Investigating the Functional Significance of the MNK1/LARP1 Interaction in Breast Cancer

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ABSTRACT (ENGLISH)

The hallmarks of cancer are governed by both intracellular and intercellular events, which ultimately depend on the activity of proteins within the cell. It is therefore intuitive that regulators of the pipeline from gene to protein, when dysregulated, would have a large impact on cancer progression. RNA-binding proteins (RBP) are one such class of proteins that can influence mRNA translation by repressing, enhancing, or stabilizing mRNA. Evidence that these proteins contribute to tumorigenesis is ever-increasing.

The evolutionarily-conserved La superfamily of proteins contain an example of one such RBP involved in tumorigenesis. There are several members that have been implicated in the progression of cancer; however LARP1 is particularly distinct in this family as it is the only La-related protein that binds its target mRNA—the 5'Terminal OligoPyrimidine (5'TOP mRNA) that encode for translational machinery—at the 5'm7G cap via the DM15 region unique to LARP1 (Stavraka and Blagden, 2015). As one of the focuses of our lab is the MNK1/2-eIF4E axis, we had sought to identify novel targets of MNK1, whose best-characterized role is the phosphorylation of the eIF4E, which in turn increases the translation of pro-oncogenic proteins (Prabhu et al., 2020). We recently identified that MNK1 co-immunoprecipitates with LARP1 in tumor cell lines. Given the pro-oncogenic role that LARP1 plays in other cancers and the data from our lab for the pro-tumor role of MNK1 in cancer, we hypothesized that MNK1 and LARP1 may cooperate in favour of breast tumorigenesis.

In the following study, we used two models of breast cancer to reveal a new role for LARP1 in driving their malignant phenotype. My results show that loss of LARP1 does not reduce clonogenic outgrowth of triple-negative 4T1 breast cancer cells. Overexpression of the short isoform of LARP1 increased clonogenic outgrowth of hormone receptor-positive MCF7. Using a

CRISPRi system to knockdown MNK1 in MCF7 cells, we show that loss of MNK1 does not alter the increased clonogenic outgrowth conferred by LARP1 overexpression. We also reveal that LARP1 expression affects the MCF7 and 4T1 cell lines' capacity to invade collagen-coated Boyden chambers. Briefly, LARP1 overexpression in MCF7 cells enhances the invasivity of the poorly invasive MCF7 cells. Moreover, LARP1 knockdown in the highly invasive 4T1 cells, reduces their ability to invade. Further, we show that loss of LARP1 expression reduces experimental lung metastasis *in vivo*, but does not impact experimental liver metastasis *in vivo*. Altogether, our data support further investigation of the role of LARP1 in breast cancer invasion and metastasis.

ABSTRACT (FRENCH)

Les caractéristiques du cancer sont régies par des événements intracellulaires et intercellulaires, qui dépendent en fin de compte de l'activité des protéines au sein de la cellule. Il est donc intuitif que les régulateurs du passage du gène à la protéine, lorsqu'ils sont déréglés, aient un impact important sur la progression du cancer. Les protéines de liaison à l'ARN (PLA) constituent l'une de ces catégories de protéines qui peuvent influencer la traduction de l'ARNm en réprimant, en renforçant ou en stabilisant l'ARNm. Les preuves que ces protéines contribuent à la tumorigenèse sont de plus en plus nombreuses.

La superfamille des protéines La, conservée au cours de l'évolution, contient un exemple de PLA impliquée dans la tumorigenèse. Plusieurs de ses membres ont été impliqués dans la progression du cancer, mais LARP1 est particulièrement distincte dans cette famille, car c'est la seule protéine apparentée à La qui se lie à son ARNm cible - l'ARNm 5'TOP (5'Terminal OligoPyrimidine) qui code pour la machinerie traductionnelle - au niveau de la coiffe 5'm7G via

la région DM15 propre à LARP1 (Stavraka et Blagden, 2015). L'axe MNK1/2-eIF4E étant l'un des centres d'intérêt de notre laboratoire, nous avons cherché à identifier de nouvelles cibles de MNK1, dont le rôle le mieux caractérisé est la phosphorylation de l'eIF4E, qui à son tour augmente la traduction des protéines pro-oncogéniques (Prabhu et al., 2020). Nous avons récemment identifié que MNK1 co-immunoprécipite avec LARP1 dans les lignées cellulaires tumorales. Compte tenu du rôle pro-oncogénique que LARP1 joue dans d'autres cancers et des données de notre laboratoire concernant le rôle pro-tumoral de MNK1 dans le cancer, nous émettons l'hypothèse que MNK1 est une protéine pro-oncogénique et que LARP1 est une protéine pro-tumorale.

Dans l'étude suivante, nous avons utilisé deux modèles de cancer du sein pour révéler un nouveau rôle pour LARP1 dans la conduite de leur phénotype malin. Mes résultats montrent que la perte de LARP1 ne réduit pas l'excroissance clonogénique des cellules de cancer du sein triple négatif 4T1. La surexpression de l'isoforme courte de LARP1 a augmenté la croissance clonogénique des cellules MCF7 à récepteurs hormonaux positifs. En utilisant un système CRISPRi pour éliminer MNK1 dans les cellules MCF7, nous montrons que la perte de MNK1 ne modifie pas l'augmentation de la croissance clonogénique conférée par la surexpression de LARP1. Nous révélons également que l'expression de LARP1 affecte la capacité des lignées cellulaires MCF7 et 4T1 à envahir les chambres de Boyden recouvertes de collagène. En bref, la surexpression de LARP1 dans les cellules MCF7 augmente l'invasivité des cellules MCF7 peu invasives. En outre, la désactivation de LARP1 dans les cellules 4T1 très invasives réduit leur capacité d'invasion. En outre, nous montrons que la perte de l'expression de LARP1 réduit les métastases pulmonaires expérimentales in vivo, mais n'a pas d'impact sur les métastases hépatiques expérimentales in vivo. Dans l'ensemble, nos données plaident en faveur d'une étude plus approfondie du rôle de LARP1 dans l'invasion et la métastase du cancer du sein.

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CONTRIBUTION OF AUTHORS

Virus production for the CRISPRi dCas9, plx317 LARP1 overexpression vector, and for two of the four vectors used in the 4T1-luc cells throughout this work were performed by Dr. Sathyen Prabhu. Luciferase tagging of the 4T1 cells was performed by Raul Flores Gonzalez.

Single-cell sorting for MCF7 cells transduced with dCas9 for the CRISPRi system was performed by Christian Young.

Tail vein injections were performed by Yvhans Chery of our animal facility staff. All splenic injections were performed by Dr. Samuel Preston and Raul Flores Gonzalez.

All other data in this thesis was acquired and analyzed by myself.

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LIST OF ABBREVIATIONS

Akt	Ak strain transforming
Bcl-2	B cell lymphoma 2
BIK	Bcl-2 interacting protein
BiP	Binding Immunoglobulin Protein
CDK 4/6	Cyclin dependent kinases 4 and 6
circRNA	Circular RNA
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
dsRBD	double-stranded RNA Binding Domain
ER+	Estrogen-Receptor positive
eIF	Eukaryotic Initiation Factor
EMT	Epithelial-to-mesenchymal transition
HER2+	Herceptin Receptor-positive
HuR	Human Antigen R
ISR	Integrated Stress Response
IVIS	*In-vivo* Imaging System
IRES	Internal Ribosomal Entry Site
KD	Knockdown
LaM	La Motif
LARP	La-Related protein
lncRNA	Long-non-coding RNA
MAPK	Mitogen-activated Protein Kinase
MDM2	Mouse Double Minute 2

miRNA	Micro RNA
MMP	Matrix metalloproteinase
MNK1 and 2	MAPK-interacting protein kinases 1 and 2
mTORC	Mammalian Target of Rapamycin Complex
OE	Overexpression
PABP	Poly-A Binding Protein
PD1/PD-L1	Programmed-Death Ligand 1
PI3K	Phosphoinositide 3-Kinase
PR+	Progesterone-receptor positive
RAPTOR	Regulatory-Associated Protein of mTOR
RBP	RNA binding protein
RNP	Ribonucleoprotein
RP	Ribosomal Protein
RRM	RNA-recognition motif
shRNA	Short-Hairpin RNA
RTK	Receptor Tyrosine Kinase
TNBC	Triple-negative Breast Cancer
UTR	Untranslated Region
VEGF	Vascular Endothelial Growth Factor
5'TOP	5'Terminal OligoPyrimidine

1. LITERATURE REVIEW AND INTRODUCTION

1.1 Breast Cancer

Breast cancer is the second-leading cause of cancer-related death in women—second only to lung cancer—and is one of the three most common cancers worldwide along with lung and colon cancer (Harbeck and Gnant, 2017). It is estimated that approximately one in ten women will experience breast cancer in their lifetime, and that one in 34 will die from the disease. Throughout medical history, great progress has been made in both understanding the disease and developing effective treatments to improve the survival and quality of life of patients affected by breast cancer. Early disease intervention and comprehensive treatment strategies have been successful in reducing mortality rates; however, the occurrence of metastasis of the disease has increased (Liang et al., 2020).

The metastasis of breast cancer is the biggest contributing factor to its mortality rates. The overall survival rate for patients without metastasis is 80% while those whose disease spreads to distal organs reduces dramatically to only 25% (Allemani et al., 2018; Valastyan and Weinberg, 2011). The disease exhibits metastatic heterogeneity; meaning it has a propensity to metastasize to specific organs; and this lends itself to highly varied responses to treatment. Metastasis to the bone comprises 75% of metastatic cases with a 22.8% 5-year survival rate(Tulotta and Ottewell, 2018; Xiong et al., 2018). The second-most common site is lung with an even lower 5-year survival rate at 16.8%(Smid et al., 2008). Metastasis to the liver is the next most common site with a dwindling 5-year survival rate of 8.5%(Pentheroudakis et al., 2006). Metastasis to the brain occurs in 10-30% of breast cancer patients with metastatic disease; however, their prognosis and quality of life remains poor as serious neurological symptoms often occur with the radiation, surgery, and targeted therapy used to control the brain metastases(Hosonaga et al., 2020). Therefore, it is

imperative that as many studies aimed to identify new targets as possible are explored to tackle the challenge that is managing the metastasis of breast cancer.

The disease of breast cancer can be categorized into four molecular subtypes: luminal A (ER+/PR+/HER2-/Ki-67 low) and luminal B (ER+/PR-/HER2+/Ki-67 high), human-epidermal growth factor positive (HER2+), and triple-negative breast cancer (TNBC) (indicating a lack of upregulated expression of ER, PR, and HER2) (Prat et al., 2015; Yersal and Barutca, 2014). The subtype at hand, in addition to the age of the patient, can help to predict the prognosis and the metastatic pattern of the disease. Patients under 50 years old with metastatic disease most commonly face multiple-organ metastasis, with the second-most common sites being metastasis to the bone (9.7%) and liver (5.6%) in Luminal A, lung (2.8%) for Luminal B and HER2+, and bone metastases (5.6%) for TNBC (Tagliabue et al., 2021). Multi-organ metastasis is also the most common metastatic outcome in patients over 50 with liver, followed by bone in Luminal A, and lung for TNBC. In a study comprised of 8831 breast cancer patients, the five-year survival rate for metastatic TNBC was by far the lowest at less than 20%; while the outcome for metastatic disease in Luminal A, B, and HER2+ were found to be 30-40% (Tagliabue et al., 2021). Women with brain metastases suffer the worst survival rates regardless of their subtype (Tagliabue et al., 2021).

There are a variety of treatment options which have greatly improved the prognosis for breast cancer patients. The development of anti-HER2 therapy—namely, the monoclonal antibody treatments trastuzumab and pertuzumab—have helped in the management of HER2+ breast cancers despite uncovering a population in HER2+ breast cancers that have a propensity to metastasize to the central nervous system (Liang et al., 2020; Lin and Winer, 2007). Other therapeutic staples of breast cancer include endocrine therapy, which is the modulation of hormone expression in HR+ cancers; chemotherapy, including Anthracycline and taxane-containing agents

which can be enhanced with neoadjuvant of platinum; and finally surgical resection depending on the size and location of the tumor load (Harbeck and Gnant, 2017). Although a complete pathological response is possible with neoadjuvant targeted and chemotherapy, surgery to remove the last remaining tumor or to simply verify the complete response typically remains necessary (Harbeck and Gnant, 2017). Inhibition of the cyclin dependent kinases 4 and 6 (CDK4/6) have also gained success in the clinic against ER+/HER2- metastatic breast cancer (Kwapisz, 2017). The revolution that CDK4/6 inhibitors, such as palbociclib, have introduced to the treatment of ER+ breast cancer has led to their study in combination with other agents. For example, our lab discovered that combined CDK4/6 and MNK1/2 inhibition cooperated to further reduce clonogenic outgrowth in HR+ breast cancer cell lines compared to either agent alone; as well as reducing outgrowth of HR+ cells which have acquired palbociclib resistance (Prabhu et al., 2023). Multigene assays such as Oncotype DX have also helped clinicians to predict chemotherapy response, recurrence risk, and if there could be a need for systemic therapy(Harbeck and Gnant, 2017). Furthermore, the use of immunotherapy is becoming more popular; in particular in cases of TNBC. Breast cancer was historically thought to be poorly immunogenic and have lower mutational burden compared to 'immune-hot' tumors; yet with increasing understanding of the different molecular subtypes, it has been found that TNBC has relatively higher genetic instability, copy number changes, and genomic rearrangement as well as greater immune cell infiltration and PD-L1 expression (Bareche et al., 2018; Loi et al., 2013; Mittendorf et al., 2014). This has led to the exploration of targeting the PD1/PDL1 axis via pembrolizumab and atezolizumab in TNBC which has yielded some encouraging results; including in cases of metastatic TNBC (Schmid et al., 2018; Schmid et al., 2022).

