                                                                                  | Translation<br>mRNA localization                                         |
| SERPINE1 mRNA binding protein 1<br>(NP_001018077)                 | SERBP1     | PRMT1                | 1 x RG, 1 x RGG<br>153RGRGGLGRGRG<br>GRGRGMGRG <sup>162</sup><br>348RGGRGGGRGGGRGG <sup>360</sup>                                                                                | Translation/<br>mRNA stability                                           |
| RNA Binding Protein 8A<br>(NP_005096)                             | Y14        | PRMT1                | 1 x GRG, 1 x RGG                                                                                                                                                                 | EJC core component<br>Translation/Splicing                               |
| Mago nashi homolog<br>(NP_002361)                                 | Magoh      | nd                   | no RG motifs                                                                                                                                                                     | EJC core component<br>Translation/Splicing                               |
| Poly(A)-Binding Protein 1<br>(AAH23520)                           | PABP1      | CARM1                |                                                                                                                                                                                  | Translation<br>mRNA processing/stability                                 |

**Table 1. List of proteins interacting with Tudor domain of TDRD3.** Summary of the proteins' putative or known functions is also shown. RG-rich motifs present in the amino acid sequence of each protein are also shown here (adapted from Goulet et al., 2008; Kashima et al., 2010).

### TDRD3 and human pathologies

Interestingly, many of the aforementioned TDRD3 interacting proteins have been associated with cancer (Xia et al., 2005; Koensgen et al., 2007; Botlagunta et al., 2008).

For example, SERBP1 has been implicated in the disease progression of epithelial ovarian cancer (Koensgen et al., 2007). Both FUS and EWS are involved in recurrent chromosome translocation that produces various fusion oncoproteins related to many sarcomas (Xia et al., 2005). In addition, DDX3 has been implicated in the oncogenic transformation of breast epithelial cells (Botlagunta et al., 2008). TDRD3 may be linked to mammary tumorigenesis, as it was among the top hits in the genes that are strongly correlated with poor prognosis in recurrent ER-negative breast cancers (Nagahata et al., 2004). In the Cancer Genome Atlas' Data Portal, TDRD3 was overexpressed in 58/158 tumors from breast cancer patients (<http://tcga-portal.nci.nih.gov>). Moreover, TDRD3 was among 14 signature genes associated with disease outcome in patients with basal-like breast cancer: genes able to distinguish good and poor outcome of patients with basal-like breast cancer (Hallett et al., 2012). This suggests that this novel gene might be implicated in the pathogenesis of breast cancer.

As stated above, TDRD3 was shown to interact with FMRP, the gene associated with Fragile X mental retardation syndrome (Goulet et al., 2008; Linder et al., 2008). FMRP has recently and unexpectedly been identified to interact with human topoisomerase 3 $\beta$  (TOP3 $\beta$ ) (Stoll et al., 2013; Xu et al., 2013). Topoisomerases are thought to be the “magicians of the DNA world” (Wang et al., 2002), solving DNA topological problems (Xu et al., 2013). TDRD3 was found to be essential for maintaining the association of TOP3 $\beta$  with FMRP (Xu et al., 2013; Stroll et al., 2013). In addition, TDRD3 copy number variants (CNVs) have been detected in autistic patients (<http://autismkb.cbi.pku.edu.cn>) (Xu et al., 2013), further proposing a role for TDRD3 in various mental disorders. All of the above supports a strong and prominent role for this

novel gene in various human pathologies, making it an intriguing gene for further investigations.

## **Rationale, Hypothesis and Objectives**

### **Rationale**

Previous work from our lab illustrated the high similarity between the Tudor domain of the SMN proteins, and that of the TDRD3. Both proteins have the ability to bind arginine-methylated peptides. Interestingly, TDRD3 was identified among genes whose overexpression is strongly correlated with poor prognosis of ER-negative breast cancers (Nagahata et al., 2004). Also, TDRD3 was proposed to be linked with highly aggressive breast cancer (Hallett et al., 2012), suggesting a possible implication in the disease's pathogenesis.

Previous work from our lab identified TDRD3 as a novel modular protein that localizes to SGs upon various cellular stresses. Identified TDRD3 interactors were also shown to localize to these SGs. SG foci represent sites of mRNA triage that can promote survival in the face of various stresses. We demonstrated that the Tudor domain of TDRD3 is both required and sufficient for its localization to SGs. A single E to K substitution (E691K) in the Tudor domain of TDRD3, which disrupts TDRD3 binding to its methylated interactors, was sufficient to significantly diminish TDRD3's recruitment to SGs. An important question that remains is whether TDRD3 is required for the proper formation (assembly/disassembly) and function of SGs. Therefore, this study is an excellent opportunity to clarify the functional role that TDRD3 plays in stress granules. With the known correlation between TDRD3 levels and breast cancer and SG's implication in cancer, both in promoting cell survival and mediating resistance to cancer

therapy, gaining insight into this novel gene in light of its role in SGs may eventually have implications on detecting, classifying, and further elucidating the pathology of breast cancer.

### **Hypothesis**

I hypothesize that TDRD3 plays a significant role in the assembly, disassembly and function of stress granules.

### **Objectives**

Elucidate the role of TDRD3 in promoting stress granule assembly/disassembly.

- I- Assess the contribution of TDRD3 in stress granule assembly, disassembly and dynamics.
- II- Identify the domain required for the function of TDRD3 in stress granules.
- III- Determine the role of TDRD3 in promoting cell survival in response to stress.

## **Materials and Methods**

## **Cell culture, DNA constructs and Transfection**

Cell lines were purchased from the American Type Culture Collection. MDA-MB-231 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 1% sodium pyruvate, 2% penicillin/streptomycin and 10% fetal bovine serum (FBS). MCF7 and Hs578T media were also supplemented with 2.75 µg/ mL insulin. TDRD3 stable knockdowns were established in the Hs578T and MDA-MB-231 cell lines, utilizing a predesigned non-inducible lentiviral short hairpin RNA (shRNA) within the GIPz vector which targeted the 3' untranslated region (UTR) of TDRD3. A scrambled shRNA (a non-targeting shRNA sequence) was used as control. The transfection process was done using Lipofectamine 2000, in accordance with the manufacturer's protocol. Cells were selected using 2µg/ml puromycin and pools of stably expressing scrambled and TDRD3 shRNA were maintained at 2µg/mL puromycin. Cells were cultured at 37°C and 5% CO<sub>2</sub>. DNA constructs of full length TDRD3, pCMV-Myc-TDRD3 and mutant TDRD3 E691K, pCMV-Myc-TDRD3 were used and the transfection process was done similarly, using 2µg of DNA via Lipofectamine 2000 procedure.

First strand cDNA synthesis was achieved using 5 µg of total RNA (extracted from Hela cells) and the avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI) with an oligo-dT primer. The cDNA was then amplified using oligonucleotides that introduced the full length TDRD3 in frame with a myc-tag into EcoRI and XhoI restriction sites of the pCMV-Myc expression vector (Clontech Laboratories Inc., Mountain View, CA). The mutant TDRD3, E691K mutation was introduced via overlap extension mutagenesis (Ho et al. 1989) with the following

mutagenic oligonucleotides: 5'-ggaaactataaagaggtgctactg-3' and 5'-cagtagcacctctttatagtttcc-3'. This was done while maintaining the use of TDRD3-specific primers as flanking oligonucleotides. This mutated fragment was subsequently inserted into the restriction sites (EcoRI and XhoI) of the pCMV-Myc.

## **Antibodies**

The mouse monoclonal anti-TDRD3 antibody was purchased from Abnova and used at dilution 1:5000 for western blot analysis. Goat polyclonal anti-TIA-1 antibody (C-20) and mouse monoclonal anti-PABP-1 (10E10) were purchased from Santa Cruz Biotechnology and used for immunofluorescence (IF) at dilution 1:200 and 1:100, respectively. Rabbit polyclonal anti-DDX3 was purchased from Bethyl Laboratories Inc. and used at dilution 1:200 for IF. Mouse monoclonal anti-RACK1 was purchased from BD Transduction Laboratories <sup>TM</sup> and used at dilution 1:300 for IF. The mouse monoclonal anti-myc 9E10 antibody was obtained from ATCC and used at dilution of 1:2 for both western blot and IF. Antibodies against  $\alpha$ -tubulin and GAPDH were from Sigma and Covance, and used at dilution 1:10000 and 1:5000, respectively. Alexa fluorophore (488, 594, 647)-coupled anti-rabbit and anti-mouse secondary antibodies were purchased from Molecular Probes and used at dilutions 1:300-1:600. Peroxidase-conjugated goat anti-mouse and goat anti-rabbit secondary antibodies were obtained from Jackson ImmunoResearch and used at 1:10000-1:20000 dilution.

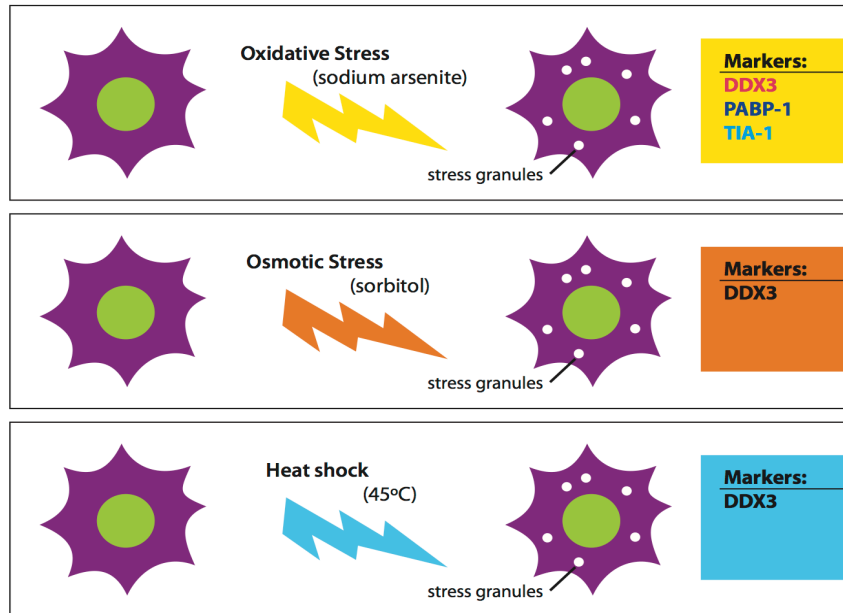
### **Cell treatment and Immunofluorescence**

For indirect IF microscopy, cells were grown in 6 well plates on glass cover slips and transfected (when indicated). Transfected cells were treated 24 h post-transfection with different stresses to induce stress granule formation. Oxidative stress was induced by treatment with 0.5mM sodium arsenite (Fluka), osmotic stress with 1M of Sorbitol (Fisher) and heat shock by incubation at 45°C for 45 minutes (**Figure 10**).

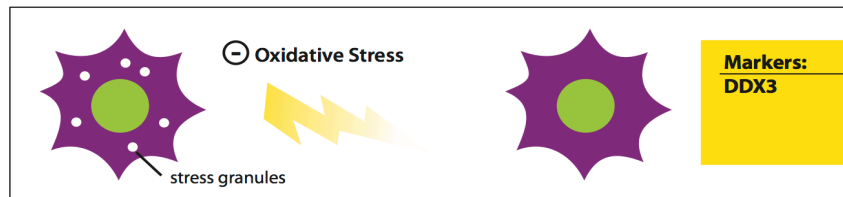
Treated and untreated cells were washed with 0.1% BSA in 1X Phosphate Buffer Saline (PBS) and then fixed with 4% paraformaldehyde for 15 minutes, permeabilized with 0.5% Triton X-100 in 1X PBS for 10 minutes. Cells were then washed 3 times with 0.1% BSA in 1X PBS and incubated with primary antibodies indicated (various SGs markers) diluted in 0.1% BSA in 1X PBS for one hour. Cells were then washed once with 0.1% Triton X-100 in 1X PBS and three times with 0.1% BSA in 1X PBS. Finally, cells were incubated with the Alexa fluorophore-coupled secondary antibodies in 0.1% BSA in 1X PBS for one hour in the dark. The cells were washed again as above, counterstained with Vectashield mounting media containing DAPI (Vector Laboratories) and mounted onto glass slides. Cells were then visualized using a Zeiss Axio Imager.Z1 microscope using the objective indicated and an AXIOCAM HRM digital camera was used to capture images using Axiovision software. For assessing disassembly, cells were treated with 0.5mM of sodium arsenite for one hour, allowed to recover for 20, 40 and 60 minutes and fixed for the identified time. The same immunofluorescence protocol was carried out.



