The role of DICER cofactors in microRNA biogenesis and immune checkpoint regulation in cancer cells

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Abstract

MicroRNAs are intrinsic regulators of gene expression, apparent in all major cellular pathways from differentiation to apoptosis. By targeting complementary mRNA, microRNA can direct post-transcriptional downregulation of gene-expression and fine tune levels of protein expression. Disruption to microRNAs on a global or singular scale has been linked to many diseases and cancers due to reduced genetic regulation.

The biogenesis pathway of microRNAs is well documented, however the functions of proteins such as the DICER co-factors TRBP and PACT are not yet fully understood. Within this project, these two proteins were investigated in the context of microRNA biogenesis, with exploration into novel links to cellular immunity, with a focus on the immune checkpoint PD-L1. It is known that PD-L1 is directly regulated by microRNAs and has also been shown to be upregulated during dsRNA stress.

The roles of TRBP and PACT were scrutinized through transient protein knockdown, where the impact of their depletion on selected microRNAs and PD-L1 was assessed through RT-qPCR and western blot. Endogenous interaction of TRBP and PACT was discovered within three different human cancer cell lines, where concurrent depletion of these DICER co-factors resulted in disrupted biogenesis of selected ubiquitous microRNAs.

Both TRBP and PACT function in microRNA biogenesis, and PACT was also identified as a possible influencer of PD-L1 expression. Potential cross talk between miRNA biogenesis and immunity is further discussed through association with Protein Kinase R (PKR).
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Declaration

I declare that this thesis is a presentation of original work and I am the sole author. This work has not previously been presented for an award at this, or any other, university. All sources are acknowledged as references.
1. Introduction

1.1 MicroRNAs and their biogenesis

MicroRNA (miRNA / miR) are short, non-coding RNA (ncRNA) that post-transcriptionally down-regulate gene expression through association with Argonaute proteins and targeting of complementary messenger RNA (mRNA). They function to fine tune gene expression, adding an extra layer of control further to the complex signalling mechanisms mediating transcription and translation. There are more than 1917 miRNA sequences identified in *Homo sapiens* to date, regulating cellular processes from differentiation to apoptosis (miRbase, 2018). Moreover, microRNAs are degenerate, meaning that one miRNA can have multiple mRNA targets, and one mRNA can be targeted by multiple miRNAs (Yi and Fuchs, 2011).

In mammalians, canonical miRNA are generated through the miRNA biogenesis pathway shown in figure 1. They are endogenously coded, transcribed from both protein coding and non-coding regions of the genome *in cis* or *in trans* with their target mRNA (Cai et al., 2009). This means that some microRNAs are transcribed at the same time as their target pre-mRNA, through shared promoters or laying in intronic locations. On the other hand, some microRNA genes are located far from their target mRNA and are independently transcribed (Cai et al., 2009).

MicroRNA transcription is carried out by RNA polymerase II (Pol II), releasing a primary miRNA transcript (pri-miRNA). (Ha and Kim, 2014; Cai et al., 2009), which self-complements to form a mismatched hairpin structure, containing a poly(A) tail and 5’ cap.

Within the nucleus, pri-miRNA is cleaved by a microprocessor complex containing Drosha, an RNase III endonuclease (Lee et al., 2003), and DGCR8 (DiGeorge syndrome critical region 8), a double-stranded RNA-binding protein (dsRBP) (Han et al., 2004). This complex releases a 60-70nt precursor microRNA (pre-miRNA) transcript which is transported out of the nucleus and into the cytoplasm by exportin 5 (Lund et al., 2004). Here, the pre-miRNA is processed by the endonuclease DICER and cofactors TRBP or PACT, cleaving the terminal loop from the miRNA transcript and releasing a mature 22-25nt RNA duplex, with 2nt3’ overhangs (Kim et al., 2016).

One strand of this RNA duplex, deemed the guide strand, is loaded onto an Argonaute (AGO) protein, forming the RNA induced silencing complex, or RISC.
(Martinez et al., 2002; Ha and Kim, 2014; Noland and Doudna, 2013). The guide strand then directs the RISC to complementary mRNA, leading to translational repression by mRNA cleavage, degradation or translational inhibition (Ha and Kim, 2014). Only AGO2 has the capacity to cleave mRNA, whilst association with AGO 1, 3 and 4 will preferentially lead to mRNA degradation or inhibition (Liu et al., 2004; Ha and Kim, 2014). MicroRNAs have also been linked with epigenetic regulation, forming a feedback loop in which miRNAs can remodel chromatin and reduce rates of gene transcription (Tao et al., 2017).

Dysregulation of miRNA biogenesis leading to impairments in mature miRNAs is linked to many cancers. For example, defects in exportin 5 can lead to accumulation of pre-miRNAs in the nucleus and a global downregulation of mature miRNAs (Sun et al., 2016). This has been associated with colon, gastric and endometrial tumours (Melo et al., 2010). Partial loss of function of DICER is also associated with human cancers, including breast, kidney, lung, ovary and stomach (Kumar et al., 2009). This is thought to be due to a global down-regulation of miRNAs and hence reduced regulation of global gene expression, or more specifically over or under expression of oncomiRs and tumour suppressor miRs.

OncomiRs, or oncogenic microRNAs, target mRNA transcripts from tumour suppressor genes (Oliveto et al., 2017). Involved in genomic integrity and cell cycle checkpoints, tumour suppressor genes help prevent uncontrolled cellular growth associated with cancer (Lee and Muller, 2010). Tumour suppressor microRNAs target transcripts of oncogenes (Oliveto et al., 2017), which promote cell growth, division and survival (Lee and Muller, 2010). Dysregulation to oncomiRs and/or tumour suppressor miRs reduce genetic regulation of the genes associated with cancer, increasing the likelihood of cancer development. This effect can also be caused by Drosha mutations (Hato and Kashima, 2017).

Aside from canonical microRNA biogenesis as detailed above, some microRNAs can also be formed non-canonically, or in a way that deviates from the ‘norm’ / generally known rules. Non-canonical microRNA biogenesis may involve DICER, DROSHA and Exportin-5 independent pathways, as reviewed by Ha and Kim (2014).

This project investigated how cancer cells respond to the depletion of two DICER cofactors, transactivation response (TAR) RNA-binding protein (TRBP) and Protein Activator of PKR (protein kinase R) (PACT). We aimed to identify if depletion of
these cofactors has a negative impact on miRNA biogenesis and thus enhance knowledge of emerging trends linking their dysregulation with cancers (Daniels and Gatignol, 2012).
Figure 1: The microRNA biogenesis pathway
1.2 TRBP and PACT are structural homologs

TRBP and PACT are double-stranded RNA-binding proteins (dsRBPs) that share structural homology. Both proteins are constructed of three domains, with each following an αββα fold as shown in figure 2 (Heyam et al., 2015).

Domains 1 and 2 of TRBP and PACT are type A double-stranded RNA binding domains (dsRBDs) and exhibit conservation of three key regions essential for the binding of dsRNA. These are found in the first α-helix, between β1 – β2, and in the final α-helix. These have a high affinity for dsRNA interactions in comparison to their counterpart, type B dsRBDs (Fierro-Monti and Mathews, 2000), with a particular emphasis on the conservation of basic amino acids in RNA binding regions 1 and 2 that are not found in type B dsRBDs (Heyam et al., 2015).

Domain 3 of both proteins is a type B dsRBD; though this shares the same structure as type A domains, it lacks the residues required for RNA binding (Banerjee and Barraud, 2014; Heyam et al., 2015). Instead, this domain provides the interface for interaction with other proteins including DICER, themselves and each other (Daniels et al., 2009, Heyam et al., 2017). dsRBD3 may also be responsible for binding of Argonaute proteins; immunoprecipitation assays have confirmed interaction of TRBP and AGO2 (Chendrimada et al., 2005), yet the interface for this interaction is yet to be confirmed.
Figure 2: TRBP and PACT share structural homology with identical domain arrangement. They exhibit conservation of key amino acids for binding RNA, at the same locus. Domains are connected through unstructured linker regions that allow a degree of flexibility and movement. Based on data from Heyam et al., 2015.
1.3 DICER co-factors in microRNA biogenesis

Both TRBP and PACT bind DICER and Argonaute proteins (Haase et al., 2005; Lee et al., 2006) and are believed to act as co-factors in the microRNA biogenesis pathway, though their exact functions are not fully understood. Through a range of in vitro and in vivo experiments, several potential cellular functions have been elucidated for these two dsRBPs.

1.3.1 DICER cleavage

Firstly, Kim et al. (2014) and Lee et al. (2013) have shown TRBP and PACT to be non-redundant proteins despite their structural homology and similar molecular interactions. TRBP, but not PACT, is able to alter the DICER cleavage site of a subset of microRNAs to form isomiRs. This has been shown endogenously, through gene knockout experimentation (Kim et al., 2014), and through in vitro reconstitution (Lee et al., 2013; Lee and Doudna, 2012). However, to explain this, we first need to understand how DICER cleaves microRNAs.

[Diagram of DICER domains]

DICER protein is comprised of eleven domains, though the function of one of these domains remains unknown (Liu et al., 2018). Figure 3 illustrates a simplified diagram of DICER, displaying the four main domain classes.

The helicase region at the base of DICER accommodates three different helicase domains (Lau et al., 2012). These bind the terminal loop of pre-miRNAs and also form the interface for interaction with proteins such as TRBP and PACT (Lee et al., 2006, Wilson et al., 2015). There is evidence to suggest that these helicase domains, particularly the DExH box domain, participate in the processing of thermodynamically unstable short hairpin RNA structures (Soifer et al., 2008).

The Piwi, Argonaute, Zwille, or ‘PAZ’ (Song and Rossi, 2017) domain, is responsible for recognition of substrates; for miRNAs this is a 2nt 3’ overhang on the pre-miRNA duplex (Park et al., 2011; Liu et al, 2018) The PAZ domain has two adjacent ‘pockets’
for binding of short RNAs: a 2nt 3’ overhang binding pocket, and also a phosphate binding pocket (Tian et al., 2014). Pre-miRNAs, like most RNAs, have a 5’ phosphate group, and hence this structure may determine orientation of loading onto DICER.

The dsRBD, or double-strand RNA binding domain, binds the stem of pre-miRNAs, and is suggested to play a ‘supporting role’ to the RNase domains (Kurzynska-Kokorniak et al., 2015). The RNase III region, compromising of RNase IIIa and RNase IIIb domains (Lau et al., 2012), performs the endonuclease activity and cleaves pre-miRNA to produce a mature miRNA duplex. This region acts as a molecular ruler, with the distance between the PAZ domain and the farthest end of the RNase III domains determining the cleavage site of the pre-miRNA (MacRae et al., 2006; Lau et al., 2012). For human DICER, this is 65 angstroms and equates to roughly 25 nucleotides (MacRae et al., 2006).