The study of breast cancer as a disease has also been pushed forward by the development of a variety of *in-vitro* and *in-vivo* models. The establishment of breast cancer cell lines derived from patients and animal models alike has allowed the study of specific signaling pathways in cancer cells via genetic and pharmacological manipulation and how it impacts various aspects of the disease. For example, our understanding of metastatic breast cancer has been supported greatly by the development of models that mimic various steps of metastasis and test relevant cellular characteristics that endow a cell with more potential to escape the primary tumor. The scratch or wound-healing assay is a well-established method to evaluate how cells respond to a 'wound' or break in the cell layer; allowing researchers to study not only the migration of cells but also their morphology as they migrate (Cory, 2011). The Boyden chamber assay is a steadfast technique that allows one to interrogate the ability of cells to invade through a variety of matrices (Chen, 2005). In terms of *in vivo* models relevant to the study of metastatic breast cancer, the injection of cancer cells directly into the bloodstream of mice is a technique that allows the interrogation of how the cells are able to survive in the vasculature, extravasate, and establish themselves in a new organ. For example, the 4T1 cell line, originally isolated from a spontaneously occurring tumor in the mammary gland of the BALB/c mouse model, tends to metastasize to the lung and has been used as both a spontaneously-occurring and intravenous model of breast cancer metastasis (Pillar et al., 2018). Models such as 4T1 are also useful in that they are syngeneic with an established murine model; meaning that they can be studied in the context of a host with a functional immune system (Moroishi et al., 2016). Injected or implanted tumor cells can be further followed within a host with the aid of luciferase-tagging; which allows localization and even an idea of general tumor burden based on the strength of bioluminescent signal acquired (Close et al., 2011; Cosette et al.,

2016). Altogether, this technology has aided researchers in painting a more detailed picture of breast cancer outside of patients.

1.2 RNA-Binding Proteins

Almost all cells in the human body share the same set of DNA, yet the expression of each of our genes differs vastly between tissues depending on their needs and functions. Organisms have developed a variety of expression regulation methods, many of which occur at the level of transcription; however, there is a large breadth of mechanisms in place at the post-transcriptional level which also contribute to the impressive differential gene expression in our cell types. RNA binding proteins (RBPs) are known for being one of the largest groups of executors of post-transcriptional regulation and account for 7.5% of protein-coding genes (Gerstberger et al., 2014b). These proteins are able to recognize all types of RNA to regulate their fate; including messenger (mRNA), micro RNA (miRNA), long non-coding RNA (lncRNA), circular RNA (circRNA), and small nuclear RNA (snRNA); and do so through recognition of specific motifs in these RNA through their binding domains (RBDs) (Figure 1) (Wang et al., 2022). All RBPs have a modular structure composed of various RBDs; the majority of which are formed from a limited and highly conserved pool. Despite this, RBPs achieve high specificity for the breadth of structural diversity of RNA by possessing multiple RBDs in a variety of structural arrangements (Lunde et al., 2007). The RNA recognition motif (RRM) is the most abundant RNA-binding domain (RBD) and is ubiquitously expressed among RBPs; which recognizes beta sheet RNA structures and interacts with 4 nucleotides of ssRNA (Lunde et al., 2007). The hnRNP K homology domain (KH domain) is capable of binding single-stranded RNA and DNA which it recognizes through hydrophobic clefts of variable loop structures; while the double-stranded RNA-binding domain (dsRBD) is capable of binding dsRNA of any sequence (Lunde et al., 2007). While there are a plethora of

other highly conserved domains, these examples demonstrate the precision through which RBPs can act.

The high specificity of RBPs lends itself to a discernible influence on the fate of the RNA. For a time, RNA was thought to serve two purposes: as either a template in the case of mRNA, or as a structural component during protein synthesis in the case of tRNA. However, the ncRNA revolution revealed other forms of RNA such as ribozymes (catalytic RNA that can commence splicing of other transcripts) or riboswitches (non-coding and intronic structures of mRNA that can modulate gene expression in response to specific, small molecules in the cell) (Baird et al., 2010; Cech and Steitz, 2014). These more 'functional' RNA often work collaboratively; that is, in complex with RBPs, which is known as a ribonucleoprotein complex. The existence of these executive forms of RNA and RNPs highlight the fact that the activity of a single RBP can have major effects on not only RNA but the fate of the cell itself. Post-transcriptional regulation is especially essential for proper embryonic development; a process which requires ultimate tissue-specific control. It thus stands to reason that dysregulation of RBP expression can have dire consequences at both the local and systemic level.

As already discussed for breast cancer, the greatest contributing factor to the mortality of cancerous disease is the metastasis of tumor cells to other sites of the body. RNA binding proteins have been extensively studied in recent years as being significant contributors to virtually all aspects of tumorigenesis. Indeed, post-transcriptional modifications and regulation contribute to the dysregulation of expression associated with cancer among a variety of other diseases. Additionally, the vast majority of RBPs are ubiquitously expressed—that is, only 6% of RBPs have a tissue specific function (Gerstberger et al., 2014a). Therefore, when a link between an RBP and a tissue-specific disease is established, its function must be critical for the function of that

particular tissue. The first RBP to be linked to cancer was the mRNA cap-binding protein eukaryotic initiation factor 4E (eIF4E), which was first shown to drive transformation in fibroblasts and mammalian epithelial cells and has since been linked to melanoma and breast cancer (Avdulov et al., 2004; Carter et al., 2016; Gong et al., 2020). The evolutionarily conserved cytoplasmic polyadenylation element binding (CPEB) family has been well-documented as having a role in tumorigenesis; with some family members being tumour-suppressive and others functioning as pro-tumorigenic. CPEB1 is thought to act as a tumor suppressor in breast cancer, where it was found that reduced levels of CPEB1 resulted in increased TGF-β-mediated EMT to support metastasis; while CPEB4 was found to enhance EMT in a ZEB-1-mediated approach in gastric cancer and to increase migration, invasion, and EMT in breast cancer cells *in vitro* by upregulating Vimentin expression (Cao et al., 2018; Lu et al., 2017; Nagaoka et al., 2016). The Human Antigen R (HuR) is also a ubiquitously expressed RBP and is one of the most well-studied RBPs in cancer; with dysregulation of its expression being linked to invasion, metastasis and poorer outcome in a variety of cancers. Breast cancer is one such diseases in which HuR expression is upregulated and increased cytoplasmic HuR levels are linked to high-grade tumors and poorer clinical outcome (Heinonen et al., 2005). Another well-known RBP implicated in metastasis is LIN28, which interferes with the processing of several miRNA that are mostly known to be tumor suppressive (Chen et al., 2012; Hu et al., 2019; Xu et al., 2013). Here, LIN28 enhances EMT in cancers such as hepatocellular carcinoma, breast, and colorectal cancer by interfering with the DICER-mediated processing of these miRNA suppressors of tumorigenesis. Altogether, it is clear from these examples that RBPs have a wide array of impacts on oncogenesis in both beneficial and detrimental ways depending on the tissue and protein at hand. Nonetheless, the fact that their

effects are frequently tissue-specific makes them attractive targets to study for potential new therapeutic approaches; particularly in the battle against metastatic disease.

With the revolution of RNA biology being implicated in cancer, a family of RBPs that is becoming increasingly apparent is the La-Related Protein (LARP) Family. Several of the family members are implicated in various cancers; usually supporting oncogenesis in epithelial malignancies; except in the case of LARP4a, which was found to be downregulated in ovarian cancer cells and to suppress tumour progression in non-small cell lung cancer cells; and LARP7, whose expression is known to be lost during tumor progression in some cancers—most notably in invasive breast cancer (Ji et al., 2014; Lu et al., 2020; Lu et al., 2023). Genuine La protein or LARP3 is known to regulate IRES-mediated genes; which include several genes associated with cancer such as BiP, MDM2, and cyclin D1; and is thought to be taken advantage of by tumor cells in a tissue-specific manner— LARP3 is associated with both head and neck cancer and cervical cancer, yet in head and neck cancer, it helps to upregulate MDM2; while in cervical cancer it upregulates cyclin D1 (Sommer et al., 2011a; Sommer et al., 2011b). Furthermore, it is also known to support epithelial-to-mesenchymal transition through its target Lamin B; implicating it as a pro-metastatic RBP (Petz et al., 2012). LARP1 is also known to be involved in the cytoskeletal changes and migration of *Drosophila* cells during embryogenesis and its high expression is not only correlated with poorer outcomes in a variety of cancers, but also in increasing their invasion, clonogenic and non-adherent cell growth, and increased tumor burden in *in* vivo models (Burrows et al., 2010; Desi et al., 2022; Mura et al., 2015). Finally, LARP6 and has also been shown to be a potential pro-oncogenic character in breast cancer as its expression was shown to be upregulated in invasive ductal carcinoma and to drive angiogenesis; potentially through regulation of expression of MMP-9 and VEGF (Shao et al., 2012). The connections of this superfamily to cancer

are becoming increasingly clear; yet there is much more characterization needed on the specifics of their roles in oncogenesis.

1.3 The La Family of Proteins and LARP1

The La-Related Protein Family are an ancient superfamily of RBPs carried through the evolution of eukaryotes; all of which share a 90-amino acid motif (the La Motif or LAM) similar to that of the Genuine La Protein (Stavraka and Blagden, 2015). LARPs are generally involved in critical processes to metabolize and use RNA in both the nucleus and cytoplasm; with the subfamilies having specialized structures and functions (Dock-Bregeon et al., 2021). In general, each of the La family members have been shown to have a role in transcription and/or translation of their targets. There are 5 subfamilies of LARP found in humans: Genuine La (originally known as SS-B and recently re-termed LARP3), LARP1 (comprised of variants LARP1a and 1b/LARP2), LARP4 (variants LARP4a and 4b), LARP6 and LARP7 (Stavraka and Blagden, 2015). LARPs typically share very high homology with mouse LARPs as well. For example, mouse LARP1 (1072aa) shares 90.4% homology with the dominant human isoform (1096aa); the missing amino acids being found near the N-terminal of the murine version of the protein; although in the case of murine LARP2, in addition to the highly homologous dominant isoform, there are also various shorter isoforms expressed in mouse that are not expressed in human (Altschul et al., 1990). Alongside the conserved LAM, all LARPs also contain an RNA Recognition Motif (RRM); which together are termed the La Module. However, the RRM domains vary between La subfamilies save for in Genuine La and LARP7; which also happen to contain a second RRM (RRM2) (Stavraka and Blagden, 2015). LARP6 also contains an additional domain known as the SUZC domain at its C terminus which is thought to be required for its subcellular localization; as has been observed with its presence in other RBPs (Marchler-Bauer et al., 2015). Additionally, LARP1 and LARP4a

and 4b are capable of binding polyadenylate binding protein (PABP); which is another evolutionarily conserved RBP that is synthetic. LARP1 and LARP1b are capable of this interaction via their DM15 region or LARP1 motif; which is unique to the protein and is comprised of triplicate amino acid repeats (Stavraka and Blagden, 2015; Tcherkezian et al., 2014). Meanwhile, LARP4a and 4b mediate this interaction via their PAM2w domain (Yang et al., 2011). This interaction is generally thought to aid PABP in maintaining the stability of their target mRNAs. As for the DM15 or LARP1 motif, this domain was also identified as being responsible for LARP1's capacity to bind the 5'cap of its targets; a function which is unique to LARP1.

LARP1 was first identified in Drosophila as being essential for embryogenesis, oogenesis, spermatogenesis, formation of spindle poles, successful segregation of mitochondria and cell cycle progression (Blagden et al., 2009; Chauvet et al., 2000; Ichihara et al., 2007). While LARP1 is predominantly cytoplasmic, it can also be found in the nucleus; although its function has yet to be linked in any way to transcription outside of being identified as a host factor for influenza A and HIV infections (Engeland et al., 2011; Karlas et al., 2010). LARP1 is highly involved in mRNA translation. LARP1 was identified to interact with poly-A binding protein (PABP) and was the first of the family to be identified as doing so in an mRNA-independent manner (Blagden et al., 2009). The first poignant clue as to its role as a 5'cap-dependent regulator of mRNA translation was when it was observed that knockdown of LARP1 significantly impacted the global protein synthesis by 15% and increased 4E-BP1 hypophosphorylation (Burrows et al., 2010). LARP1 was eventually identified as being the missing link between MTORC1 and the regulation of ribosome biogenesis; which was further solidified by the discovery that LARP1 primarily targets 5'TOP mRNAs (Aoki et al., 2013; Tcherkezian et al., 2014). The 5'TOP, or 5'Terminal OligoPyrimidine motif, is comprised of an invariable C at the +1 position (relative to the 7-methylguanosine (m7G)

cap found at the 5' terminus of most mRNA) followed by a series of 4-15 pyrimidines and, typically, a G-rich region (Yoshihama et al., 2002). The 5'TOP motif is highly conserved and 5'TOP mRNA encode proteins involved in translation; including all of the ribosomal subunits, eIF4A, eIF3, eEF2, and PABP; allowing for efficient modulation of protein synthesis machinery production in response to changes in cellular homeostasis (Iadevaia et al., 2008). Additionally, sucrose gradient velocity sedimentation of ribosomes in HEK293s revealed that LARP1 co-sedimented largely with pre-polysomes (that is, monosomes or ribosomal subunits) over polysomes (Tcherkezian et al., 2014). This indicated that LARP1 associates with mRNA during early steps of translation and, to a lesser extent, during the active translation of the transcripts. The mechanics of mTOR-mediated 5'TOP translation regulation was uncovered when LARP1 was shown to bind mTORC1 component RAPTOR, facilitating its phosphorylation by the kinase at positions S689 and T692 to dissociate LARP1 from the 5'cap while LARP1b (or LARP2) interacts only weakly with RAPTOR and mTOR; likely due to the fact that it shares only 73% similarity at the protein level (Hong et al., 2017; Jia et al., 2021). While there remains some controversy around the exact function of LARP1, the general consensus is that LARP1's association with PABP and the poly-A tail of its targets, along with its association with the cap and 5'TOP motif, helps to circularize and protect its mRNA targets when mTORC1 is inactive and translation is not ideal. Then, when sufficient nutrients are available for active translation and mTORC1 is active, mTORC1 dissociation of LARP1 from the cap via phosphorylation by mTOR frees the position for the eIF4F complex and binding of eIF4E, thus initiating translation. Meanwhile, LARP1 maintains its association to the 3'end of the transcript to continue aiding circularization and stabilization. In this model, LARP1 acts as a sort of translational gas pedal for its 5'TOP target mRNA.