## Assembly



## Disassembly



**Figure 10. Representative images of the assembly/disassembly of SGs.** The assembly of SGs is induced via various cellular stresses and assessed using different markers.

## Protein Isolation and Western Blotting

Cells were washed with 1X PBS and lysed with Radio-Immunoprecipitation Assay (RIPA) lysis buffer [10 mM Tris pH 7.4, 100 mM NaCl, 1 mM ethylene tetraacetic diamine acid (EDTA), 1% nonyl phenoxypolyethoxylethanol 40 (NP- 40), 0.5% sodium deoxycholate (NaDOC), 0.1% sodium dodecyl sulfate (SDS)]. Lysates were then incubated on ice for 15 minutes, centrifuged for 15 minutes at maximum speed and the

supernatant was collected. Protein quantification was assessed via Bradford assay according to the manufacturer's protocol. 30µg of proteins were mixed with 2X Laemmli buffer (25% glycerol, 125 mM Tris HCl pH 6.8, 4% SDS, 700 mM β-mercaptoethanol, 0.1% bromophenol Blue), boiled for 10 minutes at 95°C and subjected to 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The resolved protein extracts were then transferred onto a Polyvinylidene Fluoride (PVDF) membrane. PVDF membrane was then blocked for one hour in 5% milk in 1X PBS-T. Membranes were incubated with primary antibody diluted in 5% milk in 1X PBS-T for either one hour at room temperature or overnight at 4°C, washed three times for 5 minutes with 1X PBS-T and incubated with secondary antibody for one hour at room temperature. The membrane was then washed three times for 10 minutes in PBS-T and proteins were detected using chemiluminescent HRP substrate (Millipore) and exposed to film.

### **Cell Viability Assay**

MDA-MB-231 cells were plated on opaque 96-well plate (BrandTech Scientific). The next day the cells were stressed with different concentrations of sorbitol (1M, 2M, 3M, 4M) for one hour. The experiments were conducted in quadruplicates and according to the manufacture's protocol. For the recovery experiments, cells were stressed for one hour with 1M of sorbitol, washed, and allowed to recover in normal media for the indicated number of hours. Cell viability was monitored during and after stress using CellTiterGlo™ (Promega) assay according to the manufacturer's instructions. Luminescence was read using a GloMax® luminometer.

## **Statistical Analysis**

The data were analyzed as indicated for each experiment, expressed as mean  $\pm$  standard error of mean. Statistical significance was determined by unpaired two tailed t-test, one-way and two-way analysis of variance (ANOVA) and Tukey's multiple comparison post-tests (GraphPad Prism) where appropriate.

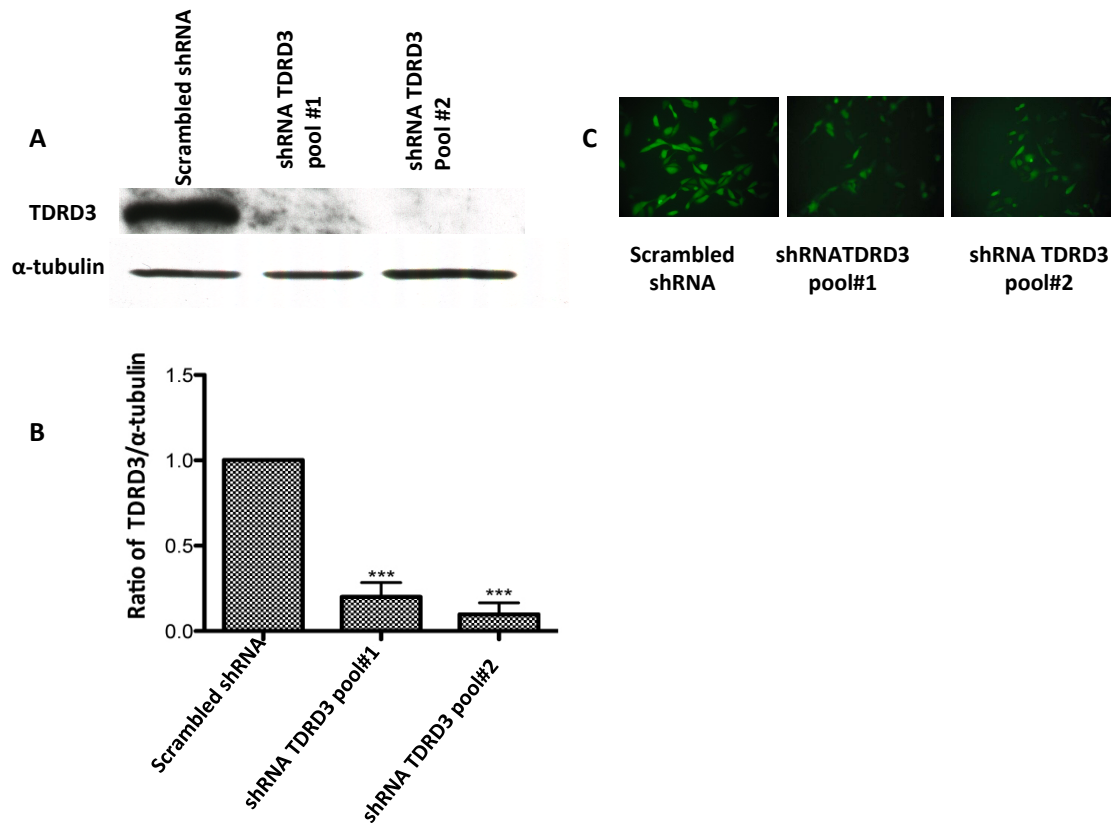
## **Results**

### **TDRD3 plays a role in stress granule assembly**

Our lab has previously demonstrated the association of TDRD3 with the translational machinery and its localization to cytoplasmic stress granules (SG) upon various cellular stresses (Goulet et al., 2008). TDRD3 localizes to stress granules and these stress granules have been hypothesized to promote cancer cell survival. The role TDRD3 plays in SG formation remains to be investigated. We reasoned that TDRD3 may be required for proper and functional formation of SGs, thus ultimately promoting survival of breast cancer cells. To assess this, we established a shRNA knockdown model of TDRD3 in MDA-MB-231 and Hs578T breast cancer cell lines. MDA-MB-231 and Hs578T cells are highly aggressive breast cancer cell lines that are estrogen-receptor negative. MDA-MB-231 and Hs578T cell lines were used, as they were the closest analogues that we have to study aggressive ER-negative breast cancer tumours in tissue culture and TDRD3 was suggested to be linked with highly aggressive breast cancer (Hallett et al., 2012).

The MDA-MB-231 cell line was transfected with shRNA vector targeting TDRD3 mRNA in its 3' untranslated region (UTR). As a control, cells were also transfected with a scrambled shRNA, a non-targeting sequence expressed from the same vector. The vector used (pGIPz) was a green fluorescent protein (GFP) expressing vector. Cells were selected for stable pools of cells expressing these shRNAs. TDRD3 protein levels in cell lines that express the shRNA targeting TDRD3 (shRNA pool#1 and pool#2) decreased by approximately 78% to 85%, as assessed using quantification from three western blots (**Figure 11A and B**). GFP expression levels of the different pools of cells, shRNA TDRD3 and scrambled are shown (**Figure 11C**). To address the

question pertaining to the requirement of TDRD3 in SG formation, both TDRD3 shRNA stable cell lines and scrambled controls were treated with various stresses and the assembly of SGs was assessed at different time points. Based on previous results from our lab, TDRD3 was demonstrated to be associated with the translation machinery and localize to SGs upon stress. This finding led us to hypothesize that the depletion of TDRD3 will likely delay or prevent SG formation. This hypothesis was in accordance with other proteins in the literature behaving like TDRD3, such as FMRP, where its depletion reduces SG formation (Didiot et al., 2009). Interestingly, FMRP was shown to also interact with TDRD3 (Linder et al., 2008).

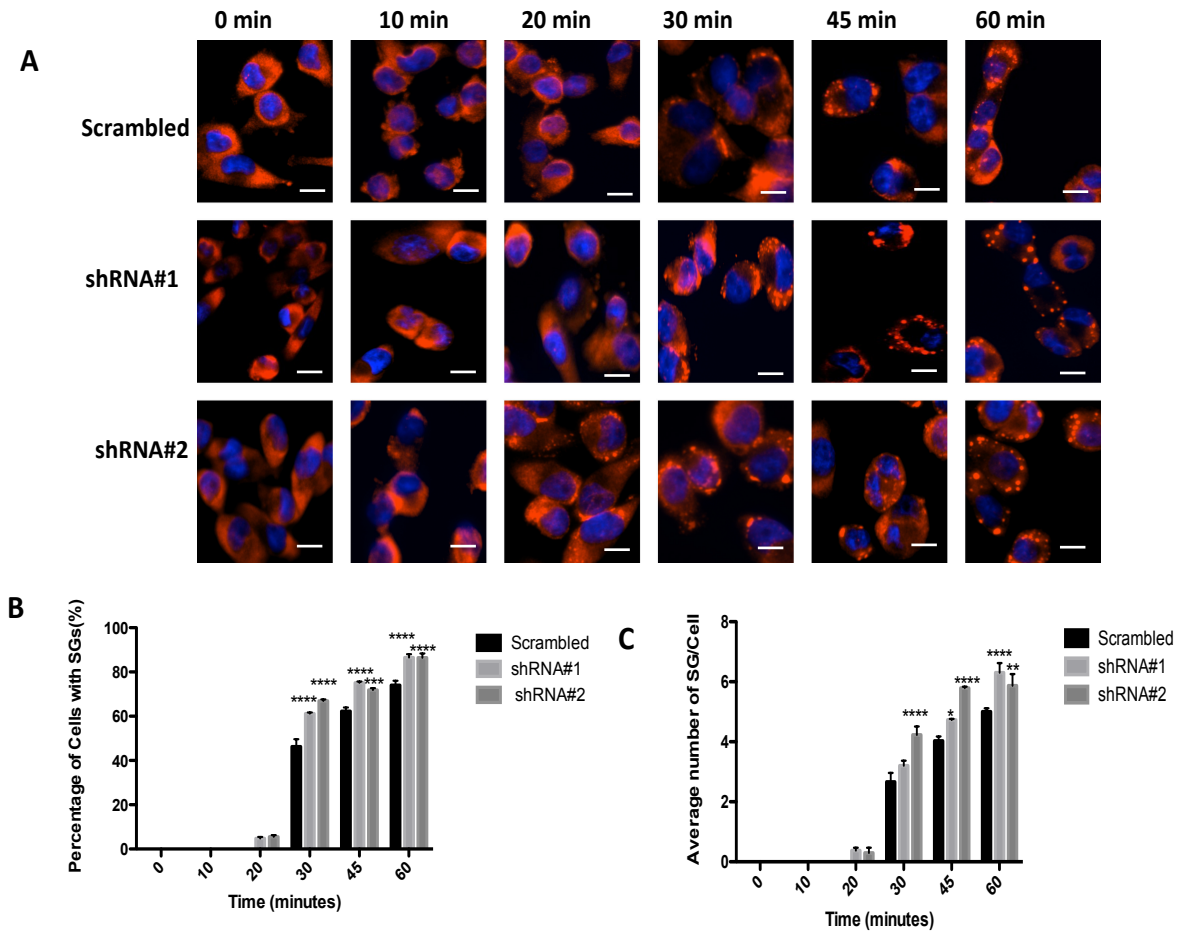


**Figure 11. Stable knockdown of TDRD3 using a shRNA-expressing vector in MDA-MB-231 cells.** (A) Western blot demonstrating reduced levels of TDRD3 expression in MDA-MB-231 cell line that express a shRNA targeting TDRD3 mRNA in its 3'UTR. A scrambled shRNA expressed from the same vector is also shown. Tubulin serves as the loading control. (B) Quantitative analysis of the protein level of the shTDRD3, normalized to tubulin and compared to the control scrambled shRNA vector. (C) GFP-fluorescence images of scrambled and TDRD3 knockdown pools, expressing the Gipez GFP vectors, as seen through fluorescent light microscope. Values presented are mean percentages  $\pm$  SEM of an experiment carried out in triplicates and \*\*\* indicating a  $P \leq 0.001$  respectively. Data analyzed using unpaired two-tail Student's t-test.