TRBP is thought to influence cleavage site, not by altering DICER conformation, but by influencing base mismatches in the pre-miRNA stem (Zhu et al., 2018). An illustration of this is shown in figure 4. TRBP directly binds and ‘smooths out’ bulges in the pre-miRNA stem, reducing the number of nucleotides able to fit in the 65-angstrom gap, producing truncated miRs as compared to independent DICER processing. This can alter miR target sequences: miRNA require perfect complementarity of a seed sequence (up to 8 nt), and partial complementarity for the remaining 18 nt (Lewis et al., 2003). This seed sequence is found at the 5’ end of the mature miRNA (Lewis et al., 2003; Kehl et al., 2017), and even 1 nt change in cleavage site is able to shift this sequence and alter the miRNA target.

We do not directly investigate DICER cleavage site in this study due to multiple complementary studies (mentioned above) agreeing that TRBP, but not PACT, can alter DICER cleavage sites.

1.3.2 TRBP presents substrates to DICER for processing

As previously mentioned, the PAZ domain of DICER recognises pre-miRNA substrates. However, it is suggested that TRBP can also contribute to this process. It has been shown in vitro that TRBP is able to help DICER differentiate between RNA
substrates (Fareh et al., 2016, Lee and Doudna, 2012), thus creating a quicker and more efficient processing rate.

TRBPs three domains are connected via unstructured linker regions (Heyam et al., 2015) that allow movement between each domain. Fareh et al. proposes that TRBP has an arm-like mechanism, where domain three binds the helicase domain of DICER, and domains 1 and 2 bind the RNA substrate. TRBP then presents the RNA substrate in an optimum orientation to the PAZ domain of DICER, where a 2nt 3’ overhang is either detected and the RNA substrate is loaded onto DICER, or the RNA is released without entering the longer binding mode. Fareh et al. (2016) did not investigate PACT alongside TRBP in their study due to a lack of funding. It would be beneficial to also investigate PACT in this manner.

Contradicting these findings, Kim et al. (2014) found no reduction in microRNA abundance upon the production of TRBP or PACT knockout HeLa cell lines. They also found no cellular compensation for a reduction in processing efficiency by upregulation of DICER, suggesting that neither TRBP or PACT were affecting DICER processing efficiency.

Within this study, we assess transient knockdown of TRBP and/or PACT, with the hypothesis that this will give cells less time to compensate for their loss of function and we may observe similar phenomenon as seen in vitro. We also explore three different cell lines, expanding on HeLa cells which have been the basis for investigating endogenous interactions of these two proteins. Due to the age and passage number of the HeLa cell line, accumulation of many mutations is extremely likely, and thus these cells may no longer constitute a reliable representative model.

1.3.3 Strand selection

TRBP and PACT both have the potential to indirectly influence strand selection and loading of mature miRNA from DICER to Argonaute proteins by favourably binding the more thermodynamically stable end of the miRNA duplex, presenting the least stable end to Argonaute for unwinding and loading (Noland and Doudna, 2013, Lee and Doudna, 2012). Intriguingly, during in vitro reconstitution, this selection and subsequent mRNA targeting was most enhanced in the presence of PACT, and sometimes diminished in the presence of TRBP.
Alternate strand selection would give rise to different miRNAs with new target repertoires. Endogenous assays conducted by Kim et al. (2014) found no evidence of this, and again has suggested that neither TRBP or PACT play a role in strand selection at least in HeLa cells. Again, we expand on these observations within this project through assessment of selected microRNAs, aiming to bridge the gap and controversy between in vitro and in vivo experimentation.

1.4 TRBP and PACT participate in viral infection and regulation of the stress response

Outside of miRNA biogenesis, TRBP and PACT both interact with protein kinase R (PKR) as part of the viral defence system, the integrative stress response (ISR) and pro-inflammatory pathways (Patel et al., 2000; Garcia et al., 2006; Dabo and Meurs 2012). PACT is rapidly phosphorylated in stress environments such as viral infection; this in turn activates PKR (Patel et al., 2000). TRBP has the opposite effect, and instead is a PKR inhibitor (Singh et al., 2011).

Other activators of PKR include dsRNA of >30bp (Lemaire et al., 2008). Activation of PKR by dsRNA follows a bell-curve distribution, where low concentrations of dsRNA results in low PKR activation, and high dsRNA concentrations are inhibitory. One theory for this result is the ratio of PKR monomers per dsRNA strand. PKR dimerises during the activation stages, hence high dsRNA concentrations reduce the probability that >1 PKR unit will be associated with the same dsRNA strand, and reduce probability of dimerization (Lemaire et al., 2008).

Upon activation, PKR dimerises, self-phosphorylates and in turn phosphorylates eIF2α, a.k.a. the alpha unit of eukaryotic initiation factor 2. EIF2α is required for initiation of translation, being responsible for the recruitment of methionine linked tRNA to the ribosome (Gale et al., 2000). Upon complete phosphorylation, protein translation is inhibited (Patel and Sen, 1998). This constitutes a major defence against viral infection, preventing synthesis of viral particles and spread of infection.

Aside from PKR inhibition of eIF2α, activated PKR can also trigger an influx of inflammatory cytokines through activation of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) and thus actively participates in the regulation of the inflammasome (Lu et al., 2012).
However, as with most cellular processes, the activities of PKR and elf2α are not so black and white. Rather than an on-off switch, activation and inhibition of this pathway is comparable to a sliding scale that affects rate of translation. PKR has been identified as a potential tumour suppressor through the reduction of protein translation and by association, promotion of apoptosis. Evidence of this has been shown within mouse models, where upregulation of PKR has an inhibitory effect on leiomyosarcoma growth, a type of soft tissue tumour (Vorburger et al., 2005). Conversely, loss of protein expression or aberrant expression has been identified in leukaemia (Blalock et al., 2010), suggesting that dysregulation rather than simply gain or loss of function of PKR activities, is linked to tumours, cancers and other diseases.

1.5 MicroRNAs in Immunity

As intricate regulators of gene expression, microRNAs are involved in most developmental and pathological processes in the human body. Consequently, their dysregulation is tightly associated with cancer and disease. With the degenerate nature of these small regulatory molecules, dysregulation of a singular miRNA can affect expression of a variety of mRNAs across different cellular pathways.

A subset of microRNAs have been identified as onco-miRs, classified as such due to their dysregulation in most cancers. Examples include miR-21, miR-16, miR-143 and let-7 family members (Esquela-Kerscher and Slack, 2006; Yan et al., 2011; Kumarswamy et al., 2011; Buscagalia and Li, 2011).

MiR-21 is a canonical microRNA that is over expressed in most cancers, ranging from epithelial to neurological, and has hence been termed a ubiquitous oncogene (Buscagalia and Li, 2011). Predicted to have as many as 384 mRNA targets (Targetscan.org, 2018), miR-21 functions in diverse cellular roles from embryonic development to cell proliferation to apoptosis (Kumarswarmy et al., 2011; Buscagalia and Li, 2011). As a regulator of several highly important cellular pathways, disruption to the abundance or sequence of miR-21 can lead to serious repercussions, such increased cell proliferation or prevention of apoptosis, which are hallmarks of cancer.

MiR-16 on the other hand, although also a highly conserved and ubiquitously expressed canonical miRNA, is frequently deleted or downregulated in a subset of cancers (Calin et al., 2002; Bonci et al., 2008; Bandi et al., 2009). MiR-16 functions in cellular roles such as promotion of apoptosis, inhibition of proliferation and
suppression of tumourigenicity through association with oncogenes such as BCL2 (reviewed by Aqeilan, et al., 2010). BCL2 is an inhibitor of apoptosis / promoter of cell survival and is frequently overexpressed in tumours (Adams and Cory, 2007; Aqeilan et al., 2010). Depletion of miR-16 reduces regulation of such oncogenes and may increase the likelihood of cancer development.

Such examples demonstrate how dysregulation of a singular microRNA can lead to major ramifications, as miRs are associated with many mRNAs and many molecular pathways and as such are critical to cellular health. This knowledge has inspired the question: does microRNA machinery itself play a role in cellular immunity?

MiRNA biogenesis components DROSHA, DICER and AGO have already been linked to disease through global downregulation of microRNAs (Adams et al., 2014), however direct links to immune pathways and molecules are less clear and have not yet been explored.

This project investigated the cofactors of TRBP and PACT within this novel context. TRBP and PACT have been shown to have direct interaction with PKR, suggesting these proteins may also participate in immunity. We focused on the immune checkpoint programmed death ligand 1 (PD-L1) in this project; discussed in section 1.5.1.

1.5.1 PD-L1

PD-L1 is a cluster of differentiation (CD) molecule, also known as CD274 (Kataoka, 2016). In healthy individuals, PD-L1 is expressed on antigen presenting cells to prevent self-activation and targeting of the immune system. PD-L1 binds to induced PD-1 receptors on activated T cells to prevent an inflammatory and proliferative response (Kier et al., 2008).

This pathway is also hijacked by cancerous cells to evade the immune response (see figure 5). By blocking this interaction with anti-PD-L1 molecules, the immune system is able to activate and kill cancerous cells (Powles et al., 2014; Kier et al., 2008).
It is known that structural variation to the 3' UTR of PD-L1 improves the competency of a cell to escape the immune response (Kataoka et al., 2016). It is also known that PD-L1 expression is regulated post-transcriptionally by miRNAs. MiRNAs frequently target the 3'UTR of mRNAs, hence truncation or mutations in this region prevent the binding of miRs and consequently reduces post-transcriptional regulation.

MiR-155 has been identified as a regulator of PD-L1, capable of directly bind the 3'UTR (Yee et al., 2017). Within this project, we assess expression of miR-155 upon depletion of DICER co-factors. As a canonical microRNA, successful disruption to microRNA biogenesis may result in dysregulation of miR-155. This in turn would reduce regulation of PD-L1 and we may see an increase in its expression. It should be acknowledged that more than 50 further miRNAs have been identified as influencers in the cell surface expression of PD-L1 across different cell types (Wang et al., 2017), and these should not be forgotten during result interpretation.

Further to this, increases in dsRNA stress has been linked to an upregulation of PD-L1 (Sheng et al., 2018). Depletion of cofactors of the miRNA biogenesis pathway may lead to a build-up of dsRNA through reduction of processing efficiency. Increased dsRNA stress or upregulation of PACT upon TRBP knockdown may also directly activate PKR, leading to an inflammatory response and stimulation of PD-L1.
Both PKR and PD-L1 exhibit responses to stimulation by IFN-γ. IFN-γ is a cytokine predominantly secreted by activated T cells, so it makes sense that PD-L1 is upregulated upon IFN-γ stimulation (Yee et al., 2017) – in order to prevent T-cell mediated death. PKR is similarly activated upon IFN-γ stimulation (Sharma et al., 2011), subsequently leading to phosphorylation of EIF2α. PKR can also mediate effects induced by IFN-γ stimulation such as regulation and differentiation of hematopoietic cells (Sharma et al., 2011) and also initiate further transcription factors such as NF-κB (Zamanian-Daryoush et al., 2007).