LARP1 has also been cited as being able to target and regulate the translation of non-5'TOP transcripts and may have as many as 3000 mRNA targets which were identified via RNA immunoprecipitation and microarray profiling (RIP-Chip) using anti-LARP1 antibody in HeLa cells (Mura et al., 2015). Some of the most notable cancer-related pathways identified from analysis of these targets were extracellular matrix interactions, regulation of the actin cytoskeleton, focal adhesion, PI3K, and VEGF signalling. Of note, it was revealed that LARP1 post-transcriptionally regulates its regulator, mTOR, by associating with the mTOR 3'UTR (Mura et al., 2015). Among other targets identified via reverse-phase protein array, it was found that LARP1 stabilizes anti-apoptotic BCL2 mRNA while destabilizing pro-apoptotic BIK mRNA; again via association with the 3'UTR of these targets (Hopkins et al., 2016). Interestingly, it is thought that this 3'UTR-mediated regulation is also occurring through the DM15 region of LARP1. Through crystal structuring, it was shown that the DM15 region contains an mTORC1 recognition region in addition to HEAT-like motifs which may have been repurposed over their evolution for interaction with other RNA (Hopkins et al., 2016; Lahr et al., 2015). Furthermore, LARP1 has also been cited to interact with the 3'UTR of MYC mRNA in colorectal cancer cell lines in a manner that increases the translation of MYC and supports tumorigenesis (Desi et al., 2022). Most recently, LARP1 was shown to post-transcriptionally regulate the expression of several rate-limiting enzymes involved in metabolism as well as having an impact on the localization of mTOR to the surface of the lysosome in ovarian cancer cell lines; thereby playing a role in the activation of mTOR (James et al., 2022). Altogether, the data gathered so far concerning the targets of LARP1 highlight its importance for the maintenance of homeostasis in normal cells and alludes to the ways in which it could be exploited in disease by, for example, a tumour cell.

1.4 LARP1 in the context of cancer

Given the role LARP1 has in ribosome biogenesis and, to a degree, in global translation, it goes without saying that dysregulated or abnormally expressed LARP1 in the cell can have dire consequences. LARP1 is an emerging RBP in cancer and has already been deemed to be pro-oncogenic in several tumour types; mainly in epithelial malignancies. High LARP1 levels in patient tumours has been correlated with poor outcome, disease progression, and reduced overall survival in multiple cancers; namely, cervical cancer, hepatocellular carcinoma, ovarian cancer and breast cancer (Hopkins et al., 2016; Stavraka and Blagden, 2015). Of note, both cervical and hepatocellular carcinoma are associated with chronic viral infection—herpes simplex virus and hepatitis B/C, respectively—and therefore the correlation in these cases may be a result of LARP1's putative role as a viral host factor rather than as an oncogenic RBP. Nonetheless, this does not take away from the correlation of LARP1 in the outcome of non-virally-associated cancers. For example, in breast cancer, a study aiming to characterize the transcriptomic landscape of each of the subtypes of breast cancer, RNA sequencing and high-throughput analysis of tissue samples revealed a splice variant of LARP1 in 4/6 non-triple-negative patient samples; which the authors also confirmed to be found in HR+ breast cancer cell line MCF7 (Eswaran et al., 2013; Schwenzer et al., 2021). This isoform differs from the 1096aa-length dominant isoform that is usually expressed in humans at exon 1; yielding a short LARP1 isoform of 1019aa (Schwenzer et al., 2021).

Apart from correlative studies generated from patient data, there is a great deal of work highlighting the pro-oncogenic effect of LARP1 both *in vitro* and *in vivo* using a variety of models and cancer types. In ovarian cancer, LARP1 is known to have the net effect of aiding the resistance to apoptosis and supporting chemotherapy resistance by stabilizing BCL2 transcript and

destabilizing BIK transcripts (Hopkins et al., 2016). Additionally, subcutaneous injection of SKOV3 cells in which LARP1 was knocked down into SCID-beige mice resulted in significantly lower tumor volume as well as increased survival compared to mice injected with LARP1-expressing SKOV3 cells. Using HeLa cells as a model of cervical cancer, LARP1 overexpression increases invasion, colony formation, non-adherent cell growth and tumorsphere formation; and *in vivo* work using subcutaneous injection of these cells in immunodeficient mice revealed that LARP1 overexpression increased both tumor progression and mTOR levels within the tumor (Mura et al., 2015). The effects of LARP1 on resisting apoptosis were found in *in vitro* study of colorectal cancer cell lines HCT116 by identifying that LARP1 knockdown induced higher levels of caspase-3 and -7 activity; and the effect of LARP1 knockdown and LARP1 overexpression in DLD1 colorectal cancer cell line xenograft tumor progression also reflected its pro-tumorigenic effect (Desi et al., 2022). To date, very few non-xenograft mouse models of tumorigenesis have been published as being used to characterize the role of LARP1 as an oncogenic RBP. However, LARP1 was identified as being upregulated in hepatocellular carcinoma by the use of a *Mat1a* knockout mouse model; which develop the cancer spontaneously (Ramani et al., 2022). Here the authors also revealed LARP1 to be a putative target of CDK2 at its T449 site and suggest that phosphorylation at this site is responsible for increased proliferation, migration and invasion using HUH-7 and HEP-3B cell lines (both derived from human hepatomas), and translation of oncogenic 5'TOP mRNA using SAMe-D cells (derived from livers of the *Mat1a* knockout model), primary mouse hepatocytes, and HUH-7 cells (Ramani et al., 2022). Overall, LARP1 plays an important pro-tumor role in a variety of cancerous diseases, but additional work is still required on the mechanisms through which LARP1 is pro-oncogenic.

1.5 Other Factors Involved in mRNA Translation

Outside of RBPs, there are other factors that affect the fate of mRNA. The rate-limiting step of translation is initiation; which occurs when the eukaryotic initiation factor 4F (eIF4F) interacts with the mRNA 5' cap structure. eIF4F is a complex which is comprised of eIF4A, a DEAD-box RNA helicase that unwinds secondary mRNA structures; eIF4E, which binds the 5'm7G cap and is the last to be recruited to the complex; and eIF4G, a scaffold protein which interacts with both eIF4A and eIF4E (Hershey and Merrick, 2000). Given that this is a step through which all cap-dependent translation must pass, there are several key signaling pathways upstream of the recruitment of the 4F complex which serve as regulators; including the mitogen activated protein kinase (MAPK) pathway, the mammalian target of rapamycin or mTOR pathway, and the integrated stress response (ISR) pathway. MAPK signaling allows control in response to hormones, cytokines, and oxidative and microtubule stress. mTOR signaling regulates translation in response to availability of nutrients such as amino acids to control the synthesis of proteins, lipids and nucleotides as well as autophagic processes (Liu and Sabatini, 2020). Finally, ISR inhibits global translation in unfavourable conditions such as during oxidative stress or nutrient deficiency by diverting the cell from cap-dependent translation to that of eIF2-dependent mRNA in order to reduce global translation and mediate cell stress (Costa-Mattioli and Walter, 2020). Each of these signaling pathways allow for the tight regulation of translation to help ensure catabolism and anabolism occur promptly and at appropriate times.

In addition to each signaling pathway converging on translation initiation are their effectors, which also play crucial roles in determining protein synthesis of the cell. The MAPK interacting kinases 1 and 2 (MNK1 and MNK2) fall downstream of MAPK and PI3K/AKT/mTOR signaling and are activated via phosphorylation by ERK1/2 as well p38 in response to a variety of

factors; including cytokines, growth factors, pathogens, oxidative and microtubule stress (Kumar et al., 2018). To date, few targets have been validated outside of eIF4E, which is the only MNK1/2 substrate to be validated *in vivo* (Ueda et al., 2004). As a member of the translation initiation complex, eIF4E has a role in all cap-dependent mRNA translation; however, when it is phosphorylated by MNK1 or 2, a subset of pro-oncogenic mRNA are preferentially translated. In mouse embryonic fibroblasts, the translation of chemokine *CCL2*, matrix metalloproteinases 3 and 9 (*MMP3* and *MMP9*), baculoviral IAP repeat-containing protein 2 (*BIRC2*), and vascular endothelial growth factor C (*VEGFC*) was found to be reduced in phospho-eIF4E deficient cells (possessing ($eIF4E^{S209A/S209A}$), impairing the only phosphorylation site that MNK acts on) compared to wild-type (Furic et al., 2010). Additionally, in *KIT*-mutant melanoma cells, MNK1/2 knockdown resulted in reduced translation of *SNAI1* and *CCNE1* and their invasive and metastatic properties were impaired (Zhan et al., 2017). Furthermore, phosphorylated eIF4E is known to participate in the post-translational regulation of β-catenin in chronic myeloid leukemia; a pathway which is relied upon in self-renewing blast crisis cells and is thought to aid the cells in developing resistance to targeted therapy (Perrotti et al., 2010). These established roles for the MNK1/2-eIF4E axis and their translational targets in cancerous disease are corroborated in breast cancer as well, where in HR+ breast cancer, phospho-eIF4E promotes tamoxifen resistance via increased translation of RUNX2 (Geter et al., 2017). MNK1 has also been identified as an important player in the progression of ductal carcinoma *in situ* to invasive ductal carcinoma (Guo et al., 2019). Altogether, these data highlight the implication of MNK1/2 kinases and their impact on translation; particularly in the context of being hijacked by cancerous disease.

Logically, several agents have been developed and tested for their ability to target the MNK kinases. Early inhibitors of the kinases include CGP57380 and cercosporamide which initially

showed promising effects but were abandoned due to off-target effects (Bain et al., 2007; Konicek et al., 2011). More recently, ATP-competitive inhibitors of the MNK1/2 kinases have been used in several studies characterizing their impact against melanoma and breast cancer *in vitro* and *in vivo* (Guo et al., 2021; Guo et al., 2019; Huang et al., 2021; Prabhu et al., 2023). Several clinical trials are also currently exploring the efficacy of MNK inhibitors in combination with immunotherapies such as PD1/PD-L1 inhibitors and taxane-based chemotherapy, such as paclitaxel, against both solid and hematological tumors (Hubbard et al., 2019; Santag et al., 2015; Santag et al., 2017) (NCT04622007, NCT05744739). Nonetheless, more work is required to determine what other targets of MNK1/2 could potentially be impacted by MNK inhibition and in turn affecting the therapeutic action of these molecules.

1.6 Rationale, Hypothesis, and Objectives

Breast cancer is a common form of cancer and treatment availabilities for TNBC and metastatic forms of the disease are limited; both of which are known to have the poorest outcomes and overall survival. The MNK1/2 kinases have been identified as playing a role in the progression of ductal carcinoma and the mechanism through which it does so have not been greatly explored outside of their target eIF4E. We have recently identified that MNK1 co-immunoprecipitates directly with LARP1 in tumor cell lines (work pioneered by Dr. Sathyen Prabhu (MSc, PhD); unpublished data). Given the pro-oncogenic role that LARP1 plays in other cancers, the correlation between high-LARP1 tumors and poorer outcome in breast cancer, and the data from our lab for the pro-tumor role of MNK1 in cancer, we hypothesize that MNK1 and LARP1 may cooperate to modulate the translatome in favour of breast tumorigenesis. The aims of my project are as follows:
Aim 1: To characterize the role of LARP1 in breast tumorigenesis *in vitro* and to identify if its effects are impacted by the gain or loss of MNK1 activity.

Aim 2: To characterize the role of LARP1 in breast tumorigenesis *in vivo* with the use of models of primary tumor formation and metastasis.

METHODS

Generation of cancer cell lines with modified MNK1 and LARP1 expression

A. **MCF7 breast cancer CRISPRi model system**. MCF7 cells were transduced with a vector containing dCas9 conjugated to KRAB for use in a CRISPR interference (CRISPRi) system (Larson et al., 2013). Single-cell sorted clones were expanded and individually validated for a functional dCas9 by use of a previously validated sgRNA targeting MNK1 followed by immunoblot to confirm the knockdown of MNK1.

B. **Stable LARP1 overexpression in MCF7 breast cancer cells**. To generate a model of stable LARP1 overexpression, we obtained a PLX317 lentiviral vector containing the short isoform of LARP1 (1019aa) (SI-LARP1) sequence. Single-cell-sorted clones of the MCF7 breast cancer line expressing functional CRISPRi dCas9 were transduced with the PLX317 vector and underwent selection with a dose of 1μg/mL puromycin over one week before validation of SI-LARP1 overexpression by immunoblot and seeding for experiments.

