Defining the role of Afadin in Breast Cancer metastasis

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TABLE OF CONTENTS

ABSTRACT

Breast cancer is the most commonly diagnosed cancer in women. The loss of cell-to-cell adhesions between adjacent epithelial cells has been associated with cancer metastasis, the deadliest aspect of cancer. Claudin-2 and afadin are cell-cell adhesion proteins that interact with one another and influence cancer progression. Claudin-2 is a tight junctional protein that has been shown to have pro-tumorigenic functions in breast cancer lung and liver metastasis. Afadin is a multi-domain scaffold protein that is commonly found in both adherens and tight junctions, where it plays both structural and signal-modulating roles. Furthermore, afadin has been shown to have both tumor suppressive and pro-tumorigenic functions.

To gain a better understanding of how these proteins interact and to better define the role that afadin plays in promoting cancer metastasis, afadin was knocked out in a breast cancer cell line. Mutant forms of afadin were then generated where each individual functional domain was deleted. These mutants were then re-expressed in breast cancer cells lacking endogenous afadin. These afadin mutants were assessed *in vivo* for their impact on primary mammary tumor growth and subsequent metastasis to the liver and lung. Most afadin mutants slightly delayed the growth of primary mammary tumors relative to the parental cell line but exhibited similar growth when compared to breast cancer cells reconstituted with the s+lAF6 isoforms. Loss of each afadin domain led to decreased lung-metastatic burden. Loss of each afadin domain also had some degree of impact on liver metastasis, where the RA2 domain had the least effect. ZO-1 was also investigated as a protein that may interact with afadin and/or claudin-2 to affect cancer progression. Stable knockdown of ZO-1 inhibited lung metastasis, but had no effect on liver metastasis. These findings help to elucidate the complex roles exerted by afadin during cancer progression and suggest that afadin and claudin-2 may interact in different ways to promote this process depending on the site of metastasis. However, it is possible that afadin and claudin-2 may also interact through another protein or instead function in parallel signaling pathways to promote metastasis.

RÉSUMÉ

Le cancer du sein est le plus fréquemment diagnostiqué chez les femmes. La perte d'adhérence cellulaire entre cellules épithéliales adjacentes a été associée à la formation de métastases cancéreuses. Ce sont ces dernières qui représentent l'aspect le plus mortel du cancer. Claudin-2 et afadin sont deux protéines impliquées dans l'adhésion cellule-cellule qui interagissent ensemble et pour lesquelles une implication dans la régulation de la progression cancéreuse a été démontrée. Claudin-2 est un constituant des jonctions serrées qui s'est avéré avoir des fonctions pro-tumorigènes dans l'établissement de métastases pulmonaires et hépatiques du cancer du sein. Pour sa part, afadin est une protéine d'échafaudage multi-domaine que l'on retrouve couramment au sein des jonctions d'adhérence et des jonctions serrées. Elle y joue à la fois des rôles structurels et de modulation des signaux. Il a également été démontré que afadin peut, en fonction du contexte, avoir à la fois des fonctions promotrices ou suppressives de tumeur.

Pour mieux appréhender comment ces protéines interagissent ainsi que le rôle joué par afadin dans les métastases cancéreuses, son expression a été éliminée dans une lignée cellulaire du cancer du sein. De plus, différentes formes mutantes de afadin ont été générées dans lesquelles chaque domaine fonctionnel a été supprimé individuellement. Par la suite, ces versions mutantes ont été réexprimés dans le modèle knock-out pour afadin. Ce panel de mutants a été évalué in vivo pour étudier les effets sur la croissance tumorale et la capacité à établir des métastases hépatiques et/ou pulmonaires. En comparaison au contrôle parental, l'expression de la majorité des mutants a entraîné un léger retard dans la croissance des tumeurs primaires. En revanche, tous avaient une courbe de croissance similaire par rapport au contrôle réexprimant les deux isoformes de afadin (s+lAF6). De façon intéressante, la perte de chacun des domaines a résulté en une diminution de la charge métastatique pulmonaire. De façon similaire, la perte de chacun des domaines a baissé la charge métastatique hépatique, le domaine RA2 ayant le moins d'effet. ZO-1 a également été étudiée étant donné sa capacité à interagir avec afadin et/ou claudin-2 pour promouvoir la progression cancéreuse. Ainsi, le knockdown de ZO-1 à diminuer la charge métastatique pulmonaire, mais n'a eu aucun effet sur la formation des métastases hépatiques. L'ensemble de nos résultats permettent une meilleure compréhension du rôle de afadin et démontrent que afadin et claudin-2 peuvent interagir pour affecter la progression du cancer du sein, mais ils peuvent interagir par ZO-1 dépendant le site métastatique. Cependant, il est possible que l'interaction entre