Summarised in figure 6, we know that PKR can interact with components of miRNA biogenesis, the inflammatory response through IFN-γ signalling and influence nuclear transcription factors. IFN-γ and miRNAs can also influence PD-L1 expression, we propose that PKR could potentially act as a mediating molecule between these different pathways. It would be interesting to determine if PKR also has a direct relationship with PD-L1.

![Figure 6: Visualisation of PKR interactions with miRNA biogenesis, IFN-γ and PD-L1.](image)

1.6 Rationale for use of different cell lines

Three different cell lines were used throughout this project: HeLa, RCC4 and MDA-MB-231. HeLa cells originate from a cancerous cervical tumour and were the first immortalised human cell line, initially isolated in the 1950s. HeLa cells are used by researchers around the world and have been the basis for many miRNA analyses,
including the study by Kim et al. (2016). Therefore, we decided to utilise HeLa cells in order to reflect these previous studies.

However, we decided to expand upon this and include a further two cell lines. Due to the age of HeLa cells, these have accrued many mutations and hence may no longer constitute a reliable cell model. Further to this, we decided to explore different tissues, using cell lines from breast cancers (MDA-MB-231) and renal cell carcinoma (RCC4). These cell lines were readily available to us and allowed more reliable exploration into the roles of TRBP and PACT in ‘general’ miRNA biogenesis and immunity, by reducing tissue-type bias.

RCC4 and MDA-MB-231 cell lines were also chosen due to their expression of PD-L1 protein. All three chosen cell lines are readily transfectable, hence were ideal options for performing RNAi and CRISPR-CAS9 experiments.
1.7 Project aims and hypothesis

The aims of this project were to investigate transient knockdown versus stable knockout of TRBP and PACT across three cancer cell lines with a focus on their roles in miRNA biogenesis and immunity. We aimed to investigate discrepancies between in vitro and in vivo results to date and explore links between the seemingly separate pathways of miRNA biogenesis and cellular immunity.

Individual aims were as follows:

I. Use RNA interference (RNAi) to transiently knock down TRBP and/or PACT
II. Assess selected miRNAs to investigate effects on miRNA biogenesis
III. Evaluate effects on PD-L1, as a means for assessing a novel role of TRBP, PACT and microRNA in immunity
IV. Utilise and design CRISPR / Cas9 genome editing approaches to produce TRBP and PACT knockout cell lines

It was hypothesised that depletion of TRBP alone or in combination with PACT would disrupt the biogenesis of microRNAs. We also hypothesised that depletion of TRBP and PACT could affect PD-L1 expression through miRNA dysregulation and/or PKR mediated mechanisms.

It was further predicted that stable knockout of TRBP protein would not exhibit a reduced DICER processing efficiency, with the suggestion that cancer cells have time to compensate for loss of function over time.
2. Materials and Methods

2.1 Mammalian Cell Culture

HeLa, RCC4 and MDA-MB-231 (MDA231) cells were cultured in Dulbecco’s modified Eagle’s Medium (DMEM) (Gibco, Life Technologies), supplemented with 10% foetal calf serum (FCS) (Hyclone), 1% 200 mM L-glutamine (Gibco, Life Technologies), and 1% penicillin/streptomycin (Gibco, Life Technologies). Cells were washed with 1x PBS (Gibco, Life Technologies) detached with 0.05% Trypsin-EDTA (1x, Gibco) once 70-80% confluent, split 1 in 10 and maintained at 37°C in a humidified atmosphere of 5% CO2.

For cryogenic storage, ~1 million cells were gently resuspended in 1 mL freeze media (see appendix 6.1) and incubated at -80°C for 48 hours. Cells were then transferred to liquid nitrogen for permanent storage.

For cell thaws, cells were collected from liquid nitrogen and thawed for 1-2 min at 37°C. Cells were then transferred slowly into pre-warmed DMEM (37°C) and spun at 259 g to remove residual freeze media. Cells were then resuspended and plated in DMEM.

2.2 RNA Interference (RNAi)

16 hours prior to transfection, cells were seeded at a density of 50,000 cells per mL per well in 6 well plates in antibiotic-free DMEM. Short interfering RNA (siRNA) targeting TRBP, PACT or DICER (On-TargetPlus Smartpools, GE Dharmacon) were transfected at a final concentration of 50 nM using TransIT-siQuest transfection reagent (Mirus Bio), alongside a non-targeting control (NTC) (50 nM, GE Dharmacon), in Opti-MEM reduced serum medium (Gibco, Life Technologies). 5-6 hours later, 1 mL complete DMEM was added to each well and incubated for 16 hours. Cells were washed with 1 mL PBS and media replaced with fresh DMEM; samples were harvested 48 hours later.

2.3 RNA Isolation

Samples were collected with 700 µL QIAzol lysis reagent (Qiagen) directly from 6 well culture plates and frozen in -80°C until RNA extraction.
Total RNA was isolated using the miRNeasy Kit (Qiagen) according to manufacturer’s instructions and eluted in 30 µL nuclease free water. RNA concentration and purity was determined using a Nanodrop 2000c UV/vis spectrophotometer (Thermo Fisher Scientific).

2.4 RT-qPCR

2.4.1 Reverse transcription / cDNA synthesis

Isolated RNA was reverse transcribed to cDNA with random hexamers or oligoDT primers in a c1000 Touch Thermal Cycler (BIO-RAD).

**Random Hexamers:** ≥200 ng of RNA sample was added to 1 µL random hexamers (50 ng/µL, Promega or Applied Biosystems), 1 µL dNTP mix (10 mM, Thermo Scientific) and made up to 10 µL volume with nuclease-free H₂O. Samples were pulsed in a centrifuge then incubated at 65°C for 5 min, cooled to 4°C for 1 min and kept on ice. 4 µL first strand buffer (Invitrogen), 2 µL 0.1M DTT (Invitrogen), 0.5 µL RNaseOUT (40 U/µL, Invitrogen), 0.5 µL superscript III (200 U/µL, Invitrogen) and 3 µL nuclease-free H₂O were added to each sample and pulsed in a centrifuge. Samples were placed back into the thermal cycler and incubated for 10 min at 25°C, 50 min at 50°C then 5 min at 85°C to terminate the reaction. Samples were chilled and kept on ice before proceeding with qPCR analysis, or stored in -20°C.

**OligoDT primers:** ≥200 ng of RNA sample was added to 1 µL anchored oligoDT primers (70 µM, Sigma), 1 µL dNTP mix and made up to 12 µL volume with DEPC H₂O (Ambien). Samples were spun briefly, incubated at 70°C for 6 min then cooled to 4°C for 2 min. 4 µL 5x first-strand buffer (Invitrogen), 2 µL DTT (0.1 M, Invitrogen), 1 µL RNase OUT (Invitrogen) and 1 µL Superscript II reverse transcriptase (200 U, Invitrogen) were added to each sample, spun briefly and incubated at 42°C for 1 hour. Samples were heated to 70°C to terminate the reaction, then chilled and kept on ice before further analysis, or stored in -20°C.

2.4.2 Quantitative PCR (qPCR)

mRNA expression was quantified using the quantitative polymerase chain reaction (qPCR), with Sybr Green master max (Applied Biosystems).
2.4.2.1 Primer Design

Primers were designed using BLAST against confirmed mRNA sequences of intended targets; primer sequences are shown in table 1. To confirm suitability for use, primers were tested on serial diluted cDNA to assess single target amplification (single peak melt curve) and amplification efficiency. An amplification efficiency of 100% suggests the DNA copy number is doubling with each cycle. To obtain primer efficiency values, log copy values are plotted against cycle threshold (CT). The slope of the standard curve is then input into a qPCR efficiency calculator such as that provided by ThermoFisher Scientific, to obtain an amplification factor value and efficiency; these are shown in results (3.1).

Primers were used at a working concentration of 10 µM.

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRBP</td>
<td>GGGAGAGAGCCTGTGTACGA</td>
<td>GGTAACCCGGGAGGTGAAA</td>
</tr>
<tr>
<td>PACT</td>
<td>TTACACGAATACGGCATGAAGAC</td>
<td>CAACGGTTACTCTGAAGGTGAAA</td>
</tr>
<tr>
<td>PD-L1</td>
<td>CATCTTATTATGCTTGGTGTAGCA</td>
<td>GGATTACGTCTCTCAAAATGTG</td>
</tr>
<tr>
<td>DICER</td>
<td>CACATGCCTCCTACCACCTACAA</td>
<td>GCTTGGTATGAGGTAGTCCAAA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GGAAGCTTGTTGGTGCTCGTA</td>
<td>GGCAAATATCCACTTACCAGAGT</td>
</tr>
</tbody>
</table>

Table 1: Primers were ordered as custom desalted oligonucleotides from Sigma Aldrich.

2.4.2.2 qPCR

1 µL cDNA was added to 10 µL SYBR Green Master Mix (Applied Biosystems), 0.6 µL custom forward and reverse primers (10 µM) and 7.8 µL nuclease-free H$_2$O in a MicroAmp Fast Optical 96 well reaction plate (Applied Biosystems). Plates were sealed with MicroAmp Optical adhesive film (Applied Biosystems), before centrifugation at 1200g for 1.5 min.

Plates were run in StepOnePlus™ Real-Time PCR Systems (Applied Biosystems) for 40 cycles (95°C - 60°C), with a set cycle threshold of 0.1 ΔRn (ΔRn (Δreaction) refers to change in fluorescent signal). Base thresholds were set automatically by StepOnePlus Software (V2.3).
All experimental samples had experimental controls, therefore relative gene expression was calculated using the comparative CT method (ΔΔCT). GAPDH was used as a housekeeping gene.

\[ ΔCT = (Average\ Test\ CT − Average\ Housekeeping\ CT) \]

\[ ΔΔCT = (Test\ sample\ ΔCT − Experimental\ Control\ ΔCT) \]

\[ mRNA\ fold\ change = 2^{−ΔΔCT} \]

2.5 MicroRNA RT-qPCR

2.5.1 MicroRNA reverse transcription / cDNA synthesis

1-5ng of total isolated RNA was reverse transcribed to miRNA cDNA using TaqMan® miRNA reverse transcription Kit (Applied Biosystems) according to manufacturer instructions. cDNA was made in separate PCR strips for each target.

2.5.2 MicroRNA qPCR

miRNA expression was quantified by qPCR using TaqMan Universal Master Mix II (Applied Biosystems) and relative gene expression calculated using the ΔΔCT method as described in 2.4.2.

2.6 Western blot

2.6.1 Sample collection

Cells were washed with ice cold PBS and lysed with 30-40µL radioimmunoprecipitation assay (RIPA) buffer (see appendix 6.1) containing protease and phosphatase mixture inhibitors P8340, P5726, and P0044 (1:100) (Sigma). Cells were scraped from the plate with a cell scraper and kept on ice before centrifugation at 10,000 g for 15 min at 4°C. Pelleted cell debris was removed, and protein was stored at -20°C.