First, the effect of oxidative stress was induced by treating the MDA-MB-231 cell lines expressing both scrambled shRNA and shRNA TDRD3 with 0.5 mM sodium arsenite for 0 (untreated), 15, 30, 45 and 60 minutes. Cells were then stained for immunofluorescence using DDX3 as a SG marker (Shih et al., 2012) at the indicated time points (**Figure 12A**). This experiment was repeated 3 times and statistical analysis of the “percentage of SG-positive” cells and “number of SGs per cell” revealed an alteration in SG assembly with reduced levels of TDRD3. Specifically, the TDRD3 shRNA expressing cell line exhibited a higher percentage of cells with SGs and consequently higher number of SG per cell with oxidative stress (**Figure 12B**). It is also important to note that SGs formed earlier, at the 20-minute time point, in TDRD3 shRNA cell lines compared with the control scrambled shRNA expressing cell line where they formed at 30 minutes. The most obvious difference is observed between the 30 to 45 minutes time points (**Figure 12B and 12C**). At the 30-minute time point, there was an increase in percentage of cells with SGs approximately 31% and 25% greater in the shRNA TDRD3 cells (pool #1 and pool #2) respectively, in comparison with scrambled control. At 45 minutes, the increase was 17% and 13% in shRNA TDRD3 cells (pool #1 and pool #2) respectively. The numbers of SGs per cell was also altered in the TDRD3 depleted cells and statistically significant at the 30 and 45 minutes time points. At 30 minutes, there was an increase of 19% and 25% in the numbers of SGs per cell in the shRNA TDRD3 (pool #1 and pool #2) respectively, in comparison to scrambled control. At 45 minutes, the increase was approximately 15% and 29% in the number of SGs per cell in the shRNA TDRD3 cells (pool #1 and pool #2) respectively, in comparison to the control. Also, specifically using DDX3 as a marker, there was a



statistical difference at the 60 minutes time point, with an increase in the percentage of cells with SGs by approximately 15% in the shRNA TDRD3 (pool #1 and pool #2) and increase average number of SGs per cell by 20% and 14% in shRNA TDRD3 (pool #1 and pool #2) respectively. Altogether, these results indicate that SG assembly is in fact enhanced and accelerated upon reduced TDRD3 level. This opposes our original hypothesis and suggests that TDRD3 regulates SG formation or could be involved in promoting SG disassembly.



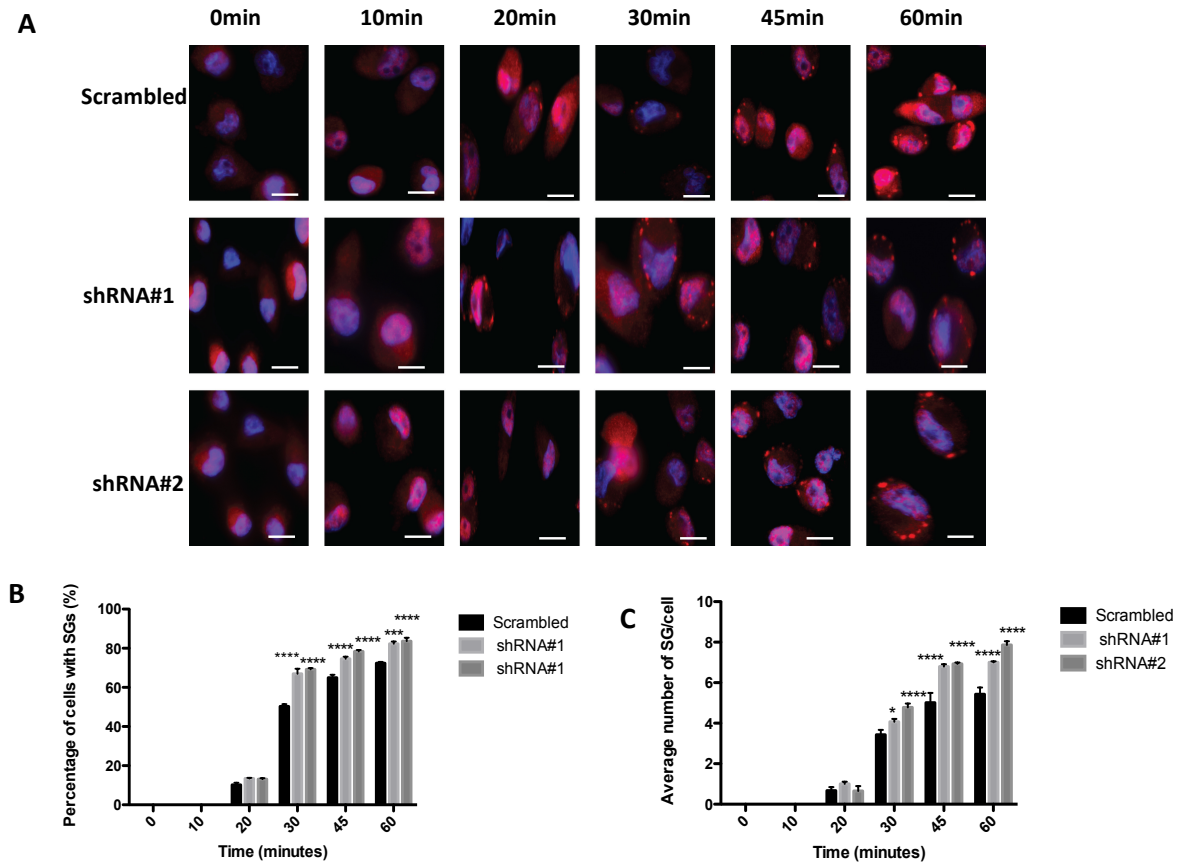
**Figure 12. Depletion of TDRD3 alters SG assembly in MDA-MB-231 cells upon oxidative stress using DDX3 as a marker.** MDA-MB-231 cell lines expressing scrambled shRNA and shRNA TDRD3 were incubated with 0.5mM sodium arsenite for the specified time. Cells were then fixed, permeabilized and processed for immunofluorescence using anti-DDX3 as a marker. DAPI (blue) was used as a nuclear stain. **(A)** Representative immunofluorescence images showing accelerated SG assembly in shRNA TDRD3 when compared to scrambled TDRD3. Scale bars represent 10µm. **(B)** Percentage of cells harboring SGs for each time point from at least 5 different fields representing at least 200 cells and 3 independent experiments. **(C)** Average number of SGs per cell for each time point from at least 5 different fields and 3 independent experiments. Error bars represent SEM of an experiment carried out in triplicates. Statistically significant difference was determined by two-way ANOVA and Tukey's post-hoc test (\*\*\*\*P< 0.0001, \*\*\* P<0.001, \*\*P<0.01).

To ensure that these results were not specific to an effect on DDX3 upon TDRD3 depletion, the experiment was repeated with two other well-established SG markers, TIA-1 and PABP-1 (Kedersha et al., 2000; Kedersha and Anderson, 2007). Cells were treated as previously described and processed for immunofluorescence using either anti-TIA-1 or anti-PABP-1 antibodies. Consistent results were seen when examining SG formation and number with both TIA-1 and PABP-1 markers (**Figure 13 and 14** respectively). Using TIA-1 as a SG marker, at 30 minutes, there was a 25% and 27% increase in percentage of cells with SGs in the shRNA TDRD3 cells (pool#1 and pool#2), respectively, when compared with scrambled. At 45 minutes, there was an increase of 13% and 17% in both the shRNA TDRD3 pool#1 and pool#2, respectively. At 60 minutes, there was an increase of 13% in both pools of shRNA TDRD3.

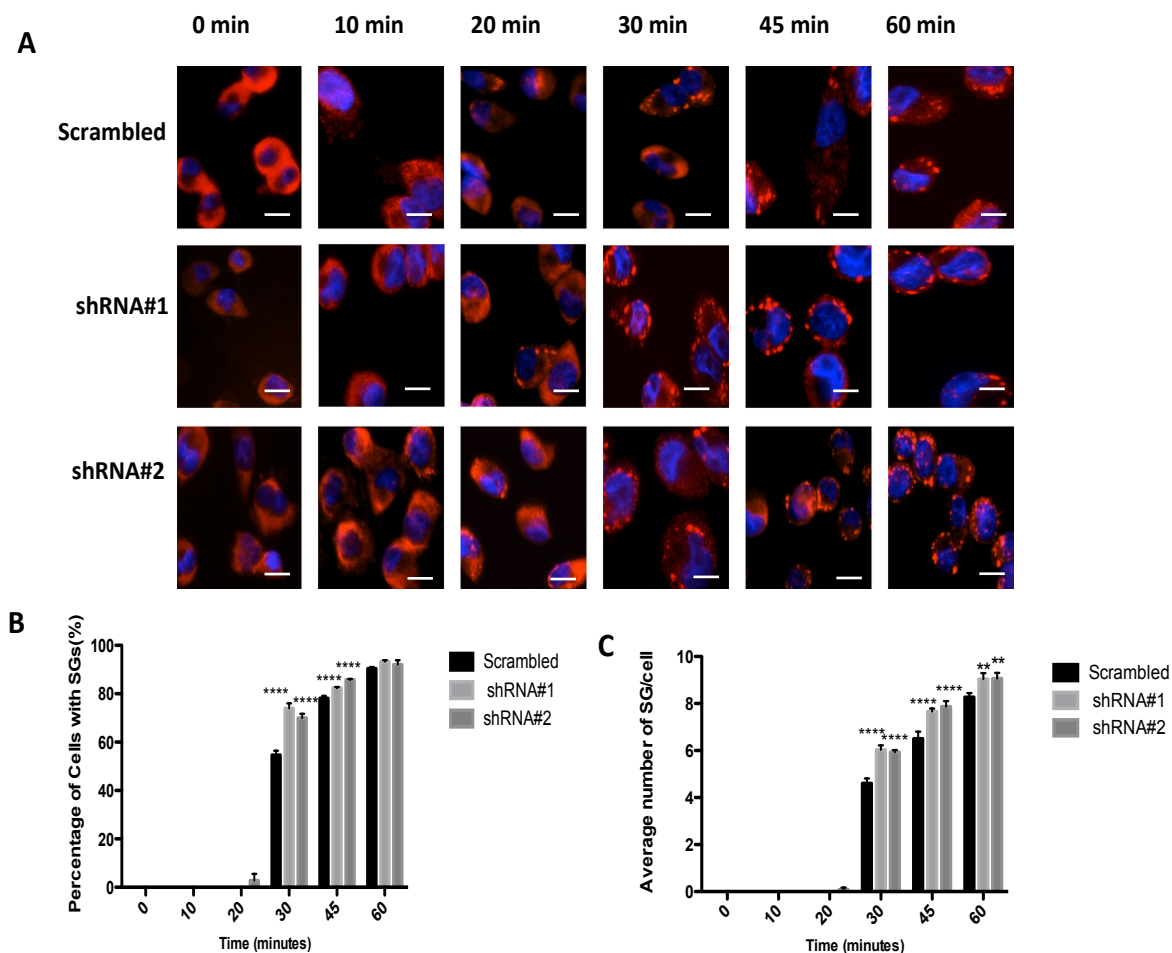
The number of SGs per cell were also increased in TDRD3 depleted cells. A statistical difference was observed at 30, 45, and 60 minutes. At 30 minutes, the shRNA TDRD3 cells (pool#2) exhibited a greater number of SGs per cell with a 27% increase, in comparison with the scrambled shRNA. At 45 minutes, shRNA TDRD3 cells (pool#1) showed a 28% increase in the number of cells with SGs, compared to the scrambled shRNA. At 60 minutes, shRNA TDRD3 pool#1 and pool#2 demonstrated an increase of 23% and 30%, respectively, when compared to the scrambled.