C. **Stable knockdown of LARP1 in 4T1 murine breast cancer cells**. To generate a model of stable LARP1 knockdown, 4T1 murine breast cancer cells were transduced with lentivirus harboring pLKO control or independent shRNAs targeting LARP1, as previously described by other groups(Papadakis et al., 2015; Wang et al., 2017). Cells were selected with puromycin over one week before validation of LARP1 knockdown and seeding for experiments.

D. **Lentivirus production and transduction**. Lentiviral plasmids were co-transfected with the packaging plasmids Pax2 and MD2G into HEK293T cells using calcium phosphate precipitation. Viral supernatant was harvested 72 hours post transfection, spun down at 500xg for 5 minutes, and filtered through a 0.45 μm filter. 500 μl of viral supernatant were used to transduce 100,000 cells in the presence of 8 μg/mL polybrene for 24 hours. The following day, media was changed, and transduced cells were selected using 1-4 μg/mL of puromycin depending on the cell line to be transduced.

Boyden Chamber Assay

4T1 cells seeded in 15cm petri dishes at 75% confluency were starved in serum-free media for 18 hours. Polycarbonate transwell inserts of 8μm pore size in 12-well plates were coated with 300μl collagen at 20μg/mL and left at 37ºC for at least 30 minutes. The transwell inserts were then carefully aspirated and serum-starved cells were immediately seeded at a density of 200 000 cells per well in serum-free media. 1.5mL of complete media (10% FBS) or with 2.5μM SEL201 was added to the bottom of the wells and cells were allowed to invade for 16 hours or 24 hours. The transwell inserts were then washed with PBS, fixed with 10% glutaraldehyde for 30 minutes, washed with distilled water, and stained with crystal violet for 30 minutes. Any cells that did not migrate were vigorously removed with a cotton swab in distilled water. Transwell inserts were left to dry overnight, then imaged for manual quantification on ImageJ. For each cell line, 3 biological replicates were performed with 3 technical replicates per experiment, and four images were quantified per technical replicate. Two-way ANOVA was performed on data with three biological replicates.

Wound Healing Assay

4T1 murine breast cancer cells were seeded into 6-well plates at a density of 350 000 cells per well. After adhering overnight, triplicate wells were scratched using a P20 pipet tip. Media was changed and images of each wound were taken immediately, then 24 hours later. Quantification of migration was taken as a percentage of the width of the wound at 0 hours compared to the width of the wound at 24 hours. Three biological replicates were performed for each cell line. Wound healing assays with MCF7 cells were conducted in the same manner but with a seeding density of 700 000 cells per well to account for slower doubling time.

Immunoblotting

Lysates were prepared using RIPA containing 10mM Tris (pH 7.4), 1% NP40, 0.1% SDS, 0.1% sodium deoxycholate,150mM sodium chloride, and protease and phosphatase inhibitors (Roche #11697498001 & #4906845001). Cells were lysed using 75-100 ul of complete RIPA and sonicated at 50% power for 4 seconds before centrifuging at 15000g for 15 minutes. Protein concentration was measured by Bradford assay and 50µg of protein per sample was loaded and separated on 10% SDS-PAGE. Protein was transferred onto a PVDF membrane (Roche #0301004001). Membranes were blocked for one hour in 5% non-fat milk then incubated with primary antibody overnight at 4°C. The following day the membranes were washed and incubated with secondary antibody for one hour. The membranes were developed using Amersham ECL Prime Western Blotting Detection Reagent and Immobilon Western Chemiluminescent HRP Substrate.

Antibodies:

Target	Antibody source and catalog number	Dilution
LARP1 (murine)	Protein Tech #67810-1-Ig	1 in 1000
LARP1 (human)	Cell Signaling #14763S	1 in 1000
RPL4	Santa Cruz Biotechnology #sc-100838	1 in 1000
RPS3	Santa Cruz Biotechnology #sc-376008	1 in 1000

RPS6	Cell Signaling #2217	1 in 1000
MNK1	Cell Signaling #2195	1 in 1000
p-eIF4E	Cell Signaling #9741	1 in 1000
eIF4E	BD Biosciences #610270	1 in 1000
Beta actin	Sigma Aldrich #A5441	1 in 5000
Alpha actinin	Santa Cruz Biotechnology #sc-17829	1 in 1000

Proliferation assay

5000 cells were seeded in a 96 well plate and allowed to adhere for 2 hours before being live imaged every 3 hours in the Incucyte SX5 for a duration of 72-96 hours. Images were analyzed for confluency using the Incucyte AI-driven confluence analysis software and proliferation was plotted as a percentage of confluency over time.

Clonogenic assay

Cells were seeded in 12-well plates at densities of 2,000 to 5,000 cells per well and allowed to adhere overnight. Media was changed the next day and 1μM and 2.5μM SEL 201 was added. At experimental endpoint, the cells were stained with 0.5% crystal violet in 70% ethanol for 1 hour. The plates were scanned and clonogenic outgrowth was quantified manually using ImageJ, or were dissolved using 1mL of 10% glacial acetic acid per well for 1 hour on a shaker. Subsequently, absorbance was measured at 590 nm and relative differences were graphed. Two-way ANOVA was performed on data with three biological replicates.

BALB/c mouse models of metastasis

A. Animal experiments were conducted according to the regulations established by the Canadian Council of Animal Care, and protocols approved by the McGill University Animal Care and Use Committee (AUP JGH-6009).**Tail vein injection:** 8-week old female BALB/c mice were ear-tagged one day before the procedure. Cells were trypsinized and resuspended in PBS and kept on ice until time of injection. Mice were

kept under a heat lamp for 5-10 minutes prior to tail vein injection to increase circulation and make the tail vein more visible. 100 000 shLARP1 or shControl 4T1-luciferase tagged cells were injected into the tail vein of each mouse using a 1mL insulin needle (aided by Yvhans Chery LDI Animal Care Facilities). After using gauze to clot the site of injection for approximately 1 minute, mice were returned to their respective cages. Mice were monitored for signs of distress or clotting periodically over the next several hours.

B. **Splenic injection:** 8-week old female BALB/c mice were ear-tagged and shaved one day before the procedure. Approximately one hour before the procedure, mice were given carpofen. Cells were trypsinized, resuspended in PBS and kept on ice until time of injection. All surgery tools were autoclaved before use. During the procedure, all mice were given isofluorane as anesthesia with oxygen. The surgical procedures of this protocol were carried out by Dr. Samuel Preston and Raul Flores. To access the spleen, a small incision was made on the skin just below the ribcage on the left side of the mouse followed by another incision on the muscle layer. Connective tissues were removed using a cotton tip. 100 000 shLARP1 or pLKO control 4T1-luciferase tagged cells were injected into the spleen with a 26_G 1mL needle and the spleen was removed using curved tweezers. After allowing the blood to clot using a cotton tip, the muscle layer was closed using a 13mm 3/8c surgical suture. Approximately 10µL of lidocaine was given before closing the epidermal layer with surgical clips. Topical antibacterial cream was put around the surgical wound to prevent infection. ~500µL of saline was given via subcutaneous injection before removing mouse from anesthesia. Mice were

put into their respective cages and into an incubator at 33°C before being returned to their housing. At 24 and 48 hours post-surgery, carpofen and saline was readministered.

Kinetic curves for IVIS imaging:

After injection of tumor cells, all mice were injected with 100µL per side of D-luciferin via intraperitoneal (IP) injection. Images were taken using the Amit-HT *In-vivo* imaging system (IVIS) machine (LDI Animal Quarters) every 3 minutes immediately after D-luciferin injection until a decline in signal emission was apparent. Luminescence signal from each image were graphed together from which the kinetic curve was generated. The timepoint at which maximum intensity was achieved was determined with the kinetic curve along with the percent total emission (photons/second) which was calculated by dividing all emission signals with the maximum emission.

2. RESULTS

2.1 The role of LARP1 and MNK1 in breast cancer *in vitro*

2.1.1 LARP1 knockdown does not cooperate with MNK1 inhibition to reduce clonogenic outgrowth of 4T1-luc cells

The role of LARP1 in breast cancer cell lines is currently understudied. Our first goal in the project was to characterize how modulating expression of LARP1 would impact the behaviour of breast cancer cells. To do so, we generated three murine TNBC 4T1 cell lines in which LARP1 was stably knocked down using shRNAs (Figure 3a). These cells were also luciferase tagged in anticipation of future *in vivo* work to facilitate live-image tracking of tumor progression in mouse models, and thus will be referred to as 4T1-luc cells from here onward.

In a variety of other cancers, loss or reduction of LARP1 expression impairs cell proliferation (Desi et al., 2022; Hopkins et al., 2016; James et al., 2022). To test if this was also the case with the 4T1-luc cell line, we performed proliferation assays using the Incucyte SX5. Images were taken every three hours over a period of 96 hours and proliferation was measured as a percentage of confluency over time based on AI-generated cell recognition in each well. Cell proliferation over the course of four days was not significantly reduced in LARP1 knockdown cell lines compared to wild-type LARP1 4T1-luc cells (Figure 3a). We next seeded wild-type and LARP1 KD 4T1-luc cells for long-term (7 days) clonogenic assays with either a DMSO control treatment or increasing doses of the MNK1/2 inhibitor SEL201 at 1μM and 2.5μM. Knockdown of LARP1 resulted in significantly reduced clonogenic outgrowth in one of three LARP1 KD cell lines compared to wild-type LARP1 4T1-luc cells; with a trend towards reduced outgrowth in another LARP1 KD cell line (Figure 3b). Additionally, the pharmacological inhibition of MNK1/2 did not significantly impact clonogenic outgrowth of either the wildtype nor the LARP1 knockdown cells (Figure 3b). These data suggest that this model of TNBC may possess some level of MNK1/2 inhibitor resistance; but that loss of LARP1 could attenuate their high proliferative rate.

2.1.2 Targeting MNK1 does not reduce clonogenic outgrowth of LARP1 (short isoform)-overexpressing MCF7 cells

The literature available on the role of LARP1 in cancer suggests that its overexpression increases proliferation and tumor progression (James et al., 2022; Sommer et al., 2011a; Sommer et al., 2011b). To date, there has been very limited information published concerning the role of LARP1 in breast cancer specifically; however, a correlation was identified between high levels of LARP1 in the tumors of breast cancer patients and poorer overall survival compared to patients

with low LARP1 expression levels in their tumors in a database analysis of 1115 breast cancer patients (Hopkins et al., 2016; Stavraka and Blagden, 2015). Therefore, we next sought to characterize the effect of LARP1 overexpression in breast cancer cells to see if its effect on proliferation holds true in our models. In anticipation of experiments wherein we would repress MNK1 expression using the CRISPRi system (Figure 4a), we transduced MCF7 breast cancer cells with a lentiviral system for dCas9 and cultured sorted cells individually. Once validated for functional dCas9 via Western blot and a test gRNA, we selected the E1 clone to move forward with in our MCF7 studies. This cell line was subsequently made to stably overexpress the short isoform of LARP1 (1019aa), which is routinely used in studies assessing the function of LARP1 (Desi et al., 2022; Fonseca et al., 2015; Mura et al., 2015)(Figure 4b).

To understand if LARP1 overexpression impacted proliferation, we made use of the Incucyte SX5 proliferation assay as was used with the 4T1-luc model. We did not observe significant differences in proliferation rates over 48 hours between the short-isoform LARP1 overexpressing MCF7 compared to their wild-type LARP1 counterpart (Figure 4c). Work from our lab, to which I contributed, including work on MCF7 showed that clonogenic outgrowth is impaired in cells treated with the MNK1/2 inhibitor SEL201 (Prabhu et al., 2023). Therefore, we next wanted to assess the impact on clonogenic outgrowth of (1) overexpressing the short isoform of LARP1 (SI-LARP1) and (2) knocking down the expression of MNK1. Performing clonogenic assays with the cell lines either expressing wild-type levels of LARP1 or those overexpressing SI-LARP1, we observed significantly increased clonogenic outgrowth in the SI-LARP1-expressing MCF7 cells. To next investigate whether genetic knockdown of MNK1 alone could alter clonogenic outgrowth of the SI-LARP1-expressing MCF7 cells, we made use of the functional dCas9 in the MCF7 E1 clone by introducing a sgRNA with specificity for the MKNK1 transcript

(encodes MNK1) via lentiviral transduction. The endonucleolytically deactivated (d) dCas9 will silence transcription of the targeted gene when bound to a sgRNA by sterically blocking transcription factors as it fused with Krüppel-associated box (KRAB) transcriptional repressor domain; thereby generating a stable knockdown as opposed to a knockout of MNK1. After selecting cells which had been successfully transduced with the sgRNA using puromycin, we validated MNK1 knockdown by Western blot (Figure 4d). Our data show that MNK1 knockdown alone does not block clonogenic outgrowth in the wild-type LARP1 cells or SI-LARP1-overexpressing MCF7 cells (Figure 4d). We posit that, in this cell line, genetic knockdown of MNK1 alone may not be sufficient to block clonogenic outgrowth as we have previously observed with pharmacologic inhibition of MNK1/2 (Prabhu et al., 2023).