2.6.2 Protein quantification

Protein samples were quantified using a Pierce bicinchoninic acid (BCA) protein assay kit (Thermo Scientific) against serially diluted bovine serum albumin standards (2000 µg – 31.25 µg). Samples were diluted 1 in 6 with PBS and 5 µL added to 95 µL.
BCA reagent mixture in a 96 well plate. These were incubated at 37°C for 30 min and absorbance was read in a VERSAmax microplate reader (Molecular Devices) at a wavelength of 562.

**2.6.3 Gel, transfer and antibody incubations**

Samples were made up to ≤10µg protein in a total volume of 20 µL with ddH₂O and 5 µL 4x loading buffer (see appendix 6.1), prior to denaturation at 95°C for 10 min. Samples were kept on ice before loading.

17 µL of sample was loaded into a stacking gel and resolved on SDSPAGE gels (8-10%, see appendix 6.1) in SDS-Page running buffer (National Diagnostics) for 1.5 hrs, 120 V using a Bio-Rad PowerPac HC and mini-PROTEAN tetra system.

Protein was transferred onto PVDF membranes (0.45 µm, Immobilon) with transfer buffer (National Diagnostics) supplemented with 10% methanol, using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad) at 25 V, 0.2 amp for 45 min. Membranes were activated in methanol for 1 min prior to transfer and held in transfer buffer.

Post-transfer, membranes were blocked in 2% Bovine Serum Albumin (BSA) (Fisher Scientific) in 0.1% TBST (see appendix 6.1) for 1 hour on a rocking platform. Primary antibodies, shown in table 2, were probed overnight at 4°C in a 50 mL falcon tube on a roller mixer. Loading controls indicated by * were incubated for 1hr at room temperature (RT).

<table>
<thead>
<tr>
<th>Target</th>
<th>Antibody</th>
<th>Suspension</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRBP</td>
<td>ProteinTech, 15753-1-AP</td>
<td>1:2000. 4% (w/v) BSA in 0.1% TBST</td>
</tr>
<tr>
<td>PACT</td>
<td>Abcam, ab31967</td>
<td>1:1000. 4% (w/v) BSA in 0.1% TBST</td>
</tr>
<tr>
<td>DICER</td>
<td>Cell Signaling Technology, D38E7</td>
<td>1:1000, 5% (w/v) Milk in 0.1% TBST</td>
</tr>
<tr>
<td>PD-L1</td>
<td>Cell Signaling Technology, E1L3N</td>
<td>1:1000, 5% (w/v) Milk in 0.1% TBST</td>
</tr>
<tr>
<td>PSTAT1</td>
<td>Cell Signaling Technology, D4A7</td>
<td>1:1000, 5% (w/v) BSA in 0.1% TBST</td>
</tr>
<tr>
<td>STAT1</td>
<td>Cell Signaling Technology, #9172</td>
<td>1:1000, 5% (w/v) BSA in 0.1% TBST</td>
</tr>
<tr>
<td>GAPDH*</td>
<td>Abcam, [6C5]</td>
<td>1:5000, 5% (w/v) Milk in 0.1% TBST</td>
</tr>
<tr>
<td>β-Actin*</td>
<td>Abcam, [AC-15] ab6276</td>
<td>1:5000, 5% (w/v) Milk in 0.1% TBST</td>
</tr>
</tbody>
</table>

Table 2: Antibodies utilised for western blot
Membranes were washed 3 x 5 min in 0.1% TBST with constant movement. Horseradish peroxidase (HRP) conjugated anti-rabbit and anti-mouse secondary antibodies (Dako) were then incubated for 1 hour at room temperature. Membranes were washed a further 3 times before visualization with Amersham ECL Western Blotting Detection Reagents (GE Healthcare). Luminol detection was either by Amersham Hyperfilm (GE Healthcare) using a compact X4 film processor (Xograph), or by ChemiDOC (BIO-RAD).

Membranes were probed for multiple primary antibodies and were consequently stripped between protein targets of similar sizes. Membranes were stripped for ~15 mins in Restore Western Blot Stripping Buffer (Thermo Fisher) at RT on a rocking platform, then blocked and probed as before.

2.6.4 Western blot quantification

Western blot images were saved as high quality TIFs and quantified using ImageJ software. All probed targets were normalised to either GAPDH or β-Actin and test samples are shown relative to experimental controls.

2.7 CRISPR / CAS9 Genome Editing

2.7.1 gRNA

Off-the-shelf crRNA was bought from GE Dharmacon and validated using BLAST software. crRNA (or CRISPR RNA) is the sequence specific region of guide RNA that directs Cas9 to the complementary region of the genome.

crRNA sequences are shown in table 3. Both sequences for each target were pooled to increase likelihood of producing a knockout cell line. crRNA1 for TARBP2 and PRKRA target coding strands, where crRNA 2 targets lag strands, though both would create double strand breaks. See figure 7 for depiction of TARBP2 crRNA targets. crRNA for PRKRA targets domain 2 (sequence 1), and a non-protein-coding region of the gene.
<table>
<thead>
<tr>
<th>Target</th>
<th>crRNA 1</th>
<th>crRNA 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>TARBP2</td>
<td>TCCTCATACCTGCTTTAT (CM-017430-02-0002)</td>
<td>TTAAAGTGGAAAGCCCCAGTG (CM-017430-01-0002)</td>
</tr>
<tr>
<td>PRKRA</td>
<td>AACGTCCGATCTCAGTAAT (CM-006426-02-0002)</td>
<td>TAAACCGTACGTTCGTTGTG (CM-006426-01-0002)</td>
</tr>
</tbody>
</table>

Table 3: crRNA sequences used in liposomal CRISPR Cas9 protocol

Figure 7: TRBP crRNA target sites (Domain 1)
2.7.2 Liposomal Delivery

See figure 8: HeLa, RCC4 or MDA-MB-231 cell lines were transfected with a Cas9 expressing plasmid (Edit-R Cas9 Expression Plasmids, hCMV promoter, puromycin resistant, GE Healthcare), crRNA as described in section 2.7.2 and trans-activating CRISPR RNA (tracrRNA) (Edit-R, GE Healthcare) according to protocol (Edit-R Cas9 Expression Plasmids and Synthetic RNAs Technical Manual). Two transfection reagents were trialled: DharmFECT Duo (GE Healthcare) and jetPRIME (Polyplus Transfection).

Figure 8: Example workflow – liposomal delivery of gene editing components (Dharmacon GE Healthcare)

2.7.3 Puromycin Kill Curve(s)

≥ 48 hours post transfection, cells were selected with puromycin (1000x (100 mg/ml)). Due to the survival of control HeLa cells, kill curves were determined to ensure the correct puromycin dosage. Alamar blue (Thermo Scientific) was used to estimate cell survival. Cells were plated in serial dilutions and exposed to 0, 2.5, 5, 7.5, 10 and 15 µg/mL of puromycin. 24-hours after the addition of puromycin, alamar blue reagent was added to wells and incubated for four hours, 37°C, away from direct light sources. Plates were then read in a VERSAmax microplate reader (Molecular Devices) at a wavelength of 570 and normalized to 600 nm. Reduction in absorbance indicates increased cell viability, based on reduction of Resazurin (Thermo Scientific – alamar blue). Results can be seen in section 3.6.
2.7.4 Transfection Efficiency

Plasmid transfection was confirmed using a fluorescent mKate 2 variant Cas9 expressing plasmid (GE Healthcare) (Excitation / Emission = 588/23 / 633 nm). Cells were transfected as described in 2.7.2, however no gRNA was inserted into the transfection medium. DharmaFECT Duo and jetPRIME transfection reagents were tested in order to determine the best reagent moving forwards.

Cells were analysed 24 hours post-transfection in order to minimalise any loss of fluorescent signal. Cells were detached with 150µL Accutase Solution (Biolegend) for 10 min, before the addition of 400 µL cold DMEM and kept on ice. Samples were then spun at 4°C, 285 g for 5 min. Supernatant was aspirated from the pellet, then the pellet was washed in 900 µL FACS buffer (see appendix 6.1). Cells were resuspended in 400 µL of FACS buffer and kept on ice before analysis.

Samples were run through a LSRFortessa X-20 (BD) Flow Cytometer and analysed by FlowJo software. Example sequential gating is shown in figure 9; fluorescence was measured using a YG610_20 filter.

2.7.5 Cell Sorting

Cells were transfected as described in 2.7.2, using DharmaFECT Duo reagent. 24 hours post transfection, cells were collected with 200µL accutase and resuspended in 300 µL DMEM. Samples were then run through a MoFlo Astrios EQ Cell Sorter, where cells with a higher fluorescence intensity were collected for further culturing. Cells were gated as described in 2.7.4, however due to differences in equipment, fluorescence was detected with a 561-614/20 filter.

Cells were collected in ~2 mL of DMEM, centrifuged for 15 min, 259 g, room temperature, then resuspended in the same media before plating into a 24-well plate.
2.7.6 Lentiviral Production and Delivery

2.7.6.1 Plasmid transformation and isolation

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>lentiCRISPR v2</td>
<td>Addgene (Plasmid #52961)</td>
</tr>
<tr>
<td>VSV.G envelope plasmid</td>
<td>Addgene (Plasmid #14888)</td>
</tr>
<tr>
<td>Δ8.14 packaging plasmid</td>
<td>Addgene (Plasmid #79047)</td>
</tr>
<tr>
<td>pUC18 control plasmid</td>
<td>Addgene (Plasmid #50004)</td>
</tr>
</tbody>
</table>

Table 4: Plasmids used throughout lentiviral CRISPR Cas9 protocol

Plasmids described in table 4 were transformed into XL1-blue competent bacterial cells (Agilent) using a heat shock method. 50 – 500 ng of plasmid was added to 30 µL XL1 cells and incubated on ice for 30 min. Samples were transferred to a 42°C water bath for 42 seconds then incubated on ice for 2 min. 700 µL of SOC media (see appendix 6.1) was added to each tube, and samples were incubated at 37°C for 1 hour with movement. Transformed bacteria were spread onto ampicillin resistant agar plates (see appendix 6.1) and incubated overnight at 37°C. Positive and negative controls were used at this point to ensure ampicillin selection results.

Single colonies were selected the next day, inoculated into 10mL LB broth and incubated at 37°C overnight with shaking (180-200 RPM). Samples were then stored in 4°C over the weekend before being grown in 50 mL LB broth for 6 hours prior to isolation (37°C with shaking).

Plasmids were re-isolated using the QIAGEN Plasmid Midi Kit according to manufacturer instructions and stored at -20°C.

2.7.6.2 Lentivirus assembly and harvest

Lentiviral particles were produced using HEK-293T cells as the packaging cell line; confluent 10cm² cell dishes were split 1:7 one day before transfection and spread evenly onto fresh culture plates. Three plasmids as described in 2.2.6.1 were transfected to each plate; 5 µg of DNA was transfected in total (2 µg lentiCRISPR, 1.5 µg VSV.G and 1.5 µg Δ8.14) and was made up to 50 mL with opti-MEM. The plasmid mixture was added to 15 µL Fugene transfection reagent (3 µL per µg DNA, Promega) in 35 µL opti-MEM, thus 100 µL of transfection mix was added to each plate containing 8 mL fresh opti-MEM.
1 plate of HEK-293T cells was transfected with GFP plasmid, VSV.G and Δ8.14 to act as a control and confirm that uptake of the plasmid into the packaging cell line was successful.