afadin et claudin-2 soit médier par une autre protéine ou qu'ils jouent un rôle dans deux voient de signalisation parallèle afin de promouvoir les métastases.

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CONTRIBUTION OF AUTHORS

The thesis was written by Jennifer Huxham and edited by Dr. Peter Siegel and Dr. Sébastien Tabariès. The experimental design was planned by Dr. Peter Siegel, Dr. Sébastien Tabariès and Jennifer Huxham. The data was gathered by Jennifer Huxham, while animal work was done by Jennifer Huxham and Dr. Sébastien Tabariès.

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LIST OF FIGURES AND TABLES

ABBREVIATIONS

AF6	Afadin
ADIP	Afadin dilute domain-interacting protein
AJ	Adherens Junctions
CFTR	Cystic fibrosis transmembrane conductance regulator
CRC	Colorectal cancer
DIL	Dilute
EMT	Epithelial-to-mesenchymal transition
ER	Estrogen receptor
FAB	F-actin binding domain
FHA	Forkhead associated
GFP	Green fluorescent protein
HER2	Human epidermal growth factor receptor 2
H&E	Hematoxylin and eosin
IVIS	*In vivo* imaging system
JAM-A	Junctional adhesion molecule A
KD	Knockdown
KO	Knockout
lAF6	Long afadin
LZ	ZsGreen luciferase construct
MFP	Mammary fat pad
MLL	Mixed lineage leukemia
PDGF	Platelet derived growth factor
PDZ-GEF2	PDZ-guanine nucleotide exchange factor 2
PR	Progesterone receptor
PDZ	PSD95/Dlg1/ZO1
PDZ BD	PSD95/Dlg1/ZO1 binding domain
PRR	Proline rich region
RA	Ras association
sAF6	Short afadin
TJ	Tight Junctions

TNBC	Triple negative breast cancer
WT	Wild Type
WT-HA	HA-tagged wild type claudin-2
ZO-1	Zonula occludens -1
$231TR^{AF6CR}$	MDA-MB-231TR Afadin CRISPR knockout
$231TR^{C2CR}$	MDA-MB-231TR claudin-2 CRISPR knockout
Δ	Deletion

CHAPTER 1: LITERATURE REVIEW

1.1 Breast Cancer

In Canada, breast cancer is the most commonly diagnosed cancer among women (CCS 2021). It is estimated that in 2021, 27,700 women will be diagnosed with breast cancer and 5,400 will die from this disease. These numbers account for 25% of all new cancer cases and 13% of all cancer deaths among women (CCS 2021). Fortunately, with screening programs the majority of patients will be diagnosed with stage I or II breast cancer, which is more easily treated (CCS 2018; 2021). Indeed, the 5-year survival rate for breast cancer in Canadian women is 89% (CCS 2021). However, patient outcomes can differ significantly from this depending on the breast cancer subtype. Despite the over all good prognosis of early stage breast tumors, as breast cancer progresses and metastases form, it becomes more and more difficult to treat (Radecka and Litwiniuk 2016). In fact, 90% of all cancer related deaths are associated with metastasis formation (Chaffer and Weinberg 2011). Therefore, a better understanding of the mechanisms controlling breast cancer metastasis will help to greatly improve outcomes for patients with already advanced disease.