Using PABP-1 as a marker, at 30 minutes, there was an increase in the percentage of cells with SGs of about 26% and 22% in shRNA TDRD3 cells (pool #1 and pool #2) respectively, in comparison with scrambled control. At 45 minutes, the increase was about 5% and 9%, in the shRNA TDRD3 (pool #1 and pool #2) respectively. The number of SGs per cell was also altered in the TDRD3 depleted cells

and statistically significant at the 30 and 45 minutes time points. At 30 minutes, the shRNA TDRD3 cells (pool#1 and pool#2) displayed an increase of 22% and 16% in the number of SGs per cell, respectively. At 45 minutes, shRNA TDRD3 cells (pool#1) showed a 15% increase in the number of cells with SGs. Thus, the accelerated formation of SGs with TDRD3 depletion is not restricted to a specific marker but it is an effect confirmed with multiple SG markers.



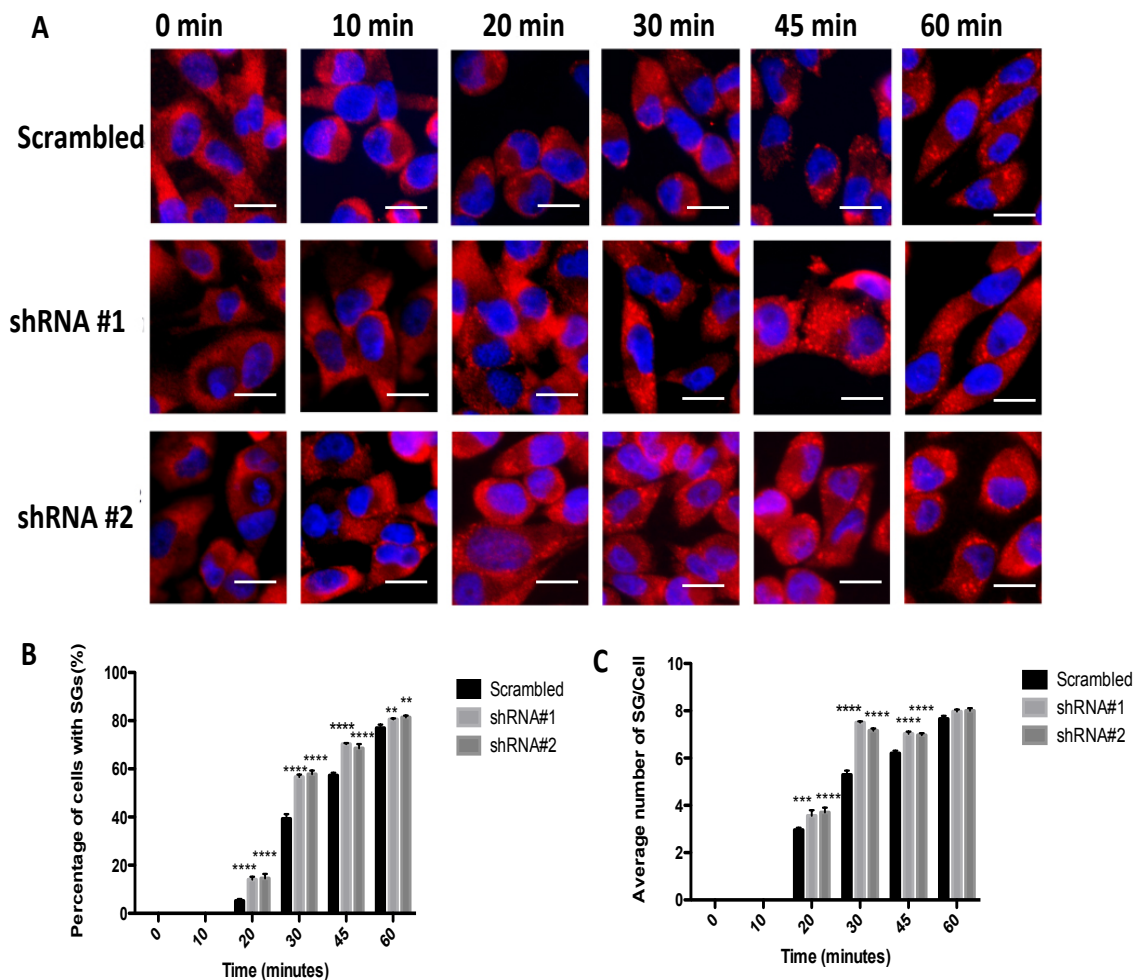
**Figure 13. Depletion of TDRD3 alters SG assembly in MDA-MB-231 cells upon oxidative stress using TIA-1 as a marker.** MDA-MB-231 cell lines expressing scrambled shRNA and shRNA TDRD3 were incubated with 0.5mM sodium arsenite for the specified time. Cells were then fixed, permeabilized and processed for immunofluorescence using anti-TIA-1 as a marker. DAPI (blue) was used as a nuclear stain. **(A)** Representative immunofluorescence images showing accelerated SG assembly in shRNA TDRD3 when compared to scrambled TDRD3. Scale bars represent 10 $\mu$ m. **(B)** Percentage of cells harboring SGs for each time point from at least 5 different fields and 3 independent experiments. **(C)** Average number of SGs per cell for each time point from at least 5 different fields and 3 independent experiments. Error bars represent SEM of an experiment carried out in triplicates. Statistically significant difference was determined by two-way ANOVA and Tukey's post-hoc test (\*\*\*\*P< 0.0001, \*\*\* P<0.001, \*\*P<0.01, \*P<0.05).



**Figure 14. Depletion of TDRD3 alters SG assembly in MDA-MB-231 cells upon oxidative stress using PABP-1 as a marker.** MDA-MB-231 cell lines expressing scrambled shRNA and shRNA TDRD3 were incubated with 0.5mM sodium arsenite for the specified time. Cells were then fixed, permeabilized and processed for immunofluorescence using anti-PABP-1 as a marker. DAPI (blue) was used as a nuclear stain. **(A)** Representative immunofluorescence images showing accelerated SG assembly in shRNA TDRD3 when compared to scrambled TDRD3. Scale bars represent 10 $\mu$ m. **(B)** Percentage of cells harboring SGs for each time point from at least 5 different fields representing at least 200 cells and 3 independent experiment. **(C)** Average number of SGs per cell for each time point from at least 5 different fields and 3 independent experiments. Statistically significant difference was determined by two-way ANOVA and Tukey's post-hoc test (\*\*\*\* $P < 0.0001$ , \*\*\*  $P < 0.001$ , \*\* $P < 0.01$ )

To confirm that the effect of TDRD3 depletion on SG formation is not specific to oxidative stress, the effect of TDRD3 depletion on additional stresses was investigated. Osmotic stress was induced by treating cells with 1M sorbitol for one hour and assessing SG assembly as aforementioned. DDX3 was used as a marker for SGs since all the markers used in the previous experiments demonstrated consistent results.

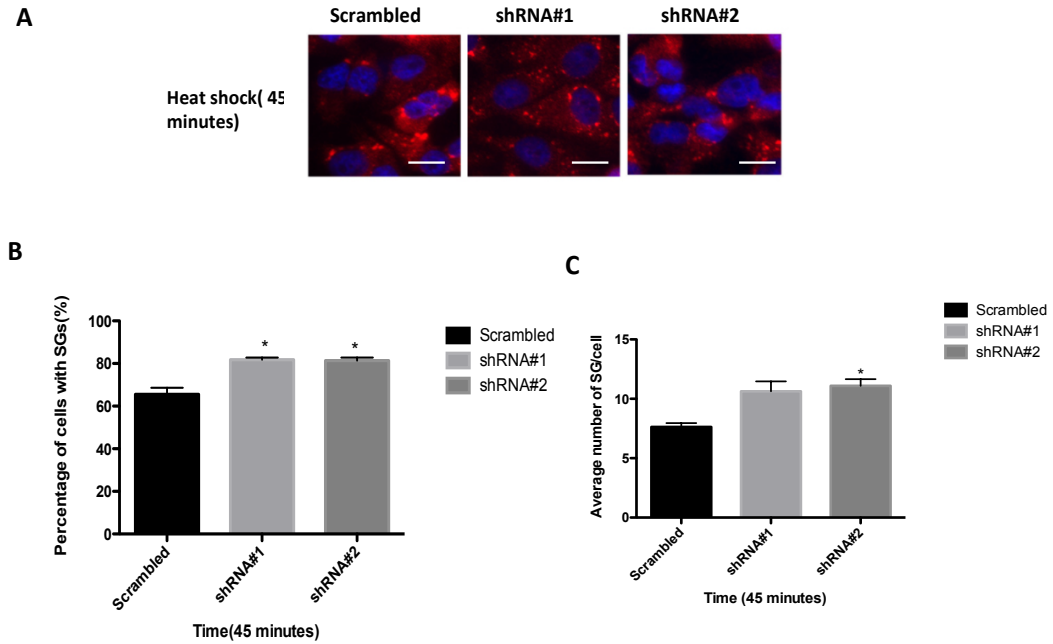
A similar trend was observed with sorbitol as that seen with sodium arsenite, where stably-expressing TDRD3 shRNA cells formed SGs earlier and this difference was emphasized at time points 30-45 minutes (**Figure 15**). At 30 minutes, there was an increase in the percentage of cells with SGs of 30% and 32% in the shRNA TDRD3 cells (pool #1 and pool #2) respectively, in comparison with scrambled control. At 45 minutes, there was an increase in the percentage of SGs of 19% and 16% in shRNA TDRD3 cells (pool #1 and pool #2) respectively, compared with scrambled control. The number of SGs per cell was also altered in the TDRD3 depleted cells and a statistically significant increase was evident at time points 30 and 45 minutes. At 30 minutes, there was an increase in the average number of SGs of 29% and 26%, in the shRNA TDRD3 cells (pool #1 and pool #2) respectively. At 45 minutes, about a 12% increase in number of SGs was evident in shRNA TDRD3 cells (pool #1 and pool #2).



**Figure 15. Depletion of TDRD3 alters SG assembly in MDA-MB-231 cells upon osmotic stress using DDX3 as a marker.** MDA-MB-231 cell lines expressing scrambled shRNA and shRNA TDRD3 were incubated with 1M sorbitol for the specified time. Cells were then fixed, permeabilized and processed for immunofluorescence using anti-DDX3 as a marker. DAPI (blue) was used as a nuclear stain. **(A)** Representative immunofluorescence images showing accelerated SG assembly in shRNA TDRD3 when compared to scrambled TDRD3. Scale bars represent 10 $\mu$ m. **(B)** Percentage of cells harboring SGs for each time point from at least 5 different fields and 3 independent experiments. **(C)** Average number of SGs per cell for each time point from at least 5 different fields and 3 independent experiments. Statistically significant difference was determined by two-way ANOVA and Tukey's post-hoc test (\*\*\*\* $P < 0.0001$ , \*\*\* $P < 0.001$ , \*\* $P < 0.01$ ).