5 hours post-transfection, the transfection medium was removed and replaced with 10mL DMEM (with 10% FCS, 1% pen-strep). Viral particles were harvested and filtered (0.45 µM) 48 hours later, and stored in -80°C.

**2.7.6.3 Infection**

RCC4 cells were seeded at a density of 75,000 cells/well in a 6-well culture plate in DMEM supplemented with 10% FCS. 16 hours later, virus collected in 2.7.6.2 was thawed on ice before being warmed to 37°C for 2-3 min. Media was aspirated from wells and replaced with 1 mL of CRISPR or GFP lentivirus. Successful lentiviral infection was confirmed by fluorescent microscopy of GFP infected cells. CRISPR infected cells were selected with puromycin 48 hours later, though due to expected cell death, half of the infected cells were kept for western blot analysis. No Cas9 was detected (results not shown).

**2.8 Co-Immunoprecipitation Assay**

Two confluent 10cm² plates of HeLa, RCC4 and MDA-MB-231 cells were washed with ice-cold PBS and lysed with 500 µL 1x Cell Lysis Buffer (Cell Signalling Technologies, #9803) supplemented with 1 mM PMSF protease inhibitor mix (Cell Signalling Technologies, #8553). Cells were scraped and both plates of each cell line pooled together before filtration with a 25 g needle. Samples were spun at 14,000 g for 10 min at 4°C, half of each supernatant was frozen in -80°C whilst the other half was taken forward to pre-clearing.

Lysates were pre-cleared for non-specific binding to beads with 2 µg normal rabbit IgG (Cell Signalling Technology, #2729) incubated at 4°C for 1-4 hours, 25 RPM. IgG was pulled down with 20 µL of pre-washed bead slurry (Protein A Agarose Beads, Cell Signalling Technologies, #9863), incubated at RT for 30 min, 25 RPM. Beads were pelleted at 8000 g for 1 min at RT and supernatant was carried forward for TRBP co-immunoprecipitation.

Supernatant for each cell line was split into 3 separate tubes (200 µL per tube), one aliquot was frozen in -80°C and is henceforth referred to as ‘input’. 3 µg of TRBP or
IgG primary antibody was added to the remaining cell lysates and incubated with gentle rotation (25 RPM) overnight at 4°C.

The next day, cell lysates were added to 20 µL bead slurry and incubated at 4°C, 25 RPM for 2.5 hours. Beads were then pelleted at 8000 g for 1 min and washed 5x with 1X Cell Lysis Buffer before resuspension in 20 µL sample buffer (see appendix 6.1). Sample buffer was also added to 5% input (10 µL) and all samples were denatured for 5 min at 95°C. Analysis was completed by western blot (10% gel), with the addition of a confirmation antibody (Cell Signalling Technologies, #3678, 1:2000 in 5% milk, 0.1% TBST) used between primary antibody incubations and secondary HRP antibody incubation. This was incubated at RT for 1 hour.

2.9 Statistics

Statistical analysis was completed using GraphPad Prism 6 software. Specific statistical analysis is indicated upon each graph legend.
3. Results

3.1 DICER co-factors TRBP and PACT do not regulate one another

Previous findings in literature suggest that TRBP and PACT do not regulate or compensate for one another (Kim et al., 2014). However, these results originate from stable loss of function, whereas within this project, we assess transient protein knockdown. Therefore, we explore the possibility that upon knockdown, cells do not have time to compensate for loss of function, and therefore we may see a regulatory or compensatory effect upon TRBP or PACT knockdown.

Messenger RNA and protein levels were assessed 48 hours post transfection of siRNA against TRBP and/or PACT. Primers used in qPCR assays were confirmed to amplify only the intended target through melt curve analysis, with efficiency assessed via standard curve as shown in figure 10. We confirm downregulation of target mRNA in figure 11, within HeLa (figure 11a), RCC4 (11b) and MDA-MB-231 (11c) cell lines, and observe no consistent regulatory effects. We identified a significant upregulation of PACT upon knockdown of TRBP in MDA231 cells, though this is less than a 0.5-fold change and not observed across any other cell line.

Figure 10: Geometric efficiency assessment of custom primer amplification

<table>
<thead>
<tr>
<th></th>
<th>TRBP</th>
<th>PACT</th>
<th>PD-L1</th>
<th>DICER</th>
<th>GAPDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplification Factor</td>
<td>2.03</td>
<td>2.05</td>
<td>2.04</td>
<td>1.98</td>
<td>2.01</td>
</tr>
<tr>
<td>Efficiency</td>
<td>102.69%</td>
<td>104.88%</td>
<td>104.02%</td>
<td>97.94%</td>
<td>100.58%</td>
</tr>
</tbody>
</table>
TRBP has been observed to ensure efficient DICER processing in vitro (Fareh et al., 2016). Therefore, upon depletion of TRBP (and/or PACT), if TRBP (and/or PACT) has a measurable contribution to DICER processing, you could expect to see either loss of function or some compensation. One possible mechanism might be an upregulation in DICER levels to compensate for reduced processing activity of the enzyme in the absence of its co-factor TRBP. We observe this trend in HeLa cells, but not in the renal or breast cancer lines and thus are unable to conclude if this is a compensatory effect.

Upon transfection of siRNA against DICER, we confirmed significant reduction of DICER mRNA (11.d), and observed limited trends of regulation between DICER and its co-factors across the three cancer lines: upon dual knockdown of TRBP and PACT an increase in DICER mRNA is seen for HeLa, RCC4 and MDA-MB-231 cells, though this is only significant in HeLa cells.

Figure 12 demonstrates successful reduction of TRBP and PACT protein in appropriate RNAi samples. Multiple bands are present for TRBP probes, indicating various states of protein phosphorylation (phosphositeplus.org). In RCC4 and MDA-MB-231 lines, a mild upregulation of TRBP is observed upon knockdown of PACT.
However, as evident from the representative western blots (figure 12a, figure 18) and biological replicates indicated by quantification (12c, 12d), this was not a unanimous trend and could merely be natural variance between samples. Alternately, this could be a knock-on effect from miRNA dysregulation, as this trend was not observed in mRNA levels (figure 11).

3.2 Individual knockdown of TRBP or PACT does not affect microRNA abundance

Four microRNAs were chosen for analysis upon knockdown of TRBP and PACT. MicroRNA (miR) 21 was identified as a ubiquitous miRNA in cancer cells, whereas miR-16 is deleted in some cancers. (Buscaglia and Li, 2011, Calin et al., 2002). Here we show that miR's 16 and 21 were consistently more abundant than the snRNA U6 in all cell lines utilised in this project (see figure 16), suggesting these are ubiquitously expressed in HeLa, RCC4 and MDA-MB-231 cell lines. These have been utilised as markers of disruption to canonical miRNA biogenesis.
MiR-155 was investigated owing to its roles as a marker of inflammation (reviewed by Tili et al., 2009) and regulator of the checkpoint protein PD-L1 (Yee et al., 2017), where it has been identified to directly target the 3’ UTR of PD-L1. MiR-221 was chosen because it is overexpressed and promotes tumorigenesis in triple negative breast cancer lines, such as the MDA231 line (Nassirpour et al., 2013). This is in comparison to ‘normal’ breast tissue; within this study we found miR-221 to be lowly expressed in MDA231 cells, though we did not have a healthy control for contrast.

Independent depletion of TRBP or PACT had no effect on the abundance of ubiquitous microRNAs (figure 14). Curiously, siDICER samples also presented with no reduction of canonical miRNAs.
Figure 14: MicroRNAs 16 (a, e, i), 21 (b, f, j), 155, (c, g, k) and 221 (d, h, l) were assessed by RT-qPCR in HeLa (a, b, c, d), RCC4 (e, f, g, h) and MDA-MB-231 (i, j, k, l) cell lines after RNAi treatment against TRBP, PACT or DICER. One-way analysis of variance was conducted with Dunnett’s multiple comparison test, with no significant results. N = 3 independent samples.
3.3 Depletion of both TRBP and PACT leads to downregulation of ubiquitous microRNAs

TRBP and PACT may function synergistically: when both DICER co-factors were depleted, a common trend emerged. Both selected ubiquitous miRNAs were downregulated across all three cell lines, with the most significant and exaggerated reduction observed in MDA-MB-231 cells (figure 15). This may suggest that although TRBP and PACT do not regulate one another on a protein or mRNA level, they may compensate for one another in certain functions.

It is already established that TRBP and PACT interact physically in vitro (Singh et al., 2011) and can form homodimers and heterodimers with each other (Heyam et al., 2017, Laraki et al., 2008). We predicted that TRBP and PACT also interact within a natural cellular environment, and that levels of interaction may vary between these cell types. Heterodimer interactions could indicate binding for protein stability, functional regulation or joint functionality. This is explored in section 3.5 and discussed in section 4.

Figure 15: miR-16 and miR-21 were assessed by RT-qPCR in HeLa, RCC4 and MDA-MB-231 cells 48 hours post-transfection with siRNA against TRBP and PACT. Statistical analysis by unpaired Student’s t test, *, p <0.05, ***, p = 0.0005, ****, p <0.0001. n = 3 independent samples
MiR-155 and miR-221 did not exhibit consistent downregulation upon depletion of TRBP and/or PACT (figure 16). With one exception (miR-155, RCC4), these microRNAs are lowly expressed in the tested cancerous cell lines, as shown by figure 13. Disruption to biogenesis may be less evident in lowly expressed miRs due to their longevity. In general, microRNAs are stable transcripts, with longevity lasting from a few hours to several weeks (reviewed by Zhang et al., 2013). Lowly expressed miRs are less likely to be processed, or exhibit lower levels of processing from pre-miRNA to mature complexes, during the 48-hour experimentation period, in comparison to highly expressed miRs. Thus, disruption to their biogenesis may be less evident.

In fact, low expression levels may reduce the reliability of these results due to sensitivity of software during the latter qPCR cycles. In contrast to HeLa and MDA231 cells, miR-155 is highly expressed in RCC4 cell lines, and again we see a similar trend of downregulation as noted for miR-16 and miR-21. This further supports the idea that TRBP and PACT can compensate for one another in microRNA processing efficiency.

Figure 16: miR-155 and miR-221 were assessed via RT-qPCR 48 hours post-transfection with siRNA against TRBP and PACT. Statistical analysis by unpaired Student’s t test, *, p = 0.0111, **, p = 0.0061 n = 3 independent samples.
3.4 PACT depletion leads to upregulation of PD-L1 in breast cancer cells

In order to explore links between microRNA biogenesis and immunity, PD-L1 abundance was also measured after knockdown of the DICER co-factors in HeLa, RCC4 and MDA231 cell lines. Change in PD-L1 mRNA was measured by RT-qPCR (figure 17) and PD-L1 protein by western blot (figures 18 and 19).