1.1.1. Molecular Subtypes of Breast Cancer: Breast cancer patient outcomes can be further stratified based on molecular subtypes. Indeed, there are four main molecular subtypes of breast cancer that are traditionally classified based on the expression of different receptors in a breast tumor. These include the hormone receptors for estrogen (ER) or progesterone (PR), or the human epidermal growth factor receptor 2 (HER2) (Dai et al. 2016; SEER 2022). The most common type of breast tumor is classified as Luminal, these tumors are ER+ and comprise 60-70% of all breast tumors (Johnson et al. 2020). These Luminal tumors are further divided into Luminal A or B, where Luminal B tumors have greater KI67 (proliferation marker) and lower PR expression than Luminal A. Patients with Luminal B tumors also have a poorer prognosis than Luminal A (Dai et al. 2016; Johnson et al. 2020). Tumors that are HER2+ and have low ER expression are called HER2+ and make up 12-20% of all breast cancers (Johnson et al. 2020; Perou et al. 2000). These tumors have a worse prognosis than Luminal tumors (Johnson et al. 2020). Next, is the triple negative subtype where tumors are ER-, PR- and HER2- and make up 10-15% of invasive breast cancers. Triple negative breast cancers (TNBC) are aggressive and have the poorest prognosis due to the lack of targeted therapy options (ie. drugs which target ER, PR or HER2 do not work in this cancer type) (Johnson et al. 2020). In fact, the 5-year relative survival for TNBC is only 76.9%, compared to 94.3% for patients with Luminal A tumors (SEER 2022). Within the TNBC subtype,

significant heterogeneity exists that is being extensively characterized in hopes of developing new targeted therapies (Marra et al. 2020). One TNBC subgroup encompasses Basal tumors, which maintain the ER-, PR-, HER2- status, but are also positive for cytokeratin 5/6 and epidermal growth factor receptor (Dai et al. 2016). The Claudin-low subtype is another subset of the TNBC, which is also characterized by low claudin 3, 4, and 7 expression and exhibits more Epithelial-to-Mesenchymal Transition (EMT) like features (Prat et al. 2010). Seven additional TNBC subtypes have been described using gene expression profiling. Indeed, basal-like 1 and 2, immunomodulatory, mesenchymal, mesenchymal stem-like, luminal androgen receptor and unstable have all been identified as distinct TNBC subgroups (Lehmann et al. 2011). An improved ability to characterize different breast cancer subtypes using gene expression profiling and transcriptomics is critical as it allows for the development of targeted therapies to better treat each breast cancer subtype (Marra et al. 2020). An in-depth understanding of the different classes of breast cancer will ultimately lead to improved outcomes for all breast cancer patients.

1.2 Cancer Metastasis

Cancer metastasis is regarded as the deadliest part of the disease, as the majority of cancer related deaths are associated with the spread of cancer throughout the body (Chaffer and Weinberg 2011). The dissemination of cancer cells in the primary tumors to other sites in the body is known as the metastatic cascade. This process begins when cancer cells in the primary tumor breach the basement membrane and begin to invade the local tumor environment (Valastyan and Weinberg 2011). They then intravasate into the blood stream. These circulating tumor cells will then extravasate and seed a distant organ, forming micro-metastases. From here, these cells may begin to adapt so that they can thrive and grow into overt metastases (Valastyan and Weinberg 2011).

Within this concept of the metastatic cascade there are two models, which attempt to explain the timing and progression of these events. The first model is known as the linear progression model. In this model it is believed that the metastatic cascade occurs in a more linear fashion. Indeed, the primary tumor will develop fully, undergoing multiple rounds of mutation and selection to become a large and highly proliferative malignant tumor (Klein 2009). After which, cancer cells may begin to break away from the primary tumor and enter circulation to form metastases. These primary tumors and resultant metastases will likely be more genetically similar due to the departure of the metastatic cancer cells from the primary tumor at a later stage of

development, when the cells have already undergone adaptive selection (Klein 2009). With this theory in mind, one would believe that treatment of a primary tumor at an early stage would be most "curative" as the cancer cells will not have had the chance to enter circulation and no metastases should develop. The second model is known as the parallel progression model. In this model, cancer cells will leave the primary site at early stages of tumor development (Klein 2009). These early disseminating tumor cells can then seed distant organs where they may begin to evolve to better thrive at the distant site. In this hypothesis, cancer cells in the primary tumor and at the metastatic sites may share some adaptive mutations but will likely differ more genetically due to the increased separation in time. The primary tumor and metastatic tumors may then grow and mutate in parallel, each developing their own mutations that give them a survival advantage in their respective sites (Gui and Bivona 2022). With this theory in mind, early intervention (ie. resection of primary tumor) may not be enough to prevent future metastasis development as the cancer cells may already be at distant sites. These two conflicting hypotheses provide the framework for understanding the importance of timing and genetic adaptions in cancer progression.