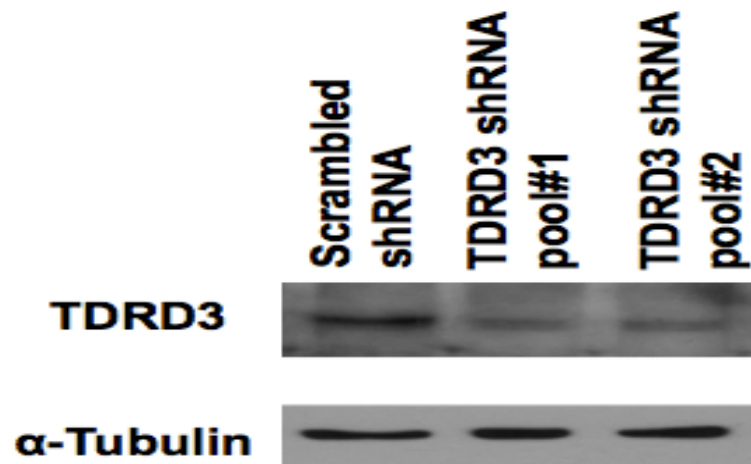


Lastly, cells were exposed to heat shock by incubation at 45°C for 45 minutes. The incubation was increased from the standard 42 to 45°C and for 45 minutes, as this is the earliest heat temperature that demonstrated SGs formation in these particular cell lines (Goulet et al., 2008). Stably-expressing shRNA TDRD3 cells form SGs more prominently than scrambled shRNA expressing cells, as measured using DDX3 as a marker for SG formation (**Figure 16**). This experiment demonstrates a similar and consistent trend as seen in the previous experiments performed. TDRD3 shRNA cells show a higher percentage of cells with SGs with a 19% increase and a higher number of SGs per cell, approximately 30%, and the SGs are shown to form earlier compared to the control scrambled shRNA. Collectively, these results strongly suggest a role for TDRD3 in regulating the assembly of SG or promoting their disassembly.



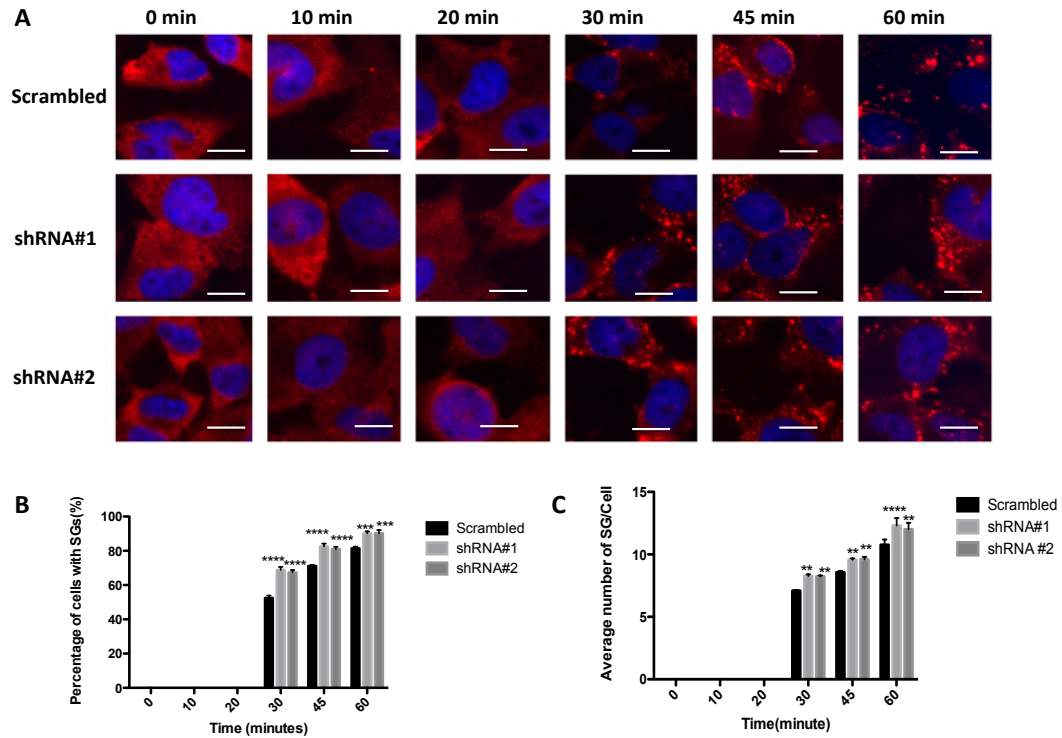
**Figure 16. Depletion of TDRD3 alters SG assembly in MDA-MB-231 cells upon heat shock using DDX3 as a marker.** MDA-MB-231 cell lines expressing scrambled shRNA and shRNA TDRD3 were heat shocked for 45 minutes at 45°C. Cells were then fixed, permeabilized and processed for immunofluorescence using anti-DDX3 as a marker. DAPI (blue) was used as a nuclear stain. **(A)** Representative immunofluorescence images showing accelerated SG assembly in shRNA TDRD3 when compared to scrambled TDRD3. Scale bars represent 10µm. **(B)** Percentage of cells harboring SGs from at least 5 different fields and 3 independent experiments. **(C)** Average number of SGs per cell for each time point from at least 5 different field and 3 independent experiments. Statistically significant difference was determined by two-way ANOVA and Tukey's post-hoc test (\*P<0.05).

To determine if TDRD3 regulates SGs in additional breast cancer cells, a second cell line was examined. The above experiment was repeated with the Hs578T breast cancer cell line, which is another highly aggressive ER negative breast cancer line. Similarly, Hs578T cell lines were transfected either with an shRNA vector targeting TDRD3 mRNA in its 3'UTR or with a scrambled shRNA expressed from the same vector. Western blot analysis demonstrated a decreased level of TDRD3 ( 75% knockdown) in cell lines expressing shRNA targeting TDRD3 (shRNA pool #1 and pool #2) (**Figure 17**).



**Figure 17. Stable knockdown of TDRD3 using a shRNA-expressing vector in Hs578T cells.** Western blot analysis showing reduced levels of TDRD3 expression in Hs578T cell line expressing shRNA targeting TDRD3 mRNA in its 3'UTR. A scrambled shRNA expressed from the same vector is also shown. Alpha tubulin ( $\alpha$ -tubulin) was used as a loading control.

Hs578T cell lines expressing either scrambled shRNA (control) or shRNA TDRD3 were treated with 0.5 mM sodium arsenite for time points of 0, 15, 30, 45 and 60 minutes. Immunofluorescence was performed using endogenous DDX3 as a SG marker (**Figure 18A**). Quantification of “percentage of SG positive cells” and “number of SGs per cell” illustrated a consistent and similar trend as observed in MDA-231 cells (**Figure 18B and 18C**). TDRD3 shRNA pools exhibit consistently higher percentage of SG positive cells. At 30 minutes, shRNA TDRD3 pool#1 and pool#2 demonstrated around 24% and 22% increase in percentage of SG positive cells, respectively, compared to scrambled control. At 45 minutes, the percentage of cells with SGs was increased by 14% and 12% in shRNA TDRD3 pool#1 and pool#2, respectively, compared to scrambled control. Higher numbers of SGs per cell were also demonstrated in the TDRD3 depleted cells. At time point 30-minutes, the number of SGs per cell was increased by 13% in the TDRD3 depleted cells compared to scrambled. Whereas at time point 45-minutes, the increase was of 12% in TDRD3 depleted cells compared to scrambled. The consistency in altered SG formation in cells that are TDRD3 depleted with various SG markers, stressors and different cell lines unveils a *bona fide* (novel) regulatory role of TDRD3 in the assembly and disassembly of SGs.



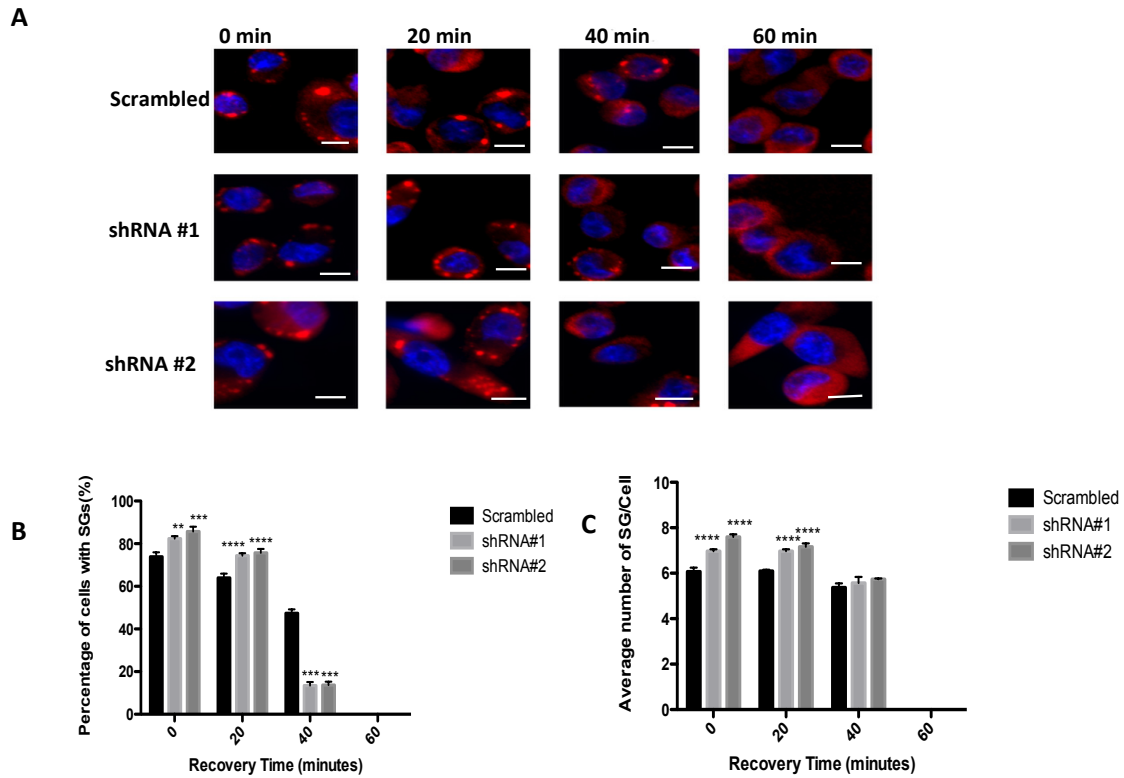
**Figure 18. Depletion of TDRD3 alters SG assembly in Hs578T cells upon oxidative stress using DDX3 as a marker.** Hs578T cell lines expressing scrambled shRNA and shRNA TDRD3 were incubated with 0.5mM sodium arsenite for the specified time. Cells were then fixed, permeabilized and processed for immunofluorescence using anti-DDX3 as a marker. DAPI (blue) was used as a nuclear stain. **(A)** Representative immunofluorescence images showing accelerated SG assembly in shRNA TDRD3 when compared to scrambled TDRD3. Scale bars represent 10µm. **(B)** Percentage of cells harboring SGs for each time point from at least 5 different fields and 3 independent experiments. **(C)** Average number of SGs per cell for each time point from at least 5 different fields and 3 independent experiments. Statistically significant difference was determined by two-way ANOVA and Tukey's post-hoc test (\*\*\*\*P< 0.0001, \*\*\*P<0.001, \*\*P<0.01).