No significant changes in steady-state levels of PD-L1 mRNA was identified, though a trend of upregulation can be seen in HeLa samples upon treatment with siTRBP and siPACT (figure 18). However, PD-L1 mRNA is very lowly expressed in HeLa cells, and even with this upregulation, no protein can be detected (figure 18a) due to the very low levels of mRNA present (shown by ΔCT values, figure 17b).
Figure 17: mRNA levels were measured by RT-qPCR 48 hours post-transfection with interfering RNA. (a,c,e) mRNA expression fold change, analysed by the comparative CT method as described in 2.5.2. (b,d,f) CT values relative to GAPDH. One-way analysis of variance with Dunnett’s multiple comparisons test result in no significant findings.
Initial western blot results identified a 2-3-fold upregulation of PD-L1 upon knockdown of PACT but not TRBP in MDA-MB-231 cells (figure 18). Though statistical testing identifies a more significant upregulation of PD-L1 upon knockdown of both TRBP and PACT, these results overlap with biological replicate experiments of sole PACT depletion, and thus the results seen are likely due to reduction of PACT alone. Multiple bands are present for PD-L1 and indicate post-translational modifications such as glycosylation of PD-L1 protein (Li et al., 2016).

It was proposed that stimulation with interferon gamma (IFN-γ) could enhance or exaggerate this result, by upregulating PD-L1. STAT1 is phosphorylated to phospho-STAT1 (PSTAT1) upstream of PD-L1 during stimulation with IFN-γ (Garcia-Diaz et al., 2017). These were both measured by western blot after TRBP and PACT knockdown to identify whether changes in PD-L1 expression were due to upstream effects upon the IFN pathway.

As shown in figure 19, without stimulation we observe a mild upregulation of STAT1 in RCC4 cells upon dual knockdown of TRBP and PACT. This trend is also observed in MDA-MB-231 cells, however this is not statistically significant. Across both cell lines, this upregulation is mild and variable, ranging from 1.4 (RCC4, 18b) to 3 (MDA231, not on representative blot). Independent knockdown of PACT results in upregulation of STAT1 in MDA231 cells. This is discussed later in section 4.

Upon stimulation with IFN-γ, we observe the expected upregulation of STAT1, PSTAT1 and PD-L1, shown in figure 18. No reproducible effects upon PD-L1 were observed in these cell lines when treated with siRNAs and IFN-γ; interferon stimulation eclipses any previously observed results. Therefore, we can propose that neither TRBP or PACT are directly involved in the cellular pathways from IFN-γ stimulation through to PD-L1 upregulation.

Of note, we again observe cell line variability between the cancer lines utilised. Intriguingly, it is the MDA-MB-231 cell line that has exhibited the most pronounced effects of TRBP or PACT depletion related to both microRNA abundance and PD-L1 upregulation. This is explored further in section 3.5.
Figure 18: Western blot analysis of Non-Stimulated (NS) and IFN-γ treated HeLa (a), RCC4 (b) and MDA-MB-231 cells. N = non-targeting control, T = siTRBP, P = siPACT, TP = siTRBP and siPACT
3.5 TRBP and PACT interact endogenously

Cell line specific variation was observed upon knockdown of TRBP and PACT on both microRNA and PD-L1 expression, with the MDA231 breast cancer line exhibiting more pronounced effects than the HeLa or RCC4 lines. Reduction of ubiquitous miRNAs was only apparent upon dual knockdown of both DICER co-factors suggesting that these two proteins may compensate functionally.
TRBP and PACT have already been shown to interact in vitro (Singh et al., 2011, Kok et al., 2007). We hypothesised that they also interact endogenously in these cells and that this interaction may vary dependant on cell type. A higher level of interaction would be expected to reduce the abundance of protein available for other cellular pathways such as microRNA biogenesis, hence depletion of said protein may then have more pronounced effects on these pathways. In accordance with these results and hypothesis, we expected a higher interaction of TRBP and PACT in the MDA231 cell line and the lowest interaction in the RCC4 cell line.

To test this hypothesis, an endogenous co-immunoprecipitation (co-IP) assay was conducted. TRBP was immunoprecipitated from each cell line and analysed by western blot (figure 20). It is confirmed that TRBP and PACT do interact endogenously in human cell lines.

![Western blot analysis of TRBP co-immunoprecipitation assay](image)

**Figure 20:** (a) Western blot analysis of TRBP co-immunoprecipitation assay. 2.5% I = 2.5% input, IgG = IgG immunoprecipitation (IP), T = TRBP IP. (b) Western blot quantification of immunoprecipitated TRBP and PACT, assessed as a % of input into the Co-IP assay.

<table>
<thead>
<tr>
<th>Protein</th>
<th>% of input</th>
<th>RCC4</th>
<th>MDA-MB-231</th>
<th>HeLa</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRBP</td>
<td>17.54</td>
<td>9.14</td>
<td>3.65</td>
<td></td>
</tr>
<tr>
<td>PACT</td>
<td>12.40</td>
<td>6.75</td>
<td>10.15</td>
<td></td>
</tr>
<tr>
<td>Ratio (TRBP:PACT)</td>
<td>1.41:1</td>
<td>1.35:1</td>
<td>0.36:1</td>
<td></td>
</tr>
</tbody>
</table>

Enrichment of PACT compared to 2.5% of the assay input is evident for all three cell lines, meaning that more than 2.5% of cellular PACT was pulled down. This was quantified using ImageJ software for an estimation of protein pulldown and displayed...
as a percentage of the total input in figure 20. MDA-MB-231 presented with the lowest level of PACT pulldown, whilst RCC4 displayed the highest level, contrary to predictions.

No negative control was used for this experiment. In future, a probe should be used that is not expected to co-immunoprecipitate with the protein of interest.

3.6 CRISPR / CAS9 Editing

This project utilised transient knockdown to assess protein function, however the gold standard for assessing endogenous gene and protein function is to produce a stable gene knockout cell line for analysis. This is because there will be residual levels of protein during transient knockdown and often the abundance of protein required for individual cellular functions is unknown.

To this extent, we utilised gene editing technology to begin the process of producing TRBP and PACT knockout cell lines. Another aim of using this technology was to enable comparison of transient vs stable depletion of protein within the same project, where it would be expected that stable knockout would complement studies already present in literature.

HeLa and RCC4 cells were initially transfected with genome editing components described in 2.7 as per protocol. One batch of HeLa cells displayed resistance to puromycin (figure 21); these were discontinued, and a fresh stock that were not resistant to puromycin were taken from cryogenic storage. A puromycin concentration of 2.5µg/mL was sufficient to kill HeLa, RCC4 and MDA-MB-231 cells at both high and low cell densities (figure 21).
Figure 21: Alamar blue cell-based survival assay of HeLa, RCC4 and MDA-MB-231 cells following exposure to puromycin titrations (0 to 15 µg/mL). A baseline absorbency reading is indicated on each kill curve.

Due to complete cell death post-transfection of genome editing components and after selection with appropriate concentrations of puromycin, HeLa and RCC4 cells were instead transfected with an MKate2 fluorescent Cas9 expressing plasmid, to ensure that transfection was taking place successfully.

A shift in fluorescence was observed between each control and test sample (figure 22), suggesting that transfection was successful. These results were quantified, using counts of individual cells that presented with an increased level of fluorescence over those that fell within the gate of the control samples (figure 23). This in turn was compared with cell survival, estimated from sequential gating described in 2.7.4. It was observed that Jetprime transfection reagent resulted in a higher transfection efficiency than Dharmacon transfection reagents, however Jetprime reagents presented with a higher level of cell death.

15 to 30% transfection efficiency (figure 23) was lower than expected. Transfection optimisation should have been conducted with positive controls to obtain the highest genomic editing with >70% post-transfection cell viability as recommended.
(Dharmacon Edit-R CRISPR-Cas9). Low transfection efficiency could be a result of improper cell confluency, or plasmid / gRNA to transfection reagent ratio.
Figure 22: HeLa and RCC4 cells were transfected with a far-red fluorescent mKate2 plasmid expressing Cas9, using DharmaFECT Duo transfection reagent or Jetprime transfection reagent. Sequential gating based on FFS and SSC as described in figure 9 (section 2.7.4) was used to determine live, single cell populations, where fluorescence was measured with the YG610_20 filter (excitation/emission for mKate2 = 588/633 nm). Control samples without plasmid were used to set gates to determine shifts in fluorescence.
Figure 23: (a) Transfection efficiency of a MKate 2 far red fluorescent Cas9 plasmid utilising Dharmacon or Jetprime transfection mediums. Efficiencies based on percentage of live cells with an increased fluorescence intensity. (b) Cell survival (% Live cells) 24 hrs post-transfection with an MKate 2 plasmid for HeLa and RCC4 cell lines

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total Cells Sorted</th>
<th>mKate Positive Cells (Count)</th>
<th>mKate Positive Cells (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa, Cas9 only</td>
<td>172,520</td>
<td>329</td>
<td>0.19</td>
</tr>
<tr>
<td>HeLa, Cas9 + gRNA against TRBP</td>
<td>192,199</td>
<td>811</td>
<td>0.42</td>
</tr>
<tr>
<td>HeLa, Cas9 + gRNA against PACT</td>
<td>3556</td>
<td>203</td>
<td>5.71</td>
</tr>
</tbody>
</table>

Table 5: Cells transfected with a fluorescent mKate2 plasmid expressing Cas9 were suspended in DMEM and sorted using a MoFlo Cell Sorter. Populations were sequentially gated into live singlets as described in figure 9 (section 2.8.4), and fluorescence measured with a 561-614/20 filter. A baseline was set using a control sample with no plasmid; only cells positive for mKate were sorted and cultured.
However, due to time restraints, upon successful (but low efficiency) transfection with the fluorescent MKate 2 plasmid, HeLa cells were again transfected and run through a MoFlo Astrios EQ Cell Sorter with intent to culture the cells with enhanced fluorescence. Owing to differences in filters between the LSRFortessa X-20 (BD) Flow Cytometer and MoFlo Astrios EQ Cell Sorter (see section 2.7.4), the enhanced fluorescence was not easily detectable, and of the cells isolated for re-culturing (table 5), none survived. Cells were checked on the LSRFortessa on the same day, showing stable protein expression of the fluorescent plasmid (results not collected).

As a control, cells were transfected using the MDA-MB-231 cell line and harvested for western blot analysis before puromycin selection to assess whether any reduction in target protein was achieved. CAS9 was also probed. Figure 24 confirms that no identifiable reduction in TRBP or PACT was present as compared to the CAS9 control lane. No CAS9 protein could be identified in any sample, however it would have been ideal to run a positive control to ensure correct exposures of the membranes; though the antibody was confirmed to recognise Cas9 by other members of the laboratory.