1.3 The Role of Junctional Complexes in Cancer Progression

The vast majority of human cancers are epithelial in origin (McCaffrey and Macara 2011). Thus, the protein complexes that control the formation of cellular junctions in epithelial cells (e.g. tight junctions, adherens junctions), which maintain cellular polarity, have an important role to play in modulating cellular transformation and progression towards metastatic disease. In certain contexts, cancer cells retain functional junctional complexes and exhibit collective cell migration, which contributes to metastasis (Cheung and Ewald 2016; Friedl et al. 2012; Friedl et al. 1995). In other instances, loss of cell-cell adhesions has been associated with cancer progression, which is exemplified by an EMT that is associated with single cell migration and invasion (Friedl et al. 2012). The precise role of an EMT in cancer is complicated, and it is believed to be a transient process in which a mesenchymal-to-epithelial transition occurs at metastatic sites (Chao et al. 2012; Williams et al. 2019). It is easy to see how the role of adhesion proteins in cancer progression to metastatic disease can be complex and, in some cases, contradictory.

1.3.1. Cell-cell Adhesions: There are four types of junctions that link epithelial cells together including; tight junctions (TJs), adherens junctions (AJs), gap junctions, and desmosomes

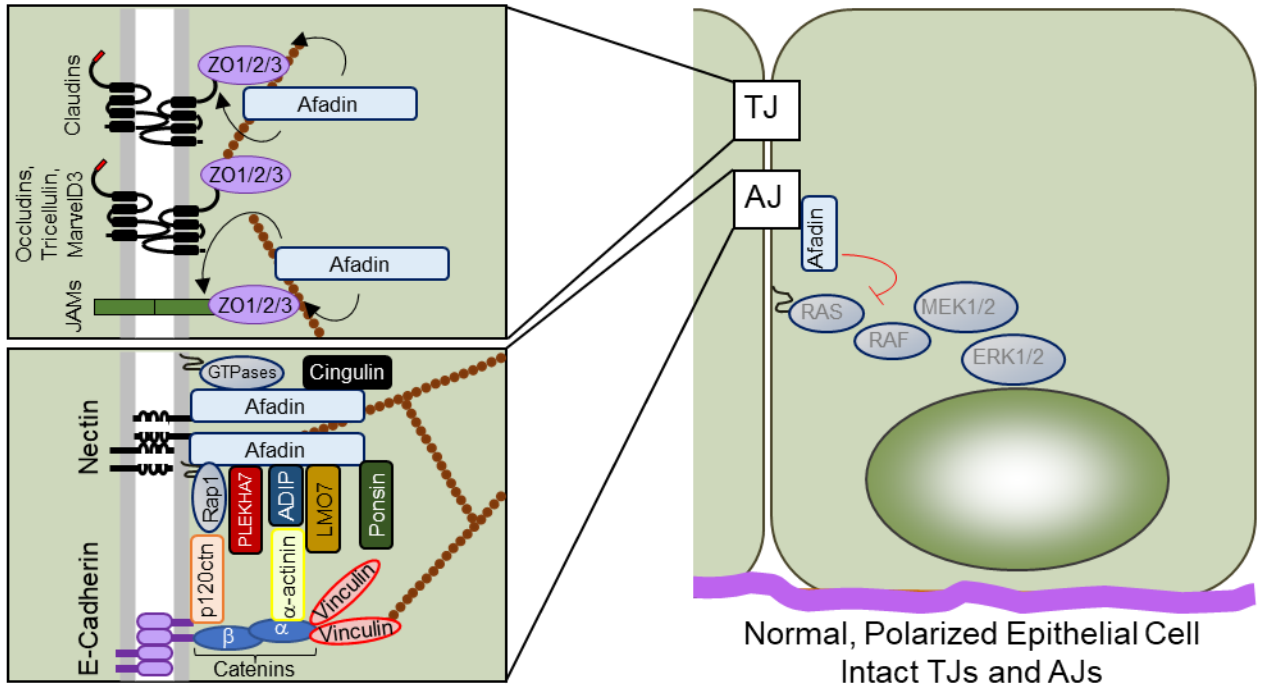
(Desai et al. 2009). Each junctional complex plays a unique role in maintaining cell-cell adhesions. TJs help to maintain polarity and regulate intercellular solute/ion movement that confers a barrier function to epithelial tissues (Zihni et al. 2016). AJs play a critical role in the initiation and maintenance of cell-cell adhesions, where they help to connect neighboring cells (Hartsock and Nelson 2008; Meng and Takeichi 2009). Gap junctions are channels that connect adjacent cells, which enables the transfer of ions and small molecules from one cell to the other (Goodenough and Paul 2009). Desmosomes join adjacent cells and link the plasma membrane to intermediate filaments (Delva et al. 2009). Together, these adhesion systems play a crucial role at the cellular and tissue levels to regulate normal physiological functions (Janiszewska et al. 2020). However, dysregulation of these junctional complexes and their constituent proteins can lead to diseases such as cancer (Janiszewska et al. 2020). One such protein that has been implicated in cancer progression as well as in the assembly/function of TJs and AJs is the multi-domain scaffold protein, afadin. Indeed, afadin has been shown to have a context dependent role in cancer progression where it exerts both growth suppressive and pro-metastatic functions in diverse cancer types.