2.1.3 LARP1 modulates invasion of breast cancer cells *in vitro*

LARP1 has been shown to localize in the lamellipodia and to the leading edge of migrating HeLa cells, and its overexpression in cancer cell lines has been shown to increase migration *in vitro* (Burrows et al., 2010). Therefore, we decided to test whether LARP1 contributed to migration *in vitro* by performing a series of wound-healing assays on our 4T1-luc LARP1 KD cell lines. There were no significant differences in migration of cells to close the wound between wild-type LARP1 expressing cells compared to LARP1 KD counterparts (Figure 5a). Addition of SEL201 to these cell lines also did not significantly impact the closure of the wound in these conditions compared to untreated cells (Figure 5a). The 4T1-luc triple-negative breast cancer cell line is highly invasive and we therefore hypothesized that loss of LARP1 may block their invasive phenotype and that addition of pharmacologic MNK1/2 inhibition could potentially cooperate with low LARP1 expression to further reduce invasion. We then performed Boyden chamber assays on these cell lines and found significantly reduced invasion of the 4T1 cells in which LARP1 was

knocked down using 3 different shRNAs (Figure 5b). Furthermore, we did not observe any effect on invasion in any of the 4T1 cell lines tested with the addition of 2.5μM SEL201 (Figure 5b). Knowing that the MCF7 cell line does not readily invade (Ziegler et al., 2014), we performed Boyden chamber assays (Figure 5c) to determine if short-isoform LARP1 overexpression could induce the MCF7 cells to invade a collagen-coated membrane. We observed a significant increase in invasion in the LARP1 overexpressing cells compared to the control MCF7 cell line; indicating that LARP1 overexpression promotes the MCF7 cells to a more invasive state (Figure 5c). In summary, it appears that LARP1 plays a role in promoting cell invasion *in vitro* (Figures 5b and 5c).

Having found that LARP1 has an effect on the invasion of the 4T1-luc and MCF7 cell lines, we sought to verify whether LARP1 impacts invasion of a colorectal cell line, which is known to rely on LARP1 for migration (Desi et al., 2022). We generated a LARP1-knockdown HCT116 colorectal cancer cell line using shRNA (Figure 6a) and repeated our Boyden chamber assay. Here, we saw a robust reduction in invasion in the LARP1 KD HCT116 cells compared to their control counterpart cells; which further supports that LARP1 controls invasiveness of cancer cells (Figure 6b). We also performed wound healing assays on the HCT116 LARP1 KD cells which were treated with 2.5μM SEL 201. Here, SEL201 only inhibited wound closure when LARP1 was knocked down and we did not observe any other significant differences between the LARP1 KD cells and their pLKO control counterparts (Figure 6c).

2.2 The role of LARP1 in breast cancer *in vivo*: Characterizing the impact of LARP1 knockdown in 4T1 cells in models of metastasis in BALB/c.

2.2.1 LARP1 knockdown in 4T1-luc cells reduces their experimental lung metastasis in BALB/c mice

We have been able to elucidate a reliance on LARP1 by breast cancer cells for their invasion *in vitro* by use of the Boyden chamber assay. However, this model of invasion does not depict more the complex steps of metastasis that cancer cells undergo to colonize a secondary organ site, such as the lung and liver. Given that the lung is a major site of metastasis for breast cancer (Tagliabue et al., 2021), we performed tail vein injection of our 3 LARP1 KD 4T1-luc cell lines and the control cell line (Figure 7a) in BALB/c mice; which results in preferential metastasis in the lung. Because the cell line is luciferase-tagged, we monitored lung metastasis development by the use of live-imaging with the Ami-HT system in our animal quarters. Live images were taken every 3 days once a lung-specific signal was established, and tumors were allowed to develop over 14 days. Regular live-imaging revealed that the shLARP1 4T1-luc cell lines were less successful at establishing in the lung than the control counterparts, resulting in lower luciferase emissions across the two-week timeline (Figure 7b). At endpoint, emission levels in the shLARP1 group remained lower that the control group with significantly lower levels in two out of the three shLARP1 cell lines (Figure 7c). These data, coupled with the cell invasion data in Figure 5, show that LARP1 promotes the ability of 4T1-luc breast cancer cells to form experimental lung metastases.

2.2.2 LARP1 knockdown of splenic-injected 4T1-luc cells does not affect experimental liver metastasis in BALB/c

The liver is another significant site of metastasis in breast cancer and, notably, results in a low survival rate for patients who experience metastasis to this site (Pentheroudakis et al., 2006). Therefore, we employed use of another model available in our lab, where tumor cells are injected into the spleen to yield preferential metastasis to the liver. Here, 4T1-luc pLKO or LARP1 KD cells were injected and allowed to anchor in the liver and proliferate for 21 days. Regular live-imaging was used for this model as well every few days to monitor progression. Interestingly, at endpoint, there was no significant difference between the groups neither by luciferase emissions via live-imaging, ex-vivo imaging of the livers, nor by surface metastasis counts (Figure 8a-c). In this model, LARP1 knockdown in 4T1-luc cells does not impact their metastasis the liver.

DISCUSSION

The study of RBPs in cancer is an ever-growing field as their relevance in the initiation and progression of the disease becomes increasingly apparent. Our lab has had a long-standing interest in the impact of the MNK1/2 kinases in affecting the activity of the RBP eIF4E in cancer, and we were quickly intrigued at our finding that the MNK1 kinase also interacts with the RBP LARP1 (unpublished data), which has been established as a pro-oncogenic RBP in several cancers but whose mechanistic role in supporting tumour progression is still cloudy. In this project, we set out to determine if there was biological relevance to this interaction in the context of cancer using models of breast cancer; one of which being a cell line that we validated the MNK1/LARP1 interaction in. We generated models of LARP1 overexpression and LARP1 knockdown which we also challenged with pharmacological MNK 1/2 inhibition via SEL 201 in a series of *in vitro* assays

to measure proliferation, clonogenic outgrowth, migration and invasion. Upon determining that loss of LARP1 expression inhibits cell invasion, we next interrogated whether loss of LARP1 expression could reduce metastasis in the BALB/c mouse model with both tail vein injection and splenic injection of the tumor cells to emulate metastasis to the lung and the liver, respectively. Here, we found that LARP1 knockdown reduces successful metastasis to the lung compared to pLKO control cells; yet does not change metastasis to the liver. My thesis project has revealed LARP1 as a potential player in the invasiveness of breast cancer cells.

Short isoform vs long isoform LARP1

One of the most perplexing aspects of the data gathered for this project is the lack of effect in proliferation when LARP1 was overexpressed or knocked down despite this phenotype being quite consistently conserved across other publications in other cancer models. As discussed, there have been two LARP1 isoforms identified to be expressed, particularly in the MCF7 cell line and in breast cancer—the 1096aa-length isoform, and the 1019aa-length isoform (Eswaran et al., 2013; Schwenzer et al., 2021). The constructs that have been widely used in the literature until very recently (Ramani et al., 2022) are those containing the short-isoform LARP1 sequence (Desi et al., 2022; Fonseca et al., 2015). This is contrary to the fact that the long isoform of LARP1 is documented to be the dominant form of the protein expressed in cancer cell lines and in humans (Schwenzer et al., 2021). Our lab obtained a construct containing the long isoform sequence which I attempted to transfect into both MCF7 and 4T1-luc cells; however, did not find success in overexpressing either cell line using this construct (Table 1). In my own experience, the band for the short isoform can be observed in cancer cell lines other than MCF7 which indicates that its expression may be more widespread than was originally suggested. It is possible that these cell lines preferentially make use of the long isoform and that overexpression of the short isoform is

not enough to change the functions already being carried out by the dominant form in terms of migration and proliferation alone; as in other studies LARP1 expression has a greater impact on these characteristics than was observed in our studies. Additionally, this would explain why we did observe differences in proliferation with the use of some of the LARP1-specific shRNA; as the short and long LARP1 isoforms only differ at the beginning of the first exon (Figure 2), whereas our hairpins were designed to target regions of the transcript that are shared by both isoforms. While it has not been published, it would be important to look into pan-cancer expression of both the long and short isoforms of LARP1 to have a clearer understanding of the levels of each of these isoforms and how they could be relied upon by different types of tumor cells.

Murine vs. human LARP1

The cell models used in this project were of human and murine origin, which means that another point of contention in this study is whether there are significant differences between murine and human LARP1. For each of the shRNA used in our 4T1-luc cell lines to generate a knockdown of LARP1, the sequences had high fidelity (Table 1) with both the murine and human variants, and as was shown in our Western blots (Figures 3, 6, 7) we were able to robustly knockdown the protein in the murine breast cancer cell line. In our overexpression model in MCF7, the construct used is for the short isoform of the human LARP1. It seems that in the context of this project, LARP1 is promoting the invasion of the breast cancer cell lines used here regardless of the species.

LARP1 vs LARP2/LARP1b

One consideration for redundancy in the models used in this project is the homology shared between LARP1 and LARP2/LARP1b. LARP2 also possesses a DM15 region and is therefore capable of binding the 5'cap of its target transcripts as well (Figure 2). However, little is known

about the target specificity of LARP2 given that very few studies have been published with any focus on the protein given that the knockout of LARP1 was first shown to produce a much stronger phenotype in drosophila (Burrows et al., 2010). Database searches show that LARP2 has been a hit in many CRISPR screens and has been correlated with increased proliferation and resistance to certain treatments such as platinum-based agents and antimetabolites such as gemcitabine (Gobbi et al., 2019; Goodspeed et al., 2019; Ramaker et al., 2021). Similar to the short isoform of LARP1, LARP2 differs from LARP1 mainly at the C terminal of the protein and has a length of 914aa (Stavraka and Blagden, 2015). LARP2 shares ~60% identity with LARP1 in terms of its amino acid composition (Altschul et al., 1997). Nevertheless, this does not necessarily indicate that its structure is identical to that of LARP1 – there could potentially be major differences in protein folding given the ~40% non-homologous components. Therefore, despite these proteins sharing the DM15 and LaM motifs (responsible for binding of the 5'cap and PABP, respectively), there may be large differences in their 3D structures that could affect their effector functions that lead to LARP1 being more largely relied upon by cells in a normal and/or oncogenic state. Alternatively, LARP2 could possesses just enough similarity that it could potentially compensate for loss of LARP1 expression in some functions; such as in the context of tumor cells relying on La-related proteins for migration in our LARP1 knockdown 4T1 cell lines given that we expected to see an effect on migration based on the effect of LARP1 in other cancers.

Steps of metastasis and how might LARP1 be involved in some steps over others

It is important to also note the limitations of our models. For example, in our Boyden chamber assays, the coating used in the chambers was collagen I-based. This coating most closely models the interstitial matrix rather than, for example, the basement membrane, which is the first major obstacle epithelial cancer must overcome in terms of metastasizing. The basement

membrane is a complex extracellular matrix composed of several proteins; mainly, collagen IV, proteoglycans, and laminin and nidogen complexes (Paulsson, 1992). In this case, it would be useful to test the cells' capacity to invade chambers with more complex coating such as Matrigel; or even to test migration through a 3D ECM model, particularly with the 4T1-luc model as it is more aggressively invasive compared to the MCF7 model. A more simple method would also be to coat wells with various components (including Matrigel, collagen, fibronectin, and laminin), either separately or as a mixture; and to allow cells to 'invade' from the surface followed by quantification of cells adherent to the bottom using crystal violet staining (Yodkeeree et al., 2010).

Furthermore, LARP1 overexpression was shown to produce more lamellipodia (which have greater protrusion forces compared to filopodia) in HeLa cells compared to their relative control; a structure known to be important not only for adhesion, migration, and angiogenesis; but also cell-cell contacts (Burrows et al., 2010). If this effect is preserved in breast cancer, it is especially interesting given the phenotype we observe with our models in invasion and metastasis. An emerging factor being characterized in metastasis is the formation of invadopodia, which are a stable structure similar to lamellipodia and contain many actin-regulating proteins, but which also can last for several hours and secrete matrix metalloproteinases capable of aiding cancer cells to invade through dense ECM (Augoff et al., 2020). Verifying these structures *in vivo* remains a challenge; however, it would be fascinating to see if LARP1 can also localize to these protrusions and to perform experiments to understand if the RBP could also be aiding translation in these structures *in vitro*. For example, executing a technique such as the RiboPuromycylation Method (RPM) to map out active translation sites within the cell while using a co-stain for LARP1 could answer the question of whether LARP1 is participating in these structures as a mediator of translation of its targets or if the possibility of LARP1 fulfilling another function here should be

explored. The main method used to distinguish invadopodia from typical cellular protrusions is fluorescent confocal microscopy staining for cortactin (whose phosphorylation by the Arp2/3 complex is thought to help recruit to invadopodia filaments to enable more actin polymerization), and actin itself to reveal their characteristic F-actin core which can further reveal the various organizations of invadopodia, which include aggregates, rosettes, dots, or linear formation (Di Martino et al., 2016; Rosenberg et al., 2017). Further staining could be done on other proteins which are localized within invadopodia such as N-WASP, Arp2/3 complex members, as well as RTKs such as ERK1/2 and Src to distinguish these structures (Ayala et al., 2008; Yamaguchi et al., 2005). These aggressive structures are also studied in ECM models; where aggressive cell lines are plated in ECM to observe the degradation of the matrix over time (Rosenberg et al., 2017). Employing these types of assays on the LARP1 KD and pLKO control 4T1-luc cells could help uncover if the RBP can contribute to the formation and/or pro-invasive function of invadopodia.

Ultimately, it is important to emphasize that in the *in vivo* models we used, we are missing the characterization of the early steps in the metastatic cascade. In breast cancer, it is known that metastatic dissemination can occur early in the disease; where few tumor cells escape the primary site and remain dormant for prolonged periods before establishing and proliferating at new, distal sites (Massagué and Ganesh, 2021). In this regard, the tail vein injection model is particularly useful as it can give an indication of how tumor cells survive in the vasculature as well as how well they can extravasate and establish in a 'new' site, such as the lung. Here, cells must survive a variety of stress sources such as oxidative and biomechanical stress as well as evading targeting by immune surveillance; and, furthermore, the extravasation process itself does involve traversing a basement membrane as vessels themselves are bordered by such a barrier (Baluk et al., 2003). From the literature, we know that LARP1 is implicated in the motility of several cell types and can

localize to leading edges of HeLa cells which are rich in actin-based structures (Burrows et al., 2010). If this localization is preserved across models, LARP1 could be participating in the maintenance of the cytoskeleton in a way that gives an advantage to LARP1-high cells in surviving the mechanical damage that can occur in the bloodstream, or by helping to facilitate junction formation with other cell types in circulation to help ensure their survival. Overall, any future work on this project would benefit from the use of a primary tumor model; for example, the orthotopic mammary fat pad injection of 4T1 cells into BALB/c mice. With this experiment, we would be able to determine if LARP1 knockdown has impacts on not only tumor initiation and outgrowth; but also monitor for lung metastasis of both the pLKO control and LARP1 KD tumors.