![Western blot analysis of MDA-MB-231 cells transfected with (C) Cas9 plasmid, (T) Cas9 + gRNA against TRBP, (P) Cas9 + gRNA against PACT](image)

Due to continued cell death after puromycin selection and no confirmation in protein reduction upon liposomal transfection, an alternative method of CRISPR editing was attempted. It was hypothesised that although transfection of the Puro and MKate2 plasmids were successful, the promoters on the plasmid were not successfully
initiating transcription. Therefore, a lentiCRISPR system was trialled as this would incorporate CAS9 into the host genome.

A GFP plasmid was utilised alongside a lentiCRISPR plasmid in order to confirm successful packaging of the virus and successful infection of chosen cell lines. GFP was successfully expressed in control samples (results not shown), but no Cas9 was detectable upon analysis of edited cells, and no edited cell survived puromycin selection.

The lentiCRISPR V2 (Addgene plasmid #52961) has been fully sequenced and documented in literature, firstly by the depositing lab (Sanjana et al., 2014) and more recently (Zhang et al., 2019; Yuan et al., 2019). It is likely that no detectable Cas9 in this circumstance was due to poor detection of this protein, or unsuccessful plasmid transfection / packaging, owing to differing properties such as size (14.8 kb) to the control GFP plasmid.
4. Discussion

This project aimed to investigate the role(s) of TRBP and PACT within microRNA biogenesis by performing transient knockdown and stable gene knockout experiments within human cancer cells. We also aimed to investigate potential crosstalk between miRNA machinery and immunity through investigation of the immune checkpoint protein PD-L1.

To fulfil these objectives, we successfully performed RNAi experimentations to deplete TRBP and PACT individually and concurrently. We expanded on previous cell work by inclusion of three different cancer cell lines, however were unable to provide comparisons between transient and stable depletion of proteins due to technical difficulties and time constraints.

4.1 Depletion of TRBP and PACT is required for suppression of miRNA expression in cancer cells

We identified a novel effect on abundance of highly expressed microRNAs after depletion of TRBP and PACT in three different cancer cell lines. These included miR-16 and miR-21 within HeLa, RCC4 and MDA-MB-231 lines, miR-155 within RCC4 lines and miR-221 in HeLa cell lines. Only upon depletion of both proteins was disruption to mature miRNA abundance observed, a finding not previously identified in cellular studies. We present the possibility that TRBP and PACT act synergistically on regulating the levels of specific miRNAs, though how conserved this mechanism is at the global level is still unknown and requires further study.

TRBP and PACT are structural homologs: both bind DICER with their third domain, both have unstructured linker regions allowing movement between domains, and both domains one and two bind RNA (Heyam et al., 2015). These qualities grant both proteins the ability the ensure efficient DICER processing in line with the theory presented by Fareh et al. (2016), whereupon these dsRBDs bind RNA first and then present substrates to DICER in an optimum orientation. This prevents full loading of unrecognised substrates onto DICER, increasing processing efficiency.

Fareh et al. (2016) did not investigate PACT’s ability to alter DICER processing during reconstitution experimentation with TRBP. However, Kim et al. (2014) report TRBP, PACT and double knockout HeLa cell lines, upon which no change in microRNA abundance was observed. This leaves ambiguity: it has been demonstrated that TRBP
has the ability to aid DICER’s ability to distinguish pre-miRNAs from other RNAs, though the effects of this are not demonstrated in a stable knockout cell line. A similar occurrence could be found for PACT, but as yet it is uncertain is PACT has the functional capacity for ensuring efficient DICER processing.

Kim et al. (2014) suggest that TRBP and PACT do not compensate for one another. Based on the findings of this project, we propose that TRBP and PACT may compensate functionally in the loading of miRNA to/from DICER. It is acknowledged that this may not be evident in a genome-edited, stable knockout of TRBP and/or PACT.

There is potential that the observed reduction in mature miRNAs within this project, upon dual knockdown, could be due to disruption to both DICER efficiency and strand selection, including unloading of miRNA from DICER and preferential loading onto AGO.

Both TRBP and PACT can influence strand selection and loading of miRs from DICER to Argonaute (Wilson et al., 2015; Noland and Doudna, 2013). The mechanism of this action, utilising the flexibility of unstructured linker regions between domains (Heyam et al., 2015), may also suggest that these proteins have the potential to boost efficiency in moving miRs from DICER to AGO.

MiRNAs are tightly associated with RISC complexes, with less than 3% of total miRNAs ‘free’ within the cytoplasm (Tang et al., 2008). It is therefore argued that a reduction in loading efficiency of miRNA onto Argonaute protein could also result in a reduction of ubiquitously expressed miRNAs. This may be due to ‘free’ or unbound miRNA being more available for degradation than those in complex with RISC. Therefore, disruption to both DICER processing efficiency and loading of argonaute could contribute to the reduction of ubiquitous miRNAs upon dual depletion of TRBP and PACT.

However, using the data generated in this project, the most likely explanation for this reduction is that TRBP and PACT are redundant in the specific function of ensuring efficient DICER processing. This is not to say that they are redundant proteins overall, as it is already known that they have opposite effects on PKR (Singh et al., 2011) and only TRBP can alter bulge mismatches in pre-miRNA stems prior to DICER cleavage (Zhu et al., 2018). If this theory is correct, it could be that Homo sapiens have two
dsRBDs that can assist DICER cleavage of pre-miRNAs as these will produce mature miRNAs of different seed sequences and length, increasing diversity of mature miRNAs without the requirement of further transcripts from the nucleus.

To elucidate the exact mechanisms of the synergistic downregulation of highly expressed miRs observed, we propose in vitro reconstitutions similar to that of Fareh et al. to determine if PACT has the capability to help DICER differentiate between cellular RNAs. Additionally, we propose in vitro reconstitution with DICER and Argonaute proteins, to determine whether TRBP and/or PACT assist strand loading onto Argonaute. This could be assessed through qPCR analysis following precipitation of protein-miRNA complexes similar to that described by Queiroz et al., 2018. Furthermore, it would be beneficial to produce double knockout cell lines, to assess whether stable depletion of TRBP and PACT together result in dysregulation of microRNA biogenesis, or whether cells are indeed able to compensate for this loss of function over time. This would be compared to further knockdown experiments for a larger array of microRNAs, and could be conducted via deep sequencing or extensive qPCR experiments. DICER knockdown and knockouts would be completed in parallel.

Overall, we disagree with Kim et al. (2014) that PACT does not play a role in miRNA biogenesis in HeLa cells and instead state that both TRBP and PACT have roles in the biogenesis pathway, at least in the three cancer lines utilised for this project. Our results identify a new finding in miRNA biogenesis, previously unseen due to dual depletion being less widely reported in literature. Further to this, when dual depletion is observed, this is through a stable gene knockout, where we are currently unsure if cells have time to compensate for functional loss.

A major limitation of this project is the lack of comparison of TRBP and PACT protein depletion to DICER protein depletion. Unfortunately, due to technical difficulties, we were unable to successfully detect DICER via western blot, though we were able to measure mRNA. Comparison to DICER knockdown would allow us to make more accurate predictions on the mechanistic effects of the DICER co-factors depletion, and whether these reflect reduced DICER processing or if there are further mechanisms of action in place. Though we were able to see a reduction of DICER mRNA after treatment with siRNA, we do not see any effects on highly expressed
canonical microRNAs. This suggests that DICER was not sufficiently reduced as to impair function.

However, this may not be the only explanation for this observation. MicroRNAs are inherently stable from a few hours to a few weeks (review by Zhang et al., 2013). Knockdown experiments over 48 hours may not constitute enough time for the measured microRNAs to diminish significantly due to lack of further biogenesis. However, this argument would suggest that dual TRBP and PACT depletion was somehow reducing microRNA levels rather than disrupting the formation of new mature RNA strands. Therefore, we suggest the most likely option that DICER depletion was not sufficient.

4.2 TRBP and PACT interact endogenously

During this project, we hypothesised that TRBP and PACT interact endogenously in human cell lines; interaction has already been identified in vitro and with transfected tagged proteins in mammalian cells (Singh et al., 2011; Laraki et al., 2008). We hypothesised that interaction may vary between different cell lines and cell types.

We confirm that TRBP and PACT bind one another in human cell lines and suggest that these complexes form heterodimers. This is because both TRBP and PACT interact with other proteins via the same domain, particularly binding DICER and each other with the same interface (Heyam et al., 2017). Binding of DICER is through mutual exclusion, meaning that both TRBP and PACT cannot be in complex with DICER at one time (Wilson et al., 2015). These two papers are in disagreement with an earlier study by Kok et al., (2007), which suggested that human TRBP, PACT and DICER and form a ternary complex. Data provided by Kok et al., (2007), in the form of immunoprecipitation assays, does not confirm that all three proteins are in one complex, but instead that they can all interact, and this may be by mutual exclusion as suggested by Wilson et al., (2015).

However, this does not exclude the possibility that both TRBP and PACT could simultaneously bind further proteins or substrates with multiple binding sites, such as RNA. TRBP domains 1 and 2 can simultaneously bind one siRNA strand at different sites (Masliah et al., 2018). With the similar structure and sequence of TRBP and PACT domains 1 and 2, this may suggest that TRBP and PACT could mutually bind a singular RNA, though this would require experimental confirmation. This also opens
up the possibility that complex interactions, such as that with PKR could also have an intermediary substrate such as RNA.

TRBP and PACT have previously been identified to form heterodimers when transfected within HeLa cells (Laraki et al., 2008); we confirm that this happens naturally without overexpression of either co-factor or adjustment of PKR pathways.

Protein-protein interactions occur for a variety of reasons including to enable performance of a function, to regulate another protein and for stability. There is potential that TRBP and PACT could interact for any of the above reasons.

Contrary to expectations proposed in 3.5, MDA-MB-231 presented with the lowest level of PACT co-IP, whilst RCC4 displayed the highest level. This could suggest that cells with a higher interaction have a surplus of protein: both TRBP and PACT bind DICER, with TRBP demonstrating enhanced levels of DICER binding over PACT (Wilson et al., 2015). If TRBP and PACT have a higher level of interaction over TRBP-DICER, it could be due to a surplus of requirement in the cell, i.e. the co-factors are not functionally required and have bound each other for stability. This theory would support our results, indicating that cells with surplus of DICER co-factors show less disruption to miRNA biogenesis during depletion of TRBP and PACT.

However, if we factor in the percentage IP of TRBP, levels of interaction between TRBP and PACT are actually very similar between the renal and breast cancer cell lines. For that reason, it is unlikely that the mild variation in miRNA and PD-L1 expression observed between cell lines upon depletion of TRBP and PACT are due to the molecular interactions of the DICER co-factors. We therefore conclude that this assay indicates no significant differences in the interaction of TRBP and PACT between the different cell lines.

Previous literature suggests that TRBP and PACT may interact as a mechanism of regulation. For example, binding of PACT to TRBP reduces association with PKR, and by weakening this interaction, PACT’s ability to activate PKR is significantly enhanced (Singh et al., 2011). There is potential that although TRBP and PACT do not regulate each other on a protein level, they may regulate each other’s activity across distinct pathways such as the stress response – i.e. where TRBP may bind PACT to prevent activation of PKR. This is not something we observed in this project, however
we would not expect to see a change in protein levels in this circumstance, unless PACT was overexpressed and TRBP may become upregulated to combat this.