1.3.2. Adherens Junctions: Afadin, also known as AF6 or AFDN, is found in AJs that form between adjacent epithelial cells in tissues throughout the body (Mandai et al. 1997; Mandai et al. 2013). AJs have two main components, the first are integral membrane proteins that include cadherins and nectins, which form homo- and heterophilic interactions with counterparts on adjacent epithelial cells (Niessen and Gottardi 2008; Takahashi et al. 1999). The second are scaffold proteins such as catenins or afadin, which couple cadherins or nectins to the actin cytoskeleton, respectively (Niessen and Gottardi 2008). Catenin proteins also facilitate interaction with microtubules (Meng and Takeichi 2009). AJ formation begins when individual epithelial cells come into contact with each other. During this phase, nectin-nectin mediated adhesions begin to form, which enhance nectin-afadin interactions. Increased nectin-afadin complex formation subsequently re-enforces nectin-nectin interactions in a positive feedback loop, promoting clustering of these proteins (Kurita et al. 2011). Afadin then recruits E-cadherin, α-catenin and β-catenin to the adhesion sites (Tachibana et al. 2000). E-cadherin forms trans-interactions with the neighboring cell completing the AJ (Honda et al. 2003; Mandai et al. 2013; Tachibana et al. 2000). These trans-interactions are promoted by binding between afadin, Rap1 and p120ctn (Sato et al. 2006). These junctions are then anchored to the actin cytoskeleton by afadin and α-catenin (Mandai et al. 1997; Rimm et al. 1995). However, it has also been demonstrated that α-catenin may not be

able to link AJs directly to the actin cytoskeleton due to the observation that α-catenin is unable to bind actin and β-catenin simultaneously *in vitro* (Yamada et al. 2005). Instead, vinculin may anchor AJs to F-actin (Figure 1a) (Rangarajan and Izard 2013). It has also been shown that binding of afadin to αE-catenin (which is bound to β-catenin), promotes F-actin binding, which could allow for anchoring to the actin cytoskeleton (Sakakibara et al. 2020). The nectin-afadin complex and cadherin-catenin complexes within AJs are connected by a variety of proteins including; LMO7, ponsin, and ADIP (Figure 1a) (Asada et al. 2003; Mandai et al. 1999; Ooshio et al. 2004; Tachibana et al. 2000). Afadin recruits PLEKHA7 to AJs and binds to its N-terminus in EpH4 mammary gland epithelial cells (Kurita et al. 2013). This interaction was found to be required for proper formation of AJs in epithelial cells, as knockdown and mutation (deletion of afadin binding region) of PLEKHA7 inhibited localization of AJ proteins (E-cadherin and p120ctn) at sites of cell-cell adhesion (Kurita et al. 2013). Afadin therefore facilitates AJs formation by coordinating interactions between multiple proteins.