Moreover, tumors could be collected and dissociated for spectral flow cytometry analysis to look for any differences in immune cell infiltration of primary and metastatic tumors between LARP1 KD and respective control cells. To date, there is no literature indicating a direct link between LARP1 and secreted factors which could facilitate or hinder immune cell recruitment in either a cancer or non-cancer context. However, given some of the prolific mRNA targets of LARP1—including mTOR itself (Mura et al., 2015)—suggests that altering its expression could have a notable impact on factors secreted by the cell. mTORC1 is a critical regulator of autophagy; a process which functions to clear the cell of unwanted material such as protein aggregates, damaged organelles, and intracellular pathogens; and thus is a crucial part of the release of immunostimulatory molecules as well as MHC presentation (Jiang et al., 2019). If loss of LARP1 and its stabilizing effect on mTORC1 transcripts in turn impacts this process, one could hypothesize that this could lead to reduced adaptive immune cell recruitment; more specifically, CD8+ cells; as it has been shown that cancers with increased autophagy harbor fewer Foxp3+ T regulatory cells and greater numbers of CD8+ cells (Ladoire et al., 2016; Zhong et al., 2016). This

would be an interesting aim to investigate in an orthotopic breast cancer model of the 4T1-luc cells injected into the mammary fat pad of female BALB/c mice.

Factors affecting 4T1-luc colonization of the lung niche compared to the liver

One of the most curious pieces of this study is the difference we observed between the impact loss of LARP1 expression has on metastasis of the 4T1-luc cells in the model for the liver compared to the model for the lung. In each of these models, the tumor cells are injected directly into the bloodstream; however, they differ at the site of tumor cell implantation. The microenvironment can have massive importance in determining the success of a tumor cell in establishing a colony in a new parenchyma and there are several notable differences between the microenvironment of the lung compared to the liver. Firstly, the liver is largely composed of hepatocytes along with nonparenchymal cells such as Kupfer cells, sinusoidal endothelial cells, Ito cells and liver associated lymphocytes (Brodt, 2016). These cells all contribute to establishing an ECM unique to the liver by secreting a variety of growth factors and proteins. Comparatively, the composition of the lung microenvironment is a more varied depending on the location within the organ. In the proximal airway, one can find ciliated cells, secretory club cells, undifferentiated basal cells, goblet cells and neuroendocrine cells. The distal airways are mainly composed of alveolar type I and type II cells; the type I cells being a platform for gas exchange and therefore lacking many organelles, while the type II serve as both structural support for the damage the type I cells undergo and being progenitor cells for both types (Ward and Nicholas, 1984). Additionally, throughout the lung one can find smooth muscle cells, endothelial cells, fibroblasts, resident alveolar macrophages and dendritic cells as well as a variety of other immune cells (Altorki et al., 2019). These differences in the tumor microenvironments, immune and stromal cell, of the liver and lung could help explain our findings that LARP1-low cells have an impaired ability to colonize

the lung from the bloodstream whereas this deficiency does not impede their colonization of the liver; particularly if loss of LARP1 has any impact on a tumor cell's ability to evade immune surveillance. We know that LARP1 supports Bcl-2 transcript stability in ovarian cancer cells which aids in their evasion of apoptosis (Hopkins et al., 2016). This also impacts the tumor cells' vulnerability to immune-mediated apoptosis—for example, it has been shown that targeting Bcl-2 using small-molecule inhibitors in high-Bcl-2-expressing lymphoma and melanoma cells increased their immune-mediated destruction in co-culture with NK cells (Lickliter et al., 2007). Nevertheless, validating the ovarian cancer cell findings of LARP1 in stabilizing this anti-apoptotic transcript in our breast cancer cell models would be an important step in elucidating its tie to the tumor-immune dynamic.

In addition to differences in the microenvironment purely related to the host and the populations within each organ, it has also been characterized that breast tumor cells themselves have distinct characteristics depending on their preferred site of metastasis. In particular, the metabolic activity of breast cancer cells can be a major factor in their organotropism. It is well-known that cancer cells in general have altered metabolism; for example, the Warburg effect, where cancer cells gain a signature increase in aerobic glycolysis (Warburg, 1956). As the field of metabolic research in cancer has grown, we have been able to gain much more insight further than the Warburg effect as to how cancer cells can reprogram themselves to accommodate their proliferative or invasive needs. Recently, it was found that metastatic breast cancer cells tend to have higher glycolytic and OXPHOS activity compared to non-metastatic tumor cells (Dupuy et al., 2015). Even more poignant to our data here from the latter work, 4T1 cells that underwent *in vivo* selection for organotropic potential were found to have altered metabolic gene expression depending on the organ from which they were cultured; with the liver-metastatic 4T1 cells showing

enrichment for the glycolytic pathway and reduction in the TCA cycle and OXPHOS pathways compared to parental, bone-metastatic, and lung-metastatic 4T1 cells (Dupuy et al., 2015). They were also able to show that the bone- and lung-metastatic 4T1s had increased glucose and glutamine usage compared to parental 4T1 cells whereas liver-metastatic cells were found to use less. Furthermore, in primary tumor cells, the Dupuy group was able to show that liver-metastatic tumor cells have higher HIF-1α activity compared to parental cells and that loss of HIF-1α via shRNA significantly increased the cells' respiration; leading them to conclude that HIF-1α is an important player in the metabolic reprogramming of liver-metastatic cells (Dupuy et al., 2015). It would be fascinating to investigate whether this organotrophic-specific metabolic reprogramming could be a factor in the LARP1 KD 4T1-luc cells' ability to colonize the liver compared to the lung in the BALB/c model and impact the translation of mRNA that lend to either a more OXPHOS-dependent cell (as was found to be more lung or bone organotrophic) or a glycolysis-dependent cell (as was found to be more liver organotrophic). Outside of its 5'TOP targets, LARP1 has been known to interact with the 3'UTR of non-5'TOP mRNA (Desi et al., 2022; Hopkins et al., 2016); in this context, one could perform an RNA-IP (RIP) followed by RT-qPCR of the RIP products with primers specific to HIF-1α and relevant target genes. This experiment could be followed up by assays to determine if LARP1 is playing a role in the stability of these transcripts by performing RT-PCR and/or reporter-based assays on wild-type and LARP1 KO cells.

Limitations of models

There are several limitations of the models we used to interrogate the relevance of LARP1 in breast cancer and what potential importance the LARP1/MNK1 interaction could hold here. Firstly, this work used limited models of breast cancer. As discussed in the introduction section, the classification of breast cancer into various subtypes helps physicians to decide and appropriate

treatment regimen and, with further characterization of the cells within a patient's tumor, can help to predict disease outcome. These facts alone demonstrate the heterogeneity of breast cancer that has long remained a challenge in the clinic and it stands to reason that one of the outcomes of such heterogeneity is variability in the oncogenes and proteins relied upon by each tumor cell subset. The MCF7 cell line is an example of a human HR+ breast cancer while the 4T1-luc cell line is an example of murine TNBC. Future research, building on this thesis work, should include multiple breast cancer subtypes. For example, it would have been interesting to have a variation in the aggressiveness of each subtype. As mentioned, the MCF7 cell line we used is not an exceptionally invasive tumor cell. It would have been interesting to see if knocking down LARP1 with our shRNA in an aggressive human breast cancer cell line would elicit a similar response to what we observed in the 4T1 model. Additionally of note, based on previous data published from our lab, we expected the MCF7 cell lines we generated to harbor greater sensitivity to MNK inhibition (Prabhu et al., 2023). It is possible that, since the cell lines generated from the MCF7 model and the 4T1 model appear to be somewhat resistant to MNK inhibition in terms of its effect on proliferation, clonogenic outgrowth, migration and invasion, it may be better to use a model that is more sensitive to MNK inhibition to elucidate the functional relevance of the MNK1/LARP1 interaction in future work.

The 4T1 model was very useful in that it was syngeneic with our BALB/c mice and made for convenient transition to *in vivo* studies; however, murine and human models do not always align in their behavior. One aggressive human TNBC cell line that could be used in the future is the MDA-MB-231 cell line; which is known to be poorly differentiated, highly invasive, and therefore could also serve as an additional model to test the impact of LARP1 loss on cell invasiveness and metastasis. It appears that LARP1 has a propensity for being relied upon in

epithelial cancers and so being able to characterize the effects of overexpressing and/or knocking down LARP1 in less differentiated cell types would be a great tool for understanding this RBP's role in cancer. As for murine cell lines, there are several examples of other TNBC and HR+ cell lines to be explored: for HR+, E0771, SSM2 and SSM3 are a few popular models, as well as the recently established B6BC which is compatible with the C57BL/6 murine model; and for TNBC, there are the Py230 and Py8119 cell lines which are both derived from the C57BL/6 murine model (Bushnell et al., 2021; Maria et al., 2023; Steenbrugge et al., 2019). Using a second murine TNBC model such as the Py230 line has the advantage in that it has been cited to possess both unique disease progression and immune cell recruitment compared to that of the 4T1 model (Steenbrugge et al., 2019). It is well known that the main challenge of targeting TNBC is how heterogeneous it is and if a common link could be made (for example, with LARP1) between two such different models of the TNBC cell type would be a step forward in the study of this most aggressive form of the disease.

Given we are studying an RBP, it is important to understand how we are impacting active translation in our cell lines rather than steady-state protein levels. This is a limitation of using the Western blot technique for target validation (for example, of ribosomal proteins) following LARP1 overexpression or LARP1 knockdown. In our models, we did not observe consistent ribosomal protein patterns that were congruent with what has been published in the literature; such as correspondent increases or decreases of RPS6, RPL14, or RPS3 with LARP1 expression which were meant to serve as a secondary confirmation for the overexpression or knockdown of the RBP (Philippe et al., 2020; Ramani et al., 2022). A qPCR approach could be more useful here in observing these changes with LARP1 knockdown or overexpression. Another technique that would give insight to the impact of LARP1 on 5'TOP mRNA expression would be polysome

profiling; which would allow us to capture the active translation of each 5'TOP mRNA in response to LARP1 knockdown with our shRNA or with overexpression of the short isoform of LARP1.

CONCLUSION

The goal of this project was to determine the relevance of the RBP LARP1 and, as was recently identified by our lab, its interaction with MNK1 in breast tumor cells given the following: Firstly, the prominent role for the activity of the MNK1/2 kinases in breast cancer already established; secondly, that breast tumor cells were one of the cancer cell lines in which we validated this interaction; and finally, the noted yet greatly underexplored correlation of high LARP1 expression and poorer outcome in breast cancer. We successfully generated a number of cell line models for in vitro and in vivo use. We generated a model of LARP1 knockdown in the 4T1 cell line which we tagged with luciferase for *in vivo* tracking of tumor cells and a model of LARP1 overexpression in MCF7 cells from which we were able to also generate a dual LARP1 overexpression/MNK1 knockdown model using the CRISPRi system. Using these cell lines, we modelled proliferation, migration, and invasion *in vitro* and, contrary to what has been noted by other groups in models of other cancers, we did not find that altering LARP1 expression had a consistent effect on either proliferation or migration of breast tumor cells. The addition of pharmacological MNK1 inhibition also did not impact nor cooperate with LARP1 loss in attenuating any of the previous phenotypes. Interestingly, we found that LARP1 had a striking effect on the invasion in both our MCF7 and 4T1-luc models in that LARP1 supported the invasion of both of these cell lines.

Having identified LARP1's implication in breast cancer cell line invasiveness *in vitro*, we utilized two different models of metastasis in BALB/c to emulate metastasis to each the lung and

the liver; two of the most common sites of metastasis for breast cancer. Here, we were intrigued to find that loss of LARP1 significantly reduced the 4T1-luc cell's capacity to anchor and proliferate in the lung following tail vein injection; and yet, in our model of liver metastasis via splenic injection, we did not observe changes in tumor burden of LARP1 knockdown cells compared to pLKO control. These experiments in particular herald further work on the effect of LARP1 on breast tumor invasiveness; more specifically, to look at primary tumor escape and subsequent metastasis as opposed to starting the tumor cells in the bloodstream.

The work of this project provides not only additional evidence that LARP1 is a noteworthy RBP to study in breast cancer; but also opens a doorway on a new facet of its functions as an oncogenic RBP in general. These data suggest that it is not necessarily for the sake of proliferation or migration that these tumor cell rely upon LARP1 for, but specifically for their invasion through the ECM. While the purpose of the LARP1/MNK1 interaction remains to be determined in the context of a tumor cell, this project provides a baseboard for the study of LARP1 as a pro-invasive, potentially targetable RBP in the disease of breast cancer.