The co-immunoprecipitation itself was limited in interpretation due to several drawbacks. This includes ‘miscellaneous’ black dots present after probing for TRBP, which hindered quantification of samples. Further to this we did not achieve high levels of TRBP pulldown, with the highest precipitation predicted to be around 18% of the input. Suggestions for improvement here could involve using antibody and bead slurry in excess and increasing incubation periods to ensure maximum binding events.

Finally, although a confirmation antibody was used between primary and secondary antibodies, we still observed bands for the heavy and light chains of the IgG and TRBP antibodies used to pull down the respective proteins. A conformation antibody will be of a different species to the original antibody used to pull down protein, in order to reduce the noise of heavy and light chains remaining in the precipitate. The heavy chain of these antibodies may be partially obscuring some of the TRBP band, and again hindered quantification. Ideally, this assay would be further optimised and then repeated to ensure reproducibility.

In summary, we identify endogenous interactions between TRBP and PACT across three human cancer cell lines but cannot confirm if interaction differs between these. We suggest that TRBP and PACT may interact for protein stability, regulation, or dual functionality, though the exact purpose remains unclear at the current time.

4.3 Depletion of PACT within breast cancer cells results in upregulation of PD-L1

In section 1 it is suggested that dysregulation of microRNA biogenesis may be linked to immunity through dsRNA stress; PKR is activated by median levels of double-stranded RNA and PACT, but inhibited by high levels of double-stranded RNA and TRBP. Upon dsRNA stimulation, such as might be caused by dysregulated microRNA biogenesis, PKR is activated and may lead to apoptosis through translational inhibition (Gil and Estabon, 2000). We suggested that dysregulation to miRNA biogenesis through disruption of co-factors TRBP and PACT may result in dsRNA stress, PKR activation and increased PD-L1 expression.

Upon depletion of PACT within breast cancer cells, we observed, on average, a 2-fold increase in PD-L1 expression. Reduction of TRBP did not affect PD-L1 expression and
depletion of both DICER co-factors did not illicit an enhanced response. Therefore, it is suggested that PD-L1 upregulation is not in response to dsRNA stress caused by a reduction in miRNA processing as first hypothesised. Rather, we propose cross-talk between miRNA biogenesis and the stress response through PACTs interaction with PKR, and thus a novel immunological function of PACT.

PACT is an activator of PKR, which actively self phosphorylates upon stimulation. In turn, activated PKR can phosphorylate eIF2α; phosphorylation of eIF2α inhibits general protein translation (Krishnamoorthy et al., 2001; Deng et al., 2004; Hershey et al., 2012, Singh and Patel, 2012). Therefore, it is logical to suggest that depletion of PACT may reduce phosphorylation of PKR, eIF2α and reduce the regulation of translation (depicted schematically in figure 24).

A reduction in translation regulation may lead to increased rates of translation, hence an increase in protein production as seen for PD-L1 and STAT1 in MDA231 cell lines. However, this would suggest a general upregulation in protein, and analysis was normalised to housekeeping proteins such as GAPDH and β-actin. Therefore, what we are seeing is either only an upregulation of STAT1 and PD-L1, or a greater upregulation of these two proteins in relation to β-actin.

To test this theory, activation of PKR could be assessed via western blot, probing for phosphorylated-PKR against total PKR. Phosphorylation of eIF2α could also be assessed to this extent, with the option of conducting plate-based assays utilising antibodies against total and phosphorylated eIF2α in the same step (example: 64EF2PEG Phospho-EIF2 alpha (Ser52) cellular kit by Cisbio, link in appendix 6.2).
It should be noted here that PKR and STAT1 also have direct interactions, though PKR does not actively phosphorylate STAT1 (Wong et al., 1997) and does not need to be activated in order to bind. Interaction occurs with the dsRBD on PKR (Wong et al., 1997), the same surface that binds with PACT, and leads to prevention of STAT1 phosphorylation (Ramana et al., 2000). Depletion of PACT reduces PKR-PACT interaction, increasing the likelihood of PKR-STAT1 interaction, preventing phosphorylation to PSTAT1. This may contribute to the upregulation of STAT1 observed in this project upon PACT depletion in MDA231 cells.

Limitations of this finding include establishing if PD-L1 upregulation is functionally significant. Targeting of mRNA transcripts may not directly affect mRNA abundance, as binding of the RISC may result in prevention of translation rather than mRNA cleavage (see section 1.1). Though from this study we can say that PD-L1 protein is upregulated upon transient knockdown of PACT in MDA-MB-231 cells, we cannot certify that this is functionally significant, i.e. whether this would affect T cell activation or other cellular interactions. Confirmation of functionality could be assessed by flow cytometry, i.e. assessing that increased levels of protein are indeed being expressed on the cell surface. Alternatively, we suggest co-culturing PACT depleted cells with activated T cells and measuring sustained T cell activation through an IL-2 ELISA as previously demonstrated by Yee (2018). IL-2 is a
proinflammatory interleukin released by T cells to promote propagation and an inflammatory response (Hoyer et al., 2008).

At the current time, the role of PACT in PD-L1 regulation remains uncertain. We only identify a significant change in breast cancer cells, with the possibility that this could be specific to this cell type. We propose expanding the current results through depletion or overexpression of PKR to determine if change in PD-L1 expression is via the stress response or other unknown pathway.

Alternatively, if this line of enquiry yields no results, it is possible that depletion of PACT alone can lead to downregulation of specific miRNAs, as yet unknown, that can regulate both PD-L1 and/or STAT1. This could be investigated through miRNA sequencing, after PACT knockdown and/or knockout.

### 4.4 Future Work

In summary, to progress from these findings, we propose confirmation of PACT’s ability to aid loading of substrates to DICER. We also suggest comparison of transient knockdown to stable knockout of both TRBP and PACT in a variety of different cell types, in order to investigate if cells compensate for the loss of co-factors over time. Finally, we propose a parallel analysis with DICER depletion, to identify if depletion of TRBP and PACT are a reflection of DICER processing disruption.

In regard to the role of PACT in PD-L1 expression, we propose that further breast cancer cell lines could be examined in collaboration with PKR depletion or overexpression experiments.
5. Conclusion

This project aimed to investigate the dsRBPs TRBP and PACT in relation to their roles in microRNA biogenesis and novel functions within immunity. We conclude that both TRBP and PACT participate in the regulation of mature microRNAs contrary to previous publications. We reveal a novel finding, that only upon depletion of both DICER co-factors is abundance of selected mature microRNAs significantly reduced.

We also reveal a novel role of PACT in the expression of immune checkpoint PD-L1 in non-IFN-γ-stimulated environments, with the suggestion that this is through interaction with PKR and the stress response.

Finally, we confirm the endogenous interaction of TRBP and PACT within HeLa, RCC4 and MDA-MB-231 human cell lines.
### 6. Appendices

#### 6.1 Solutions made in house

<table>
<thead>
<tr>
<th>Solution</th>
<th>Reagents</th>
</tr>
</thead>
</table>
| 4x Sample buffer      | 250nM Tris HCl pH 6.8  
                        | 8% (w/v) SDS  
                        | 10% (w/v) Glycerol  
                        | 5% (v/v) β-Mercaptoethanol  
                        | 0.05% (w/v) Bromophenol Blue |
| RIPA (100mL)          | 150mM / 0.876g NaCl  
                        | 10mM / 1mL 1M Tris-HCl (pH 7.2)  
                        | 0.1% / 1mL 10% w/v SDS  
                        | 0.1% / 100µL Triton X-100  
                        | 1% / 1g Sodium Deoxycholate  
                        | 5mM / 1mL 0.5M EDTA |
| 10x TBS               | 1L ddH₂O  
                        | 12.2g Tris HCl  
                        | 87.65g NaCl  
                        | pH 8.0 |
| TBST                  | 900mL dd H₂O  
                        | 100mL 10x TBS  
                        | 1mL Tween 20 |
| Freeze media          | 10% (v/v) DMSO  
                        | 90% (v/v) FCS |
| Stacking gel (5%)     | 4.1mL H₂O  
                        | 1.0mL 30% Acrylamide mix  
                        | 0.75mL 1.5M Tris (pH 6.8)  
                        | 0.06mL 10% SDS  
                        | 0.06mL 10% Ammonium persulfate  
                        | 0.006mL TEMED |
| Resolving gel (10%) | 4.0mL $\text{H}_2\text{O}$  
| 3.3mL 30% Acrylamide mix  
| 2.5mL 1.5M Tris (pH 8.8)  
| 0.1mL 10% SDS  
| 0.1mL 10% Ammonium persulfate  
| 0.004mL TEMED  |
| FACS buffer | 1L PBS  
| 0.05% Azide  
| 1% FCS |
| SOC media | 2% tryptone  
| 0.5% yeast extract  
| 10 mM NaCl  
| 2.5 mM KCl  
| 10 mM MgCl2  
| 10 mM MgSO4  
| 20 mM glucose |
| LB Broth (1L) | 1L ddH$_2$O  
| 10g Tryptone  
| 5g Yeast Extract  
| 10g NaCl  
| pH 7.5  
| (+/-) 1mL Ampicillin (100mg/ml) |
| Agar (1L) | 1L dd H$_2$O  
| 10g Tryptone  
| 5g Yeast Extract  
| 10g NaCl  
| pH 7.5  
| 15g Agar  
| (+/-) 1mL Ampicillin (100mg/ml) |

Table 6: Solutions
6.2 Product links

64EF2PEG Phospho-EIF2 alpha (Ser52) cellular kit by Cisbio

Abbreviations List

AGO: Argonaute
BCA: bicinchinonic acid
BSA: bovine serum albumin
CD: cluster of differentiation molecule e.g. CD274
Co-IP: co-immunoprecipitation
CT: cycle threshold
DGCR8: DiGeorge syndrome critical region 8
DMEM: Dulbecco’s modified Eagle’s Medium
dsRBP: double-stranded RNA-binding protein
dsRBD: double-stranded RNA-binding domain
dsRNA: double-stranded RNA
eIF2α: alpha unit of eukaryotic initiation factor 2
FCS: Foetal calf serum
GFP: green fluorescent protein
IFN-γ: Interferon gamma
IL: Interleukin
IP: Immunoprecipitation
MDA231: MDA-MB-231
miRNA: microRNA
mRNA: messenger RNA
ncRNA: non-coding RNA
NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells
nt: nucleotide
NTC: non-targeting control
PACT: Protein Activator of PKR
PBS: Phosphate buffered saline
PD-L1: Programmed Death Ligand 1
PKR: Protein Kinase R
Pre-miRNA: precursor microRNA
Pri-miRNA: primary microRNA
RIPA: radioimmunoprecipitation assay
RISC: RNA induced silencing complex
RNAi: RNA interference
siRNA: short interfering RNA
TBST: tris-buffered saline with tween
TRBP: transactivation response (TAR) RNA-binding protein
tRNA: transfer RNA
References