1.3.3. Tight Junctions: AJs help to assemble and support TJs, which are found adjacent to the AJs at sites of cell-cell adhesions (Rouaud et al. 2020; Takai and Nakanishi 2003). The main integral membrane components within TJs are claudins, occludins, tricellulin, and MARVELD3 (Zihni et al. 2016). These proteins connect to adaptors/scaffolds or membrane-associated guanylate kinases that interact with a variety of other proteins in the cell (Zihni et al. 2016). Afadin is involved in the formation of these tight junctions (Ooshio et al. 2010). Specifically, afadin has been shown to interact with the tight junctional protein, zonula occludens 1 (ZO-1) (Figure 1a) (Ooshio et al. 2010; Yamamoto et al. 1999). Indeed, afadin transiently interacts with ZO-1 in HEK293 cells, a process that is required for efficient formation of TJs in MDCK cells (Ooshio et al. 2010). Deletion of the F-actin binding domain within afadin was shown to impair TJ formation in EpH4 mouse mammary epithelial cells (Sakakibara et al. 2018). Variation in TJ protein expression has been implicated in cancer progression. This has been extensively studied with respect to the claudin family of proteins, where changes in claudin expression have been reported to be tumor suppressive or pro-tumorigenic depending on the context (Tabariès and Siegel 2017). Loss or dysregulation of TJs can affect events involved in cancer progression such as proliferation and metastasis (Matter et al. 2005; Salvador et al. 2016; Tabariès and Siegel 2017).

A


Claudins
Occludins, Tricellulin, MarvelD3
JAMs
ZO1/2/3
Afadin
ZO1/2/3
Afadin
ZO1/2/3
GTPases
Cingulin
Nectin
Afadin
Afadin
Rap1
PLEKHA7
ADIP
LMO7
Ponsin
p120ctn
α-actinin
Vinculin
Vinculin
E-Cadherin
β
α
Catenins
TJ
AJ
Afadin
RAS
RAF
MEK1/2
ERK1/2
Normal, Polarized Epithelial Cell
Intact TJs and AJs