FIGURES

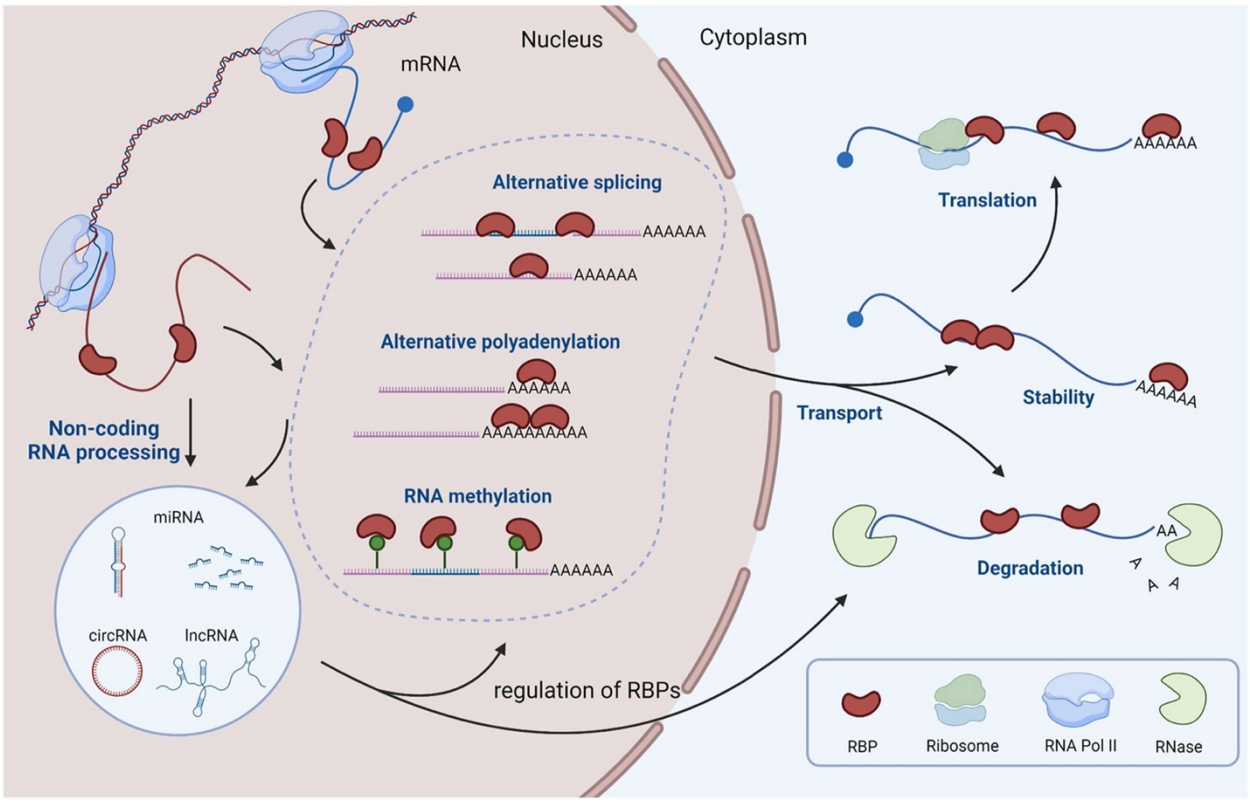

Figure 1 (Wang et al., 2022): Post-transcriptional regulation of RBPs in cancer metastasis. The synthesis of mRNAs and ncRNAs is accomplished by RNA polymerase II (Pol II). Various RBPs function as regulators of RNA maturity-associated processes, such as alternative splicing, alternative polyadenylation, stability, methylation modification, localization, and translation. ncRNAs, primarily miRNAs, lncRNAs, and circRNAs, are processed by RBPs and, in turn, interact with RBPs to influence their function.

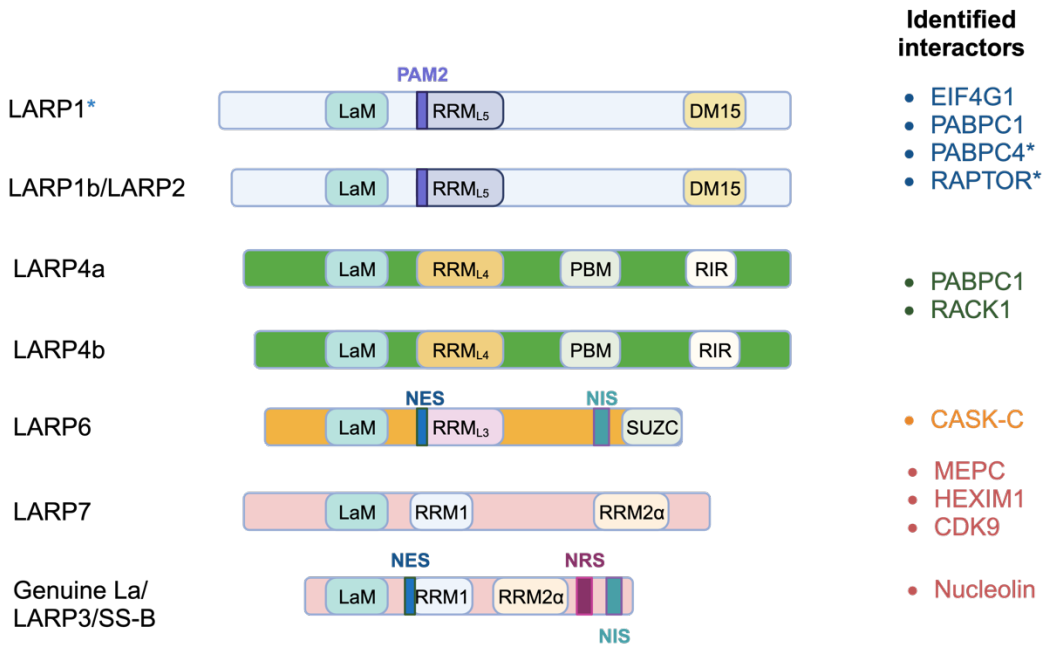

Figure 2: The La-Related Protein Superfamily and their interactors. Each of the seven LARP family members found in humans, their respective domains, and proposed interacting proteins. An asterix indicates that the given interacting protein only corresponds to one member of a subset of the family. The La Module (LaM) is conserved throughout the family while the other domains, including their RNA Recognition Motifs (RRMs), vary. Other domains include: DM15: otherwise known as the LARP1 motif; PBM: Poly-A Binding motif; RIR: RACK1 interacting motif; the SUZC domain; NES: Nuclear export sequence; NIS: Nuclear import sequence; and NRS: Nuclear retention sequence.

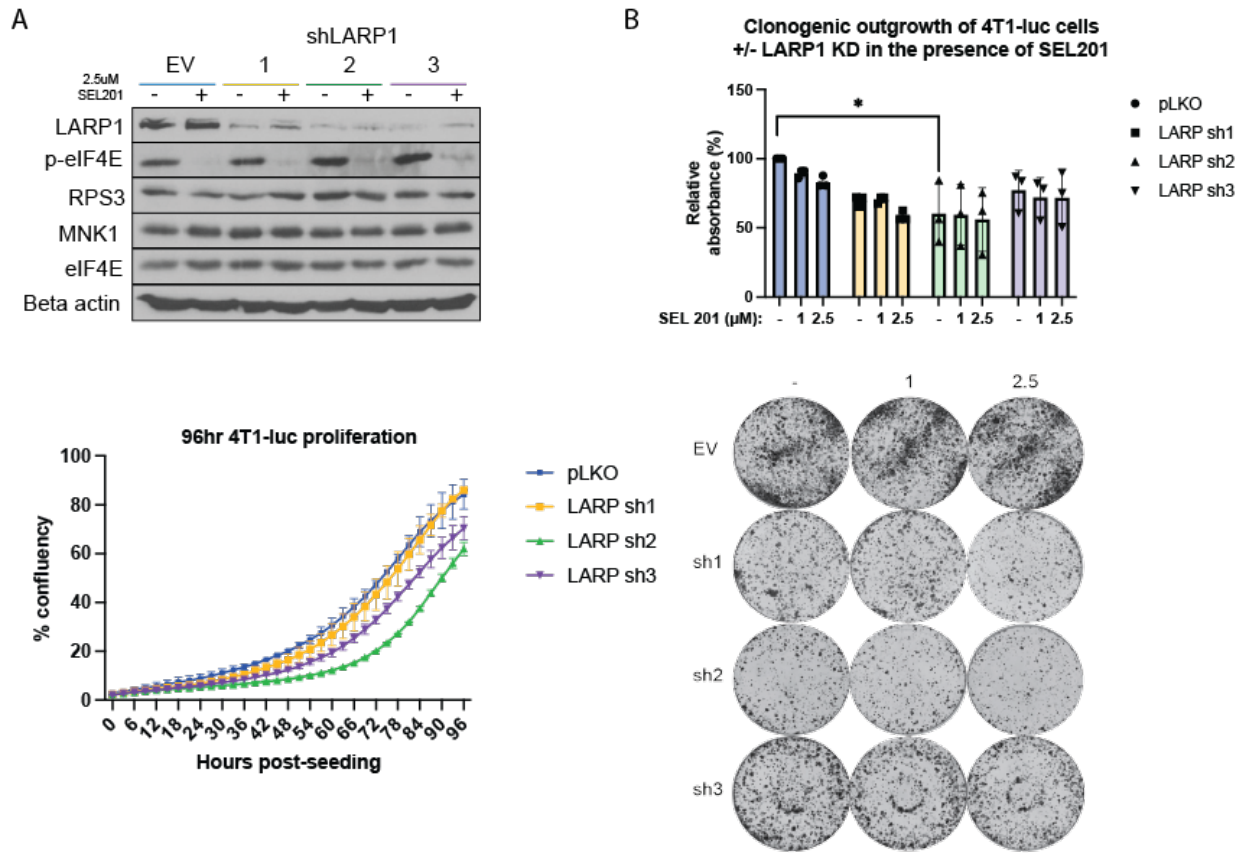

Figure 3: LARP1 knockdown does not cooperate with MNK1 inhibition to reduce clonogenic outgrowth of 4T1-luc cells. A Top panel: Confirmation of LARP1 knockdown via shRNA and confirmation of MNK1 targeting repression of p-eIF4E via 2.5μM SEL 201 treatment . **Bottom panel:** Proliferation of control pLKO 4T1-luc cells compared to shLARP1 4T1-luc cells. **B Top/Bottom**: Clonogenic assay demonstrating the effect of LARP1 knockdown (using 3 different shRNA) in combination with 1 or 2.5μM SEL 201 in 4T1-luc cells across 3 biological replicates. Two-way ANOVA pLKO vs sh2 *P*=0.0165.

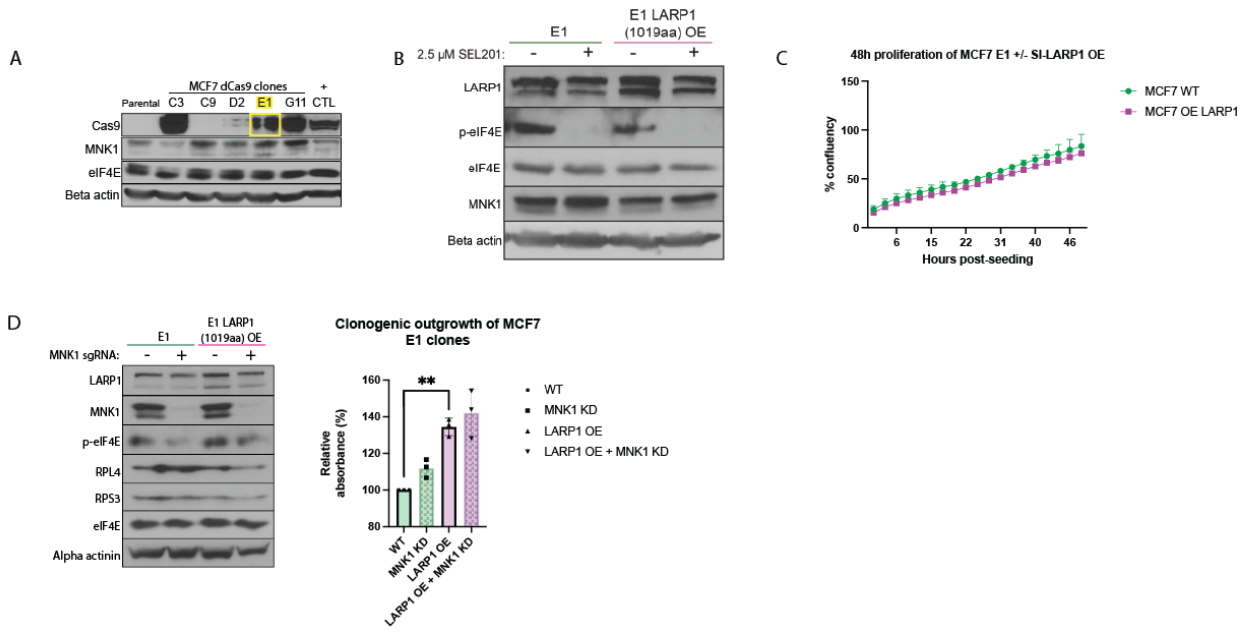

Figure 4: Targeting MNK1 does not reduce clonogenic outgrowth of LARP1 (short isoform)-overexpressing MCF7 cells. A Confirmation of dCas9 expression in MCF7 clones single cell-sorted for Cas9 positivity. **B** Western blot confirming overexpression of short isoform (1019aa) LARP1 and to confirm targeting of MNK1 via reduction of p-eIF4E following 2.5μM SEL 201 treatment. **C** Proliferation of MCF7 E1 compared to LARP1 OE counterpart across three biological replicates. **D Left Panel:** Confirmation of functionality of CRISPRi-compatible dCas9 by introduction of sgRNA targeting MNK1. **Right panel:** Clonogenic assay demonstrating that genetic loss of MNK1 via CRISPRi does not impact MCF7 WT nor LARP1 (1019aa) OE cells. LARP1 (1019aa) OE significantly increases outgrowth in MCF7 WT LARP1 cells across three biological replicates. One-way ANOVA WT vs LARP1 OE P=0.0012.