### **TDRD3 plays a role in stress granule disassembly**

Stress granules are transient dynamic entities that ultimately disassemble/disperse when stress is removed, allowing for the resumption of translation initiation of mRNAs that were harboured within SGs (Buchan and Parker, 2009). Therefore, we wanted to examine the role of TDRD3 in the disassembly of SGs. We hypothesized that TDRD3 depletion would alter SG disassembly, similar to the altered SG assembly. MDA-MB-231 cell lines expressing either scrambled shRNA (control) or shRNA TDRD3 were assessed using a stress recovery experiment. The cells were treated for one hour with 0.5 mM sodium arsenite (0 minute recovery), washed and then incubated in normal media to recover for 20, 40 or 60 minutes. Cells were processed for immunofluorescence for the specified time with antibodies against DDX3 as a SG marker (**Figure 19A**). Stress granules disassembled after approximately 60 minutes and statistical analysis of the rate of disassembly relative to time point 0 minute revealed a similar initial disassembly rate in shRNA TDRD3, at the 20 minute time point post recovery. At time point 20 minutes, the shRNA TDRD3 pool#1 and pool#2 showed a 10% and 8% decrease in the percentage of cells with SGs respective to time point 0 minute whereas scrambled shRNA revealed a 16% decrease in the percentage of cells with SGs to time point 0 minute. The average number of SG per cell is higher in shRNA TDRD3 pool#1 and pool#2 when compared to scrambled, by around 12% and 15%, respectively. However, an accelerated SG disassembly is evident in the shRNA TDRD3, at the 40 minute time point post recovery (**Figure 19B**). At 40 minutes, the rate of decrease in the percentage of SGs per cells compared to time point 0-minute is a 38% decrease in control shRNA scrambled whereas TDRD3 depleted cells demonstrated

84% and 82% (pool #1 and pool#2 respectively) rate of decrease in the percentage of SGs per cell in comparison with the respective time point 0. Consistent with altered and accelerated SG assembly with reduced TDRD3 levels, SG disassembly was also altered in TDRD3 depleted cells, with TDRD3 depletion causing an accelerated disassembly of SGs. The accelerated SG formation evident upon depletion of TDRD3 is not sustained as they disassemble more rapidly when compared to their respective scrambled control. Therefore, this suggests the requirement of TDRD3 in the cellular stress response and for the overall maintenance of SGs integrity.





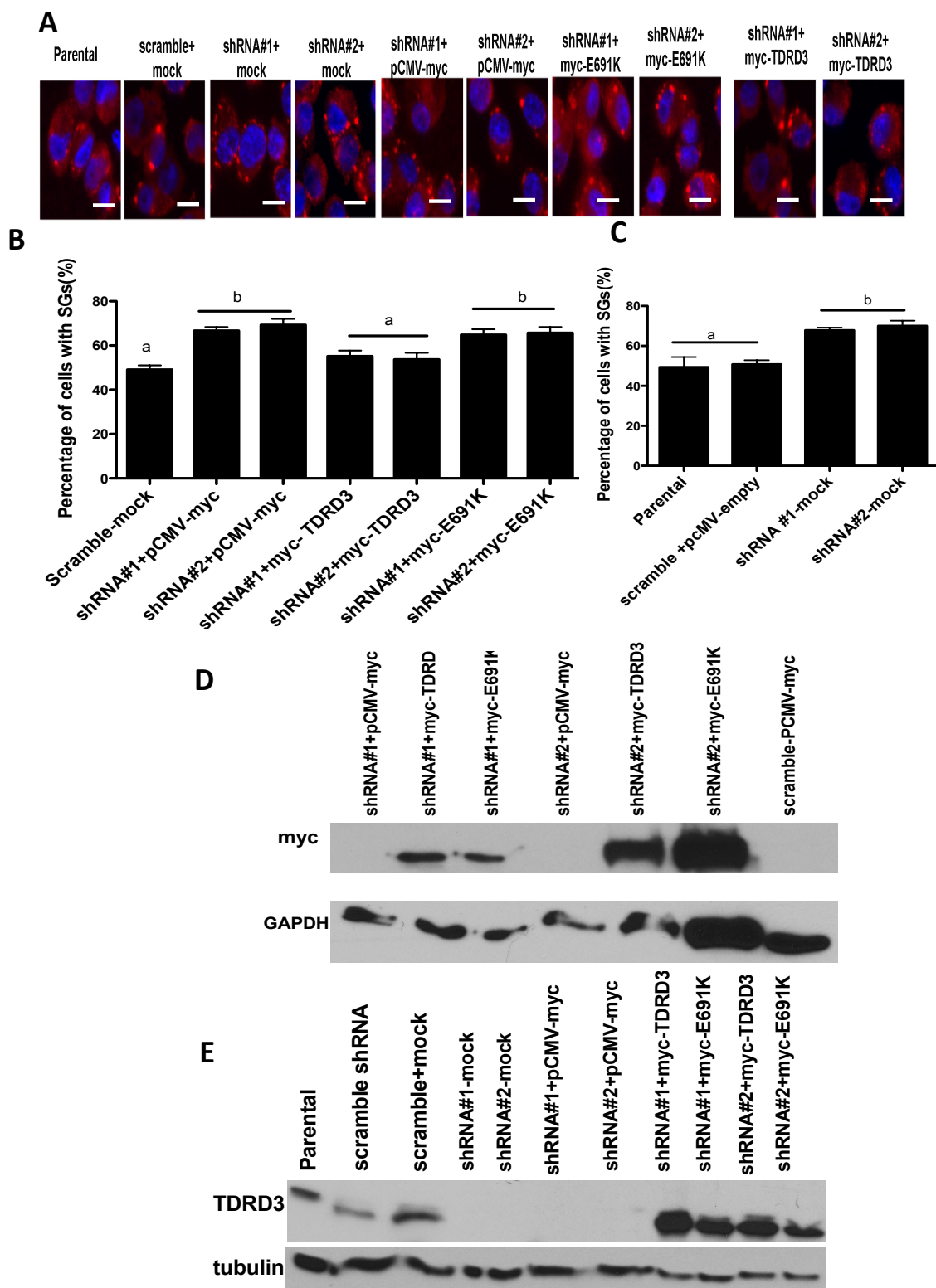
**Figure 19. Depletion of TDRD3 alters SG disassembly in MDA-MB-231 cells using DDX3 as a marker.** MDA-MB-231 cell lines expressing scrambled shRNA and shRNA TDRD3 were treated with 0.5mM sodium arsenite for one hour, media was then changed to normal media and cells were allowed to recover for up to one hour. Cells were then fixed at the specified time, permeabilized and processed for immunofluorescence using anti-DDX3 as a marker. DAPI (blue) was used as a nuclear stain. (A) Representative immunofluorescence images showing accelerated SG formation in shRNA TDRD3 when compared to scrambled TDRD3. Scale bars represent 10 $\mu$ m. (B) Percentage of cells harboring SGs for each time point from at least 5 different fields and 3 independent experiments. (C) Average number of SGs per cell for each time point from at least 5 different field and 3 independent experiments. Statistically significant difference was determined by two-way ANOVA and Tukey's post-hoc test (\*\*\*\*P< 0.0001, \*\*\*P<0.001, \*\*P<0.01).

### **Re-introduction of wild type TDRD3 rescues the defects in stress granule assembly**

Next, we wanted to define whether re-introduction of TDRD3, or TDRD3 harbouring a mutation within the Tudor domain of TDRD3 (E691K), was capable of rescuing the altered SG assembly seen in TDRD3 depleted cells. The E691K mutant inhibits the Tudor domain's recognition of arginine methylated polypeptides and abolishes the recruitment of TDRD3 to SGs upon stress (Goulet et al., 2010). Therefore, this rescue experiment investigated whether TDRD3 was responsible for the altered SG assembly as seen previously and whether the effect of TDRD3 is dependent (E691K mutant does not rescue) or independent (E691K mutant rescues) on its role in SGs. TDRD3 shRNA cells were transiently transfected with wild type myc-epitope tagged TDRD3 or the E691K mutant allele. Both MDA-MB-231 TDRD3 shRNA and scrambled shRNA were transiently transfected with myc-epitope tagged empty vector as a negative control. Twenty-four hours post transfection, cells were treated for 30 minutes with sodium arsenite and immuno-stained with DDX3 antibody as aforementioned (**Figure 20A**). A time point of 30 minutes was chosen as it consistently showed the most prominent difference in SG assembly between the shRNA TDRD3 and the control.

Statistical analysis of the percentage of SG positive cells revealed a rescue of the altered SG formation only in MDA-MB-231 shRNA TDRD3 cells transiently expressing wild type TDRD3 (myc-TDRD3) and not those expressing E691K mutant (myc-E691K) (**Figure 20B**). Furthermore, statistical analysis of the percentage of SG positive cells in the various controls is also shown (**Figure 20C**). These results indicate that TDRD3 contributes towards the alteration of SG assembly as demonstrated earlier

in the previous experiments. Furthermore, the effect of TDRD3's methyl-arginine binding capacity observed is dependent on its role in SGs, as the E691K mutant did not rescue the altered SG assembly. This corroborates our results that TDRD3 depletion is causing the alteration in SG assembly. Expression of the constructs was confirmed by immunoblotting (**Figure 20D**). Western blot analysis of the levels of TDRD3 revealed an overall increase of TDRD3 levels in the myc-TDRD3 and myc-E691K compared with scrambled (**Figure 20E**).



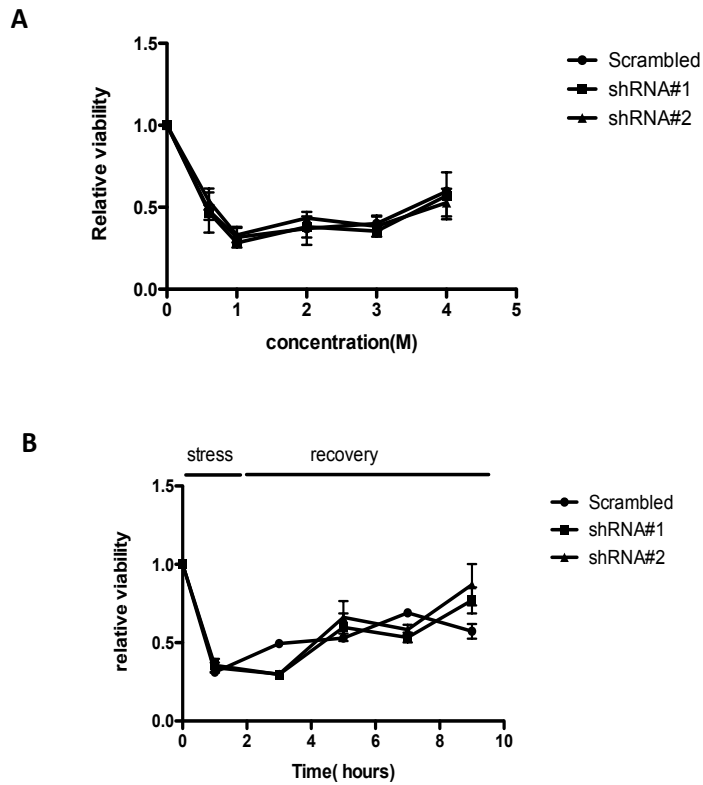
**Figure 20. Wild type but not mutated TDRD3 (E691K) rescues the effect of altered SG assembly seen in MDA-MB-231 cells with depleted TDRD3 levels.** MDA-MB-231 cell lines expressing shRNA TDRD3 were transiently transfected with myc-tagged wild type or mutated (E691K) TDRD3. Cell line expressing both scrambled shRNA and shRNA TDRD3 were transfected with empty vector (pCMV-myc). Twenty-four hours post-transfection, cells were treated with 0.5mM sodium arsenite for 30 minutes. Cells were then fixed, permeabilized and processed for immunofluorescence using anti-DDX3 as a marker. DAPI (blue) was used as a nuclear stain. (A) Representative immunofluorescence images for all conditions. Scale bars represent 10µm. (B-C) Percentage of cells harbouring SGs from at least 5 different fields and 3 independent experiments. Columns are mean percentages  $\pm$  SEM of the experiment carried out in quadruplicate. (D) Total cell lysates of MDA-MB-231 of all conditions were lysed and protein extracts were prepared and analyzed by Western blotting to detect myc and tubulin (loading control) using anti-myc and anti-tubulin antibodies. (E) Analysis of the total levels of TDRD3 in different conditions via western blotting using anti-TDRD3 and anti-tubulin specific antibodies. Values presented are mean percentages  $\pm$  SEM of an experiment carried out in quadruplicate and p values between a and b=\*\*\*\*. All data were analyzed by one-way ANOVA followed by Tukey's post hoc test.

### **TDRD3 and cell survival following stress**

SGs form in the face of adverse environmental stress as a way to promote cell survival in cancer cells (Arimoto et al., 2008; Kim et al., 2005). We hypothesized that TDRD3 contributes to cell survival and in the event of TDRD3 depletion, cell survival would be impeded. Despite the fact that SG assembly is accelerated and enhanced upon TDRD3 depletion, SGs seem to be less stable and as such more prone to disassembly. This consequently suggests that the function of SGs in promoting cell survival might be affected upon TDRD3 depletion. In order to address the physiological role TDRD3 could potentially exhibit in the stress response, control and TDRD3 knockdown MDA-MB-231 cells were treated with varying concentrations of sorbitol for one hour and cell viability was measured. Sorbitol was used here, as it is the most widely used drug to monitor survival (Baguet et al., 2007).

Cell viability was assessed using the CellTiterGlo™ assay, which measures the amount of intracellular ATP before and during stress. “The amount of ATP is directly proportional to the number of cells present in culture” (Promega). An equivalent number of cells were plated for each of scrambled control and shRNA TDRD3 pool#1 and pool#2, for all of the conditions in order to quantitatively assess cell viability. Both scrambled and shRNA TDRD3 were treated with 0.6M, 1M, 2M, 3M, 4M of sorbitol for one hour. Cell viability was assessed after one hour and statistical analysis revealed cell survival following stress between control and TDRD3 depleted cells (**Figure 21A**). Recovery from stress was monitored in both MDA-MB-231 shRNA TDRD3 and shRNA scrambled cell lines. Cells were treated with 0.6M sorbitol for one hour, washed and incubated in normal media to allow for recovery. Cell viability was then assessed via

CellTiterGlo™ for every two hours, up to eight hours. Statistical analysis from a duplicate of this experiment revealed a consistent trend in cell survival in both the control (scrambled) and TDRD3 depleted cells, implicating no effect of shTDRD3 on viability (**Figure 21B**). These results indicate that TDRD3 may not play a role in the sorbitol induced stress response. Although these results were not in accordance with our hypothesis, however this could partly be due to the choice of assay used.

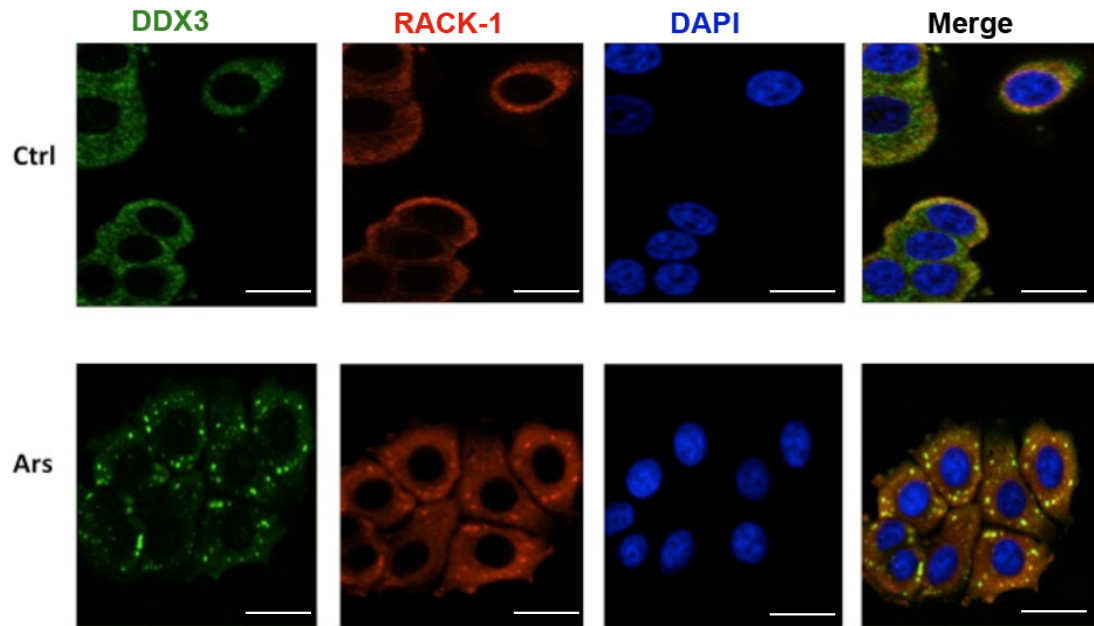


**Figure 21. Depletion of TDRD3 has no effect on cell viability.** (A) MDA-MB-231 cell lines expressing either scrambled shRNA or shRNA targeting TDRD3 were treated with different concentrations of sorbitol for one hour. (B) Knockdown shRNA TDRD3 and scrambled shRNA in MDA-MB-231 cells were treated with 0.6M sorbitol for one hour followed by an incubation with normal media where cells were allowed to recover. Cellular viability was then measured at each time point. Data were normalized to 1 for time 0. Values presented are mean  $\pm$  SEM of an experiment carried out in triplicate (A) or duplicate (B).

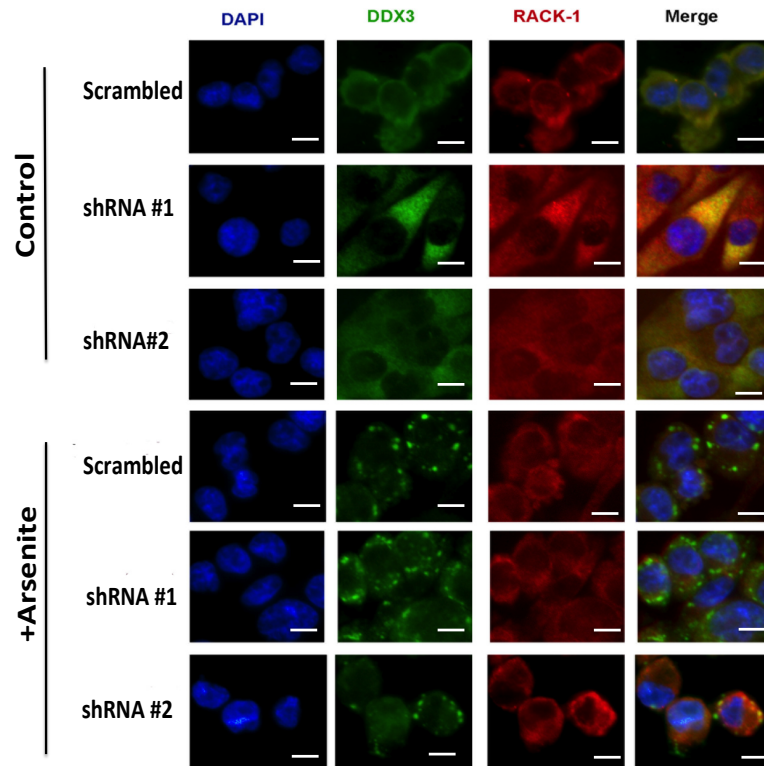


### **Composition of SGs in absence the of TDRD3**

Lastly, we wanted to obtain insights into the composition of SGs in the absence of TDRD3. Previous studies have demonstrated that RACK-1, a signalling scaffold protein, is sequestered into SGs upon exposure to hypoxia and this leads to the suppression of the stress-responsive MAP kinase apoptotic pathways (Arimoto et al., 2008). We monitored RACK-1's re-location to SG upon stress in the face of reduced TDRD3 levels. The localization of the RACK-1 kinase to SGs in MCF-7 cells was confirmed by treating cells with 0.5mM sodium arsenite for one hour and staining with DDX3 and RACK-1 (**Figure 22**), as observed in the previous study by Arimoto et al., 2008. Next, we wanted to monitor whether RACK-1 would relocate to SG in the face of reduced TDRD3 levels. MDA-MB-231 cell lines expressing either scrambled shRNA and shRNA TDRD3 were exposed to 0.5mM sodium arsenite treatment for one hour and examined by indirect IF microscopy using anti-RACK-1 and anti-DDX3 antibodies (**Figure 23**). RACK-1 was not recruited to SGs in MDA-MB-231 cell lines expressing scrambled shRNA or TDRD3 shRNA following exposure to stress. The recruitment of RACK-1 to SG upon stress may therefore be a cell line specific response. This thereby did not allow for the assessment of TDRD3 depletion on the recruitment of RACK-1 to SGs.



**Figure 22. RACK-1 is recruited into SGs by oxidative stress in MCF-7 cells. Cells were treated with 0.5 mM sodium arsenite (Ars) for one hour. Cells were then fixed, permeabilized and processed for immunofluorescence with anti-DDX3 and anti-RACK-1 followed by Alexa 594 (red; RACK-1) and Alexa 488 (green; DDX3). Scale bars represent 10 $\mu$ m.**



**Figure 23. RACK-1 is not recruited into SG by oxidative stress in MDA-MB-231 cells.** MDA-MB-231 cell lines expressing scrambled shRNA and shRNA TDRD3 were left untreated (Control) or treated with 0.5 mM sodium arsenite (Arsenite) for one hour. Cells were then fixed, permeabilized and processed for immunofluorescence with anti-DDX3 and anti-RACK-1 followed by Alexa 6 (red; RACK-1) and Alexa 488 (green; DDX3). Scale bar represents 10 $\mu$ m.

## **Discussion**

Our initial work identified the Tudor domain of TDRD3 to be highly similar to that of SMN, as both demonstrate the ability to bind arginine-methylated peptides (Goulet et al., 2008). Furthermore, the localization of TDRD3 to SGs upon various cellular stresses is now well established (Goulet et al., 2008; Linder et al., 2008). SGs are unique entities as they function to promote cell survival in the face of stress and, importantly, have been postulated to promote cancer cell survival (Kim et al., 2005; Arimoto et al., 2008; Eisinger-Mathason et al., 2008). The functional role TDRD3 plays in these SGs remains to be investigated. Therefore, in this study the novel functional role of the modular protein TDRD3 in SGs formation has been illustrated and clarified. TDRD3 depletion accelerates the formation of SGs, but also leads to faster disassembly. Based on our preliminary results however; we cannot conclude the effect of TDRD3 on cell survival.

### **TDRD3 depletion accelerates stress granules assembly and disassembly**

Tudor domains have been associated with various RNA granules including polar and germinal granules (Akrov et al., 2008; Chuma et al., 2006). Our lab identified a novel Tudor domain containing protein, TDRD3, as a component of the somatic RNA granules, cytoplasmic stress granules (Goulet et al., 2008). SG formation, whether assembly or disassembly is a regulated process (Anderson and Kedersha, 2008) and any influence on SG formation will affect homeostasis culminating in various human pathologies (Anderson and Kedersha, 2008). TDRD3 exhibits a potential role in various human pathologies (Nagahata et al., 2008; Stoll et al., 2013; Xu et al., 2013), including breast cancer, and could be participating in these pathologies via its role in SGs.

TDRD3 localizes to SGs upon various cellular stresses; which led us to speculate whether it is required for the proper and functional formation of SGs. We hypothesized that TDRD3 is required for SG formation and that TDRD3 depletion will likely decelerate and delay SG formation. It is known that depletion of other proteins behaving like TDRD3, such as FMRP, reduces SGs formation (Didiot et al., 2009). FMRP is a protein known to re-localize to SGs and was interestingly found to interact with TDRD3 (Linder et al., 2008). Furthermore, TDRD3 was shown to be present in SGs following oxidative stress treatment as early or even earlier than TIA-1 (Goulet et al., 2008). TIA-1 is a prominent component of the stress granules, where its absence impairs the ability of SGs to form (Gilks et al., 2004). The similarity exhibited in the kinetics of TDRD3 and TIA-1 in localizing to SGs and the fact that TIA-1 serves as a SG-nucleating protein (Anderson and Kedersha, 2002b) suggested that TDRD3 may be required for formation of SGs. In contrast to what we had expected, SGs formed earlier and their assembly was enhanced/accelerated upon TDRD3 depletion. TDRD3 depletion was achieved by shTDRD3 stable knockdown from two different aggressive breast cancer cell lines, MDA-MB-231 and HS578T that demonstrated consistent results (Figures 11 and 17). Moreover, the results were consistent from two different pools of TDRD3 depleted cells, eliminating any variation pertaining to selection. Thus, upon various cellular stresses (osmotic stress, oxidative stress and heat shock), using different SGs markers (TIA-1, DDX3 and PABP-1), and in different cell lines, TDRD3 depletion was found to expedite and enhance SG formation at all time points examined. This conclusively implies a prominent and crucial role for TDRD3 in SG formation. Moreover, the enhancement in SGs was evident in the increased number of SGs per cell upon TDRD3 depletion,

consistently in the different cell lines, different SG markers and upon exposure to different stresses. Therefore, TDRD3 depletion increases both the abundance and dynamics of SGs; TDRD3 seems to be regulating SG assembly. It has been reported that some proteins playing a role in SG formation also affect SG size (McDonald et al., 2011; Aulas et al., 2013). However, there was no apparent significant variation in the size of SGs upon TDRD3 depletion.