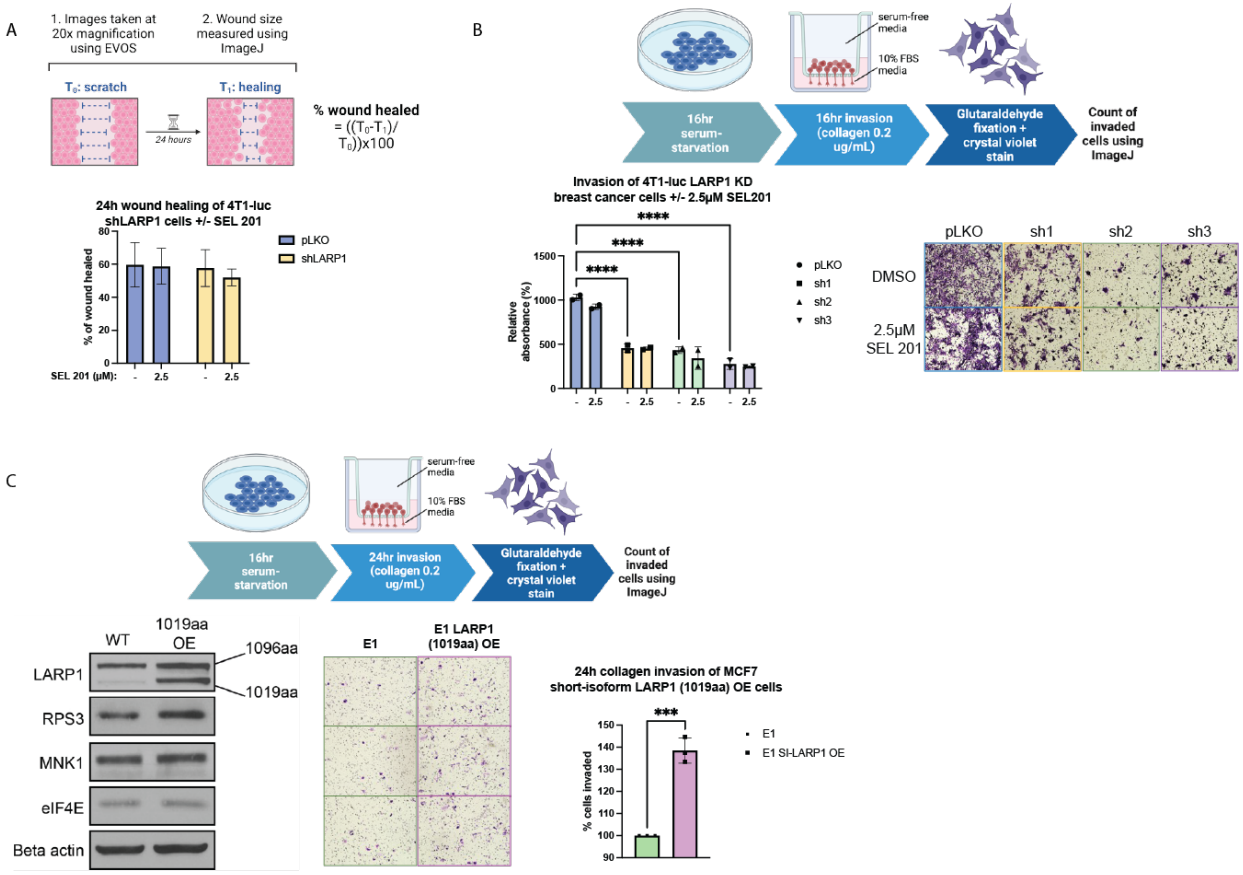

Figure 5: LARP1 modulates invasion of breast cancer cells *in vitro.* **A** Wound healing assay demonstrating that LARP1 KD in 4T1-luc cells does not change their capacity to migrate and close the wound across 3 biological replicates. Schematic generated with Biorender.com. **B** Boyden chamber assay in which 4T1-luc cells were serum-starved and allowed to invade a collagen-coated chamber for 16 hours showing that LARP1 KD using 3 different shRNA reduces their invasion while 2.5μM SEL 201 treatment had no significant effect across two biological replicates. Schematic generated with Biorender.com. Two-way ANOVA pLKO vs all shLARP1 *P*=<0.0001. **C** Boyden chamber assay of MCF7 E1 cells demonstrating that LARP1 (1019aa) OE increases their invasion through a collagen-coated membrane compared to WT LARP1 MCF7 E1 cells across three biological replicates. Unpaired t test E1 vs E1 LARP1 (1019aa) OE *P*=0.0003.

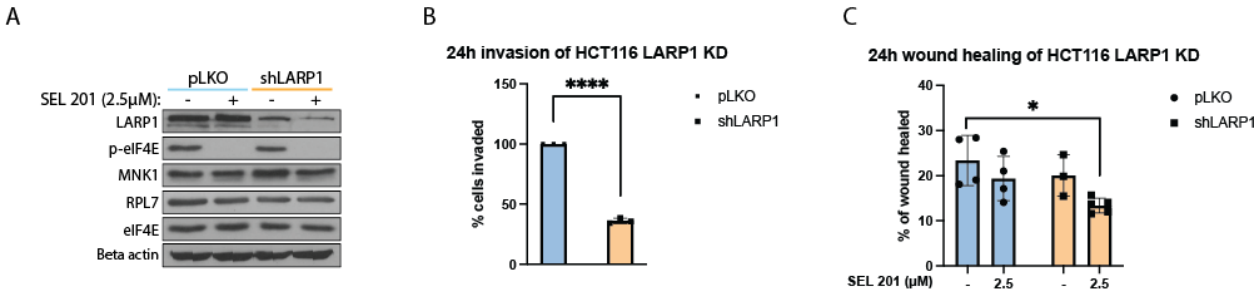

Figure 6: LARP1 KD reduces invasion of colorectal cancer cell line HCT 116. A Validation of LARP1 KD in HCT 116 using shRNA and MNK1/2 inhibition confirmed via p-eIF4E repression. **B** Boyden chamber assay of HCT 116 cells demonstrates that knockdown of LARP1 reduces their invasion over 24 hours through a collagen-coated membrane over three biological replicates. Welch's t test pLKO vs shLARP1 *P*=<0.0001. **C** Wound-healing assay demonstrating that SEL201 only significantly inhibited wound closure in the HCT116 cells when LARP1 was knocked down across three biological replicates. Two-way ANOVA pLKO vs shLARP1 2.5μM SEL 201 P=0.0133.

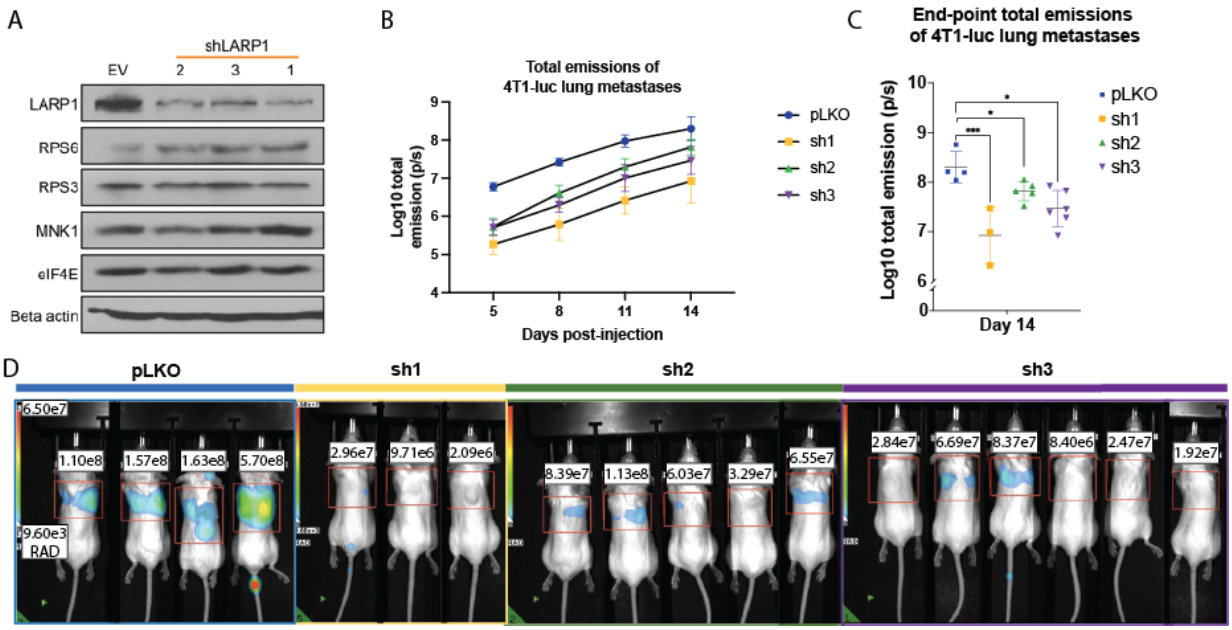

Figure 7: LARP1 knockdown in 4T1-luc cells reduces their experimental lung metastasis in BALB/c mice. A Confirmation of LARP1 knockdown in 4T1-luc cells prior to their injection into 8-week old BALB/c female mice. **B** Total emissions of 4T1-luc cells following injection of luciferin captured by live imaging using the AMI-HT system over the 2-week period following successful establishment of the cells in the lung; revealing 4T1-luc cell lines were less successful at establishing in the lung than the control counterparts. **C** Total emissions of 4T1-luc cells following injection of luciferin captured by live imaging using the AMI-HT system captured at endpoint (14 days post-injection of cells) reveal that LARP1 KD reduces outgrowth of 4T1-luc cells that metastasize to the lung following tail vein injection. One-way ANOVA pLKO vs sh1 P=0.0009; pLKO vs sh3 P=0.0133. **D** Live image data from endpoint (Day 14) revealing lower emission levels in the shLARP1 groups compared to the pLKO control group.

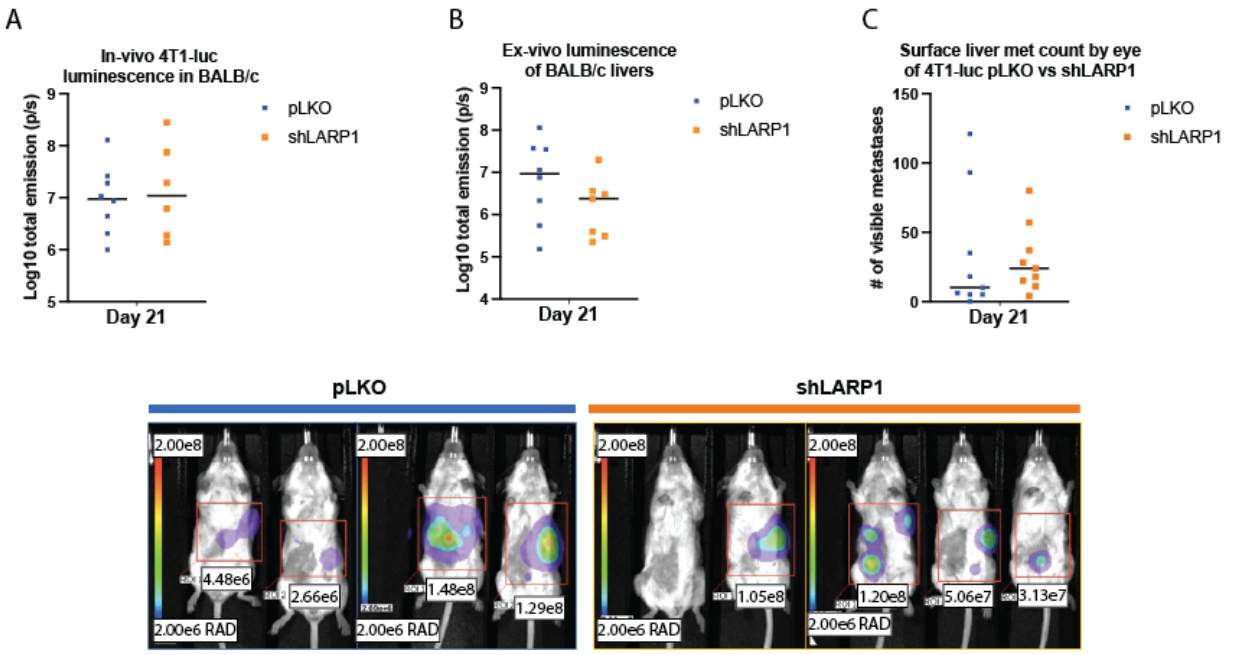

Figure 8: LARP1 knockdown of splenic-injected 4T1-luc cells does not affect experimental liver metastasis in BALB/c. A Total emissions of 4T1-luc cells following implantation in the liver reveal no differences between LARP1 KD and pLKO control cells. **B** Total emissions of ex-vivo imaged BALB/c livers shows no difference between pLKO control and LARP1 KD 4T1-luc tumor burden. **C** Metastases counted by eye on the surface of BALB/c livers also show no difference between shLARP1 and pLKO tumor burden.

		Specificity		Survival of selection			
Vector/Seq. information	shRNA target region	Mouse	Human	MCF7	4T1	KD?	OE?
FLAG-LARP1 ORF (1096aa)	N/A	N/A	N/A	N	N	N/A	N
plx317 LARP1 (1019aa)	N/A	N/A	N/A	Y	N	N/A	Y
shM1	3' UTR	100%	89%	N	Y	N	N/A
shM2	3' UTR	100%	89%	N	Y	Y	N/A
sh24	Coding sequence	89%	100%	N	Y	Y	N/A
sh91	Coding sequence	100%	100%	N	Y	Y	N/A
sh43	Coding sequence	89%	100%	N	N	N	N/A

Table 1: Tools used to generate models of LARP1 overexpression or knockdown.

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