Despite the fact that SG properties may vary slightly depending on the nature of the induced stressor (Aulas et al., 2008; Baron et al., 2008, Balagopal and Parker, 2009), the shTDRD3 stable knockdown cell lines consistently expedited SG formation irrespective of the stress. Furthermore, our results show that the disassembly of SGs in the face of depleted TDRD3 was also accelerated. Indeed, upon removal of stress, SGs disassemble to re-establish homeostasis and this reversible nature of SG is crucial for their function (Baron et al., 2008). Thus taken together, our results show that TDRD3 depletion favours SG formation but this accelerated SG formation could result in the aberrant and hasty formation of non-functional SGs that are also less stable.

Staufen-1 (Stau1) is a double-stranded RNA binding protein demonstrated to be ubiquitously present in SGs and its depletion, similarly to TDRD3, facilitated SG assembly (Thomas et al., 2009). In addition, Stau1 was found to facilitate the dissolution of SGs (Thomas et al., 2009). Likewise, DYRK3, the dual specificity tyrosine-phosphorylation-regulated kinase, has been demonstrated to allow for SG dissolution when it is in a kinase active form (Wippich et al., 2013). Both Stau1 and DYRK3 are thought to play a prominent role in facilitating the disassembly of SGs, a phenomenon that remains very poorly understood. Based on our results, it is conceivable that TDRD3

could be playing a role with DYRK3 and Stau1 in this process. Further experimentation is required to determine if TDRD3 collaborates with DYRK3 and Stau1 in mediating SGs disassembly.

One of our proposed models, the ‘organizational model’ could elucidate a potential and prominent role for TDRD3 in ‘assembling’ and ‘organizing’ together SGs formation; therefore, upon its depletion, SGs form faster but are perhaps less organized and stable, and consequently disassemble faster.

TDRD3 may function in regulating certain subsets of proteins to localize to SGs. Upon its removal, these proteins may no longer re-localize to SGs, and therefore the process of SGs formation would be expedited. An intriguing but not mutually exclusive possibility is a ‘sensitivity model’ whereby we propose the involvement of TDRD3 in the translational initiation machinery; when TDRD3 is removed, the balance is shifted away from translation initiation. This shift in balance away from translation initiation would then favour SG assembly. Consistent with this model, TDRD3 was shown to interact with a number of known translational regulators and co-fractionate with actively translating polyribosomes (Goulet et al., 2008). In order to test this hypothesis, it will be important to first clarify the potential role of TDRD3 in translation. A third interesting possibility is that of a ‘sensitization model’, involving the five stress kinases (PKR, PERK, GCN, HRI, Z-DNA), activated by their respective stress that phosphorylates eIF2 $\alpha$  on Ser51. TDRD3 may play a role upstream of these signalling kinases and upon its removal induce an increase in the level of basal eIF2 $\alpha$  phosphorylation, hence effectively lowering the threshold necessary to induce formation of SGs. More work will need to be done to rule



out the latter model, by first monitoring and comparing phosphorylated eIF2 $\alpha$  levels between control and shTDRD3 stable knockdown cell lines.

SGs measurements of one of the conditions were paralleled by another student in the lab, and yielded similar results. Furthermore, a blind subset of each experiment was carried out measuring at least 5 different fields of view for approximately 200 cells. However, live cell time-lapse microscopy could also be performed in the future to further monitor the effect of TDRD3 depletion on SG assembly and disassembly. Finally, Fluorescence Recovery After Photobleaching (FRAP) experiments enable the measurement of the flux of components shuttling in and out of SGs and the overall dynamics of SGs. Different markers would be used as different components of SGs may behave differently.

The altered SG assembly phenotype in shTDRD3 knockdown cells was rescued with wild type myc-epitope tagged TDRD3 suggesting that the defects in SG formation seen were due to the depletion of TDRD3 and not a consequence of shRNA off-target effects. Moreover, a single substitution of E to K in the Tudor domain of TDRD3 (E691K), which is known to abolish its binding to methylated arginine and hinders TDRD3 recruitment to SGs (Goulet et al., 2008), does not rescue this effect. Therefore, this suggests that the role TDRD3 plays in the regulation of SG assembly/disassembly likely involves its specific recognition, through its Tudor domain, of one or more arginine methylated proteins. Furthermore, it also suggests that TDRD3 needs to be present within SGs to exert its function. However, it's important to note that the levels of TDRD3 in transfected wild type and E691K TDRD3 is higher than the respective scrambled control. However, this increase is evident in both transfections and does not skew our comparison

between both transfections.

Further studies will be needed to elucidate the precise mechanism(s) by which TDRD3 regulates SGs. With the knowledge that the Tudor domain of TDRD3 is necessary and sufficient to localize to stress granules, it would suggest that the re-introduction of just the Tudor domain on its own should rescue the defect (Goulet et al., 2008). In the event that it does not, the same rescue experimental approach could be used by transfecting various other TDRD3 mutant alleles to identify the minimal TDRD3 domain needed to rescue the altered SG assembly effect. These experiments would provide us with potential mechanistic insights for future functional experiments. Furthermore, it would be interesting to determine the effect of TDRD3 overexpression on SG formation, and demonstrate if this overexpression would offer the opposite effect to TDRD3 depletion, and thereby inhibit SG formation.

### **TDRD3 does not appear to play a physiological role in the stress response**

SGs are known to function primarily as a protective mechanism promoting cell survival in the face of stress by, for example, sequestering various apoptotic signaling molecules (Arimoto et al. 2008; Eisinger-Mathason et al., 2008; Kim et al., 2005). Various proteins playing a role in SG formation have demonstrated a physiological role in the stress response (Baguet et al., 2008; Thomas et al., 2009; Kim et al., 2005; Arimoto et al., 2008; reviewed by Anderson and Kedersha, 2008). One could thereby hypothesize that TDRD3 is crucial for the function of SGs in promoting cell survival. However, using the CellTiterGlo™ assay, an assay measuring ATP present and thereby metabolically active cells, TDRD3 depleted and control cells demonstrated similar cell survival following exposure to stress (both oxidative and osmotic stress; Figure 21A, data not

shown). Unexpected fluctuations in cell viability were observed, with higher concentrations of stresses yielding more cell survival at times (Figure 21 A). TDRD3 depletion did not demonstrate an effect on recovery from stress for up to eight hours using the CellTiterGlo™ assay (Figure 21 B). Previously, recovery for 24 and 48 hour was also tested and no effect on recovery upon TDRD3 depletion was observed. The CellTiterGlo™ assay is widely and commonly used to assess cell survival (Baguet et al., 2007), but since SG lead to inhibition of bulk translation, essentially putting most cellular processes ‘on hold’, an assay measuring ATP levels may not have been suitable for this specific purpose.

The MTT assay is a colorimetric assay that is also widely used to monitor cell viability by measuring the reduction of yellow 3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyl tetrazolium bromide (MTT) that occurs only in metabolically active cells thus serving as a surrogate for cell viability. However a similar fluctuation in cell viability was seen in the recovery experiments using this assay (data not shown). We had lastly reverted to the use of the trypan blue Countess® Automated Cell Counter and similar fluctuations were observed. This result is not in accordance with our hypothesis but these fluctuations observed in the various assays could point to defects with the use of the pertaining assay in these particular experiments. It therefore remains unclear at present whether TDRD3 aids in cell survival following stress and this remains one of the unresolved issues of this study.

Furthermore, SGs are thought to promote survival in the face of stress by increasing resistance to apoptosis (Arimoto et al., 2008; Baguet et al., 2007; Moeller et al., 2004; Eisinger-Mathason, 2008). An example of this is evident with SG formation in

cancer cells where they offer protection against apoptosis, by sequestration of RACK-1 (Arimoto et al., 2008). Therefore, it would be interesting in the future to identify the level of apoptosis upon TDRD3 depletion. This will serve to identify if the enhancement in SG formation is offering an increased resistance to apoptosis. Apoptosis dysregulation has been suggested to be involved in resistance to chemotherapy (Grossman and Altieri, 2001; Pommier et al., 2004; Goth, 2009). A link between TDRD3 levels and apoptotic resistance via its enhancement of SGs formation could possibly allude to a role of TDRD3 in resistance to therapy. Resistance to chemotherapeutic drugs has been further associated with late-stage breast cancer, involving SGs formation (Fournier et al., 2010; Moeller et al., 2004). Therefore, and in parallel, it would be of particular significance to examine the resistance to chemotherapy between control and shTDRD3 stable knockdown cell lines (pools). Pertinent drugs to the study that can be used would be bortezomib, etoposide, camptothecin, cisplatin and doxorubicin. In addition, apoptotic cells could be monitored via Annexin V staining or TUNEL staining between control and TDRD3 depleted cell lines. Furthermore, a western blot analysis could be done to compare level of cleaved Poly (ADP-ribose) polymerase, PARP, a marker of apoptosis, between the control and TDRD3 depleted cells to determine the level of apoptosis.

### **SG composition upon depletion of TDRD3**

Another interesting aspect is the composition of SGs in the face of depleted levels of TDRD3. To obtain mechanistic insights we had monitored RACK-1's re-localization to SG upon stress in the face of reduced TDRD3 levels. We envisioned that TDRD3 depletion might affect the recruitment of this key signalling scaffold protein. Since it has

not been possible to purify SGs to date, the assessment was done via immunofluorescence microscopy. However, in the shTDRD3 stable knockdown cells lines, both in MDA-MB-231 and HS578T, RACK-1 was not recruited to SGs (Figure 23). RACK-1's recruitment to SGs was previously observed in the MCF-7 cell line (Figure 22; Arimoto et al., 2008). This could pertain to the specificity of the recruitment of RACK-1 to SGs in a particular cell line. It would be interesting in the future to generate stable knockdown TDRD3 in MCF-7 cell lines and assess the contribution of TDRD3 to the recruitment of RACK-1 to SGs. This would provide further mechanistic insights into the role TDRD3 plays in SGs and whether it contributes to cell survival. Furthermore, it would be interesting to assess the localization of proteins known to interact with TDRD3 (Goulet et al., 2008) and proteins known to regulate and mediate SGs function, upon stress in shTDRD3 knockdown cells.

## **Conclusion**

TDRD3 is a novel modular protein whose precise function remains elusive. It is an intriguing protein to study due to its potential link with various human pathologies, including breast cancer. TDRD3 was identified as one of the genes that are correlated with poor prognosis of estrogen-receptor breast cancer (Nagahata et al., 2004). Furthermore, unbiased gene expression studies links TDRD3 with highly aggressive breast cancer (Hallett et al., 2012). Elucidating the functional role of the novel TDRD3 in light of its role in SGs will help expand knowledge of its putative function and potential implications to breast cancer pathology. In this study we have identified a novel functional role of TDRD3's depletion in expediting both the assembly and disassembly of SGs. In light of a previously established correlation between TDRD3 levels with poor

prognosis of late-stage breast cancers, these results could potentially have important implications for the detection, classification and treatment of this deadly cancer.

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