B

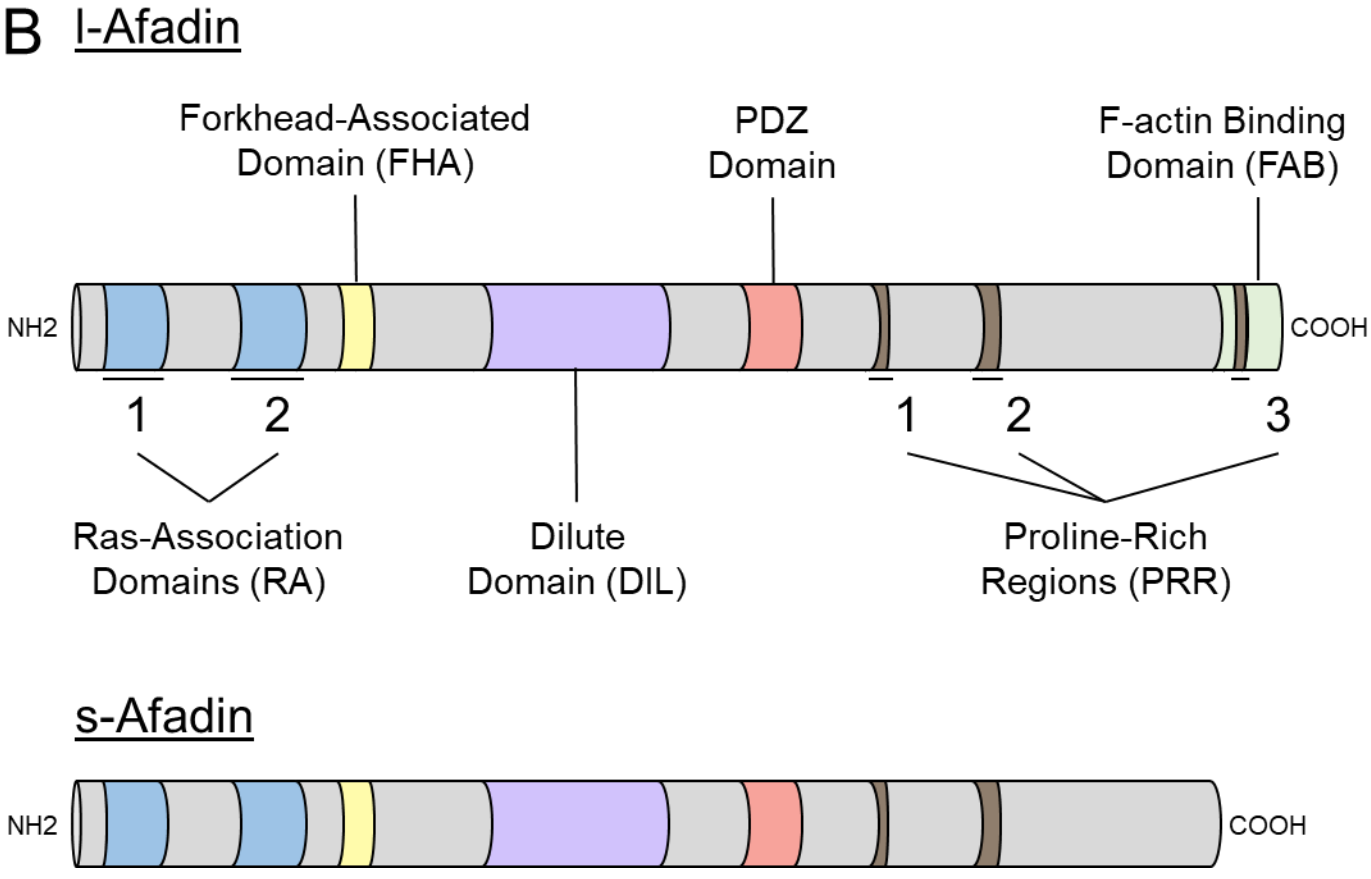

l-Afadin
Forkhead-Associated Domain (FHA)
PDZ Domain
F-actin Binding Domain (FAB)
NH2
COOH
1
2
1
2
3
Ras-Association Domains (RA)
Dilute Domain (DIL)
Proline-Rich Regions (PRR)
s-Afadin
NH2
COOH

Figure 1. Composition of adherens/tight junctions and the structural organization of afadin. **(A)** schematic representation of polarized epithelial cells depicting the location of afadin, and its associated proteins, within adherens and tight junctional complexes. Afadin binds to GTPases, such as RAS, and inhibits downstream Raf/MEK/ERK signaling. **(B)** The two main isoforms of afadin (l-afadin and s-afadin), and their domain organization, are shown.

1.3.4. Specialized Junctions: Interestingly, afadin is also part of more specialized AJ-like complexes such as puncta adherentia junctions in the brain and Sertoli-spermatid junctions in the testes (Ozaki-Kuroda et al. 2002; Toyoshima et al. 2014). Specifically, fadin is found in puncta adherentia junctions at the synapse, which joins axons and dendrites (Toyoshima et al. 2014). Loss of afadin from these structures leads to reduced incorporation of nectins and N-cadherin, which results in impaired puncta adherentia junction formation (Toyoshima et al. 2014). It also inhibits presynaptic functions in neurons in the hippocampus (Toyoshima et al. 2014). Afadin is also found at Sertoli-spermatid junctions, a heterotypic AJ found in the testes (Ozaki-Kuroda et al. 2002). Nectin-3 within spermatids interacts with nectin-2 in the Sertoli cells, which is then linked to F-actin bundles via afadin (Ozaki-Kuroda et al. 2002).

1.4. Afadin is a multi-domain scaffold protein

Afadin is an evolutionarily conserved protein, with orthologues in *Caenorhabditis elegans* and *Drosophila melanogaster* (Lynch et al. 2012; Mandai et al. 2013; Matsuo et al. 1999; Takahashi et al. 1998; Watari et al. 1998). The *Drosophila* homologue of afadin is Cno, which is involved in AJs formation (Matsuo et al. 1999; Sawyer et al. 2009; Takahashi et al. 1998). However, it is only required for AJ formation in some tissues (Sawyer et al. 2009). Cno also has a negative regulatory effect on Notch, Ras-MAPK, and Wingless/Wnt signaling (Carmena et al. 2006; Miyamoto et al. 1995). Specifically, Cno can regulate crosstalk between these pathways during progenitor specification (Carmena et al. 2006). The *C. elegans* ortholog (or AFD-1) is involved in the cadherin-catenin complex and is a putative Ras effector (Lynch et al. 2012; Watari et al. 1998).

1.4.1 Expression pattern and splice isoforms: Afadin was first isolated from rat brain and since then 7 different splice variants have been identified, which are grouped into 2 main isoforms referred to as either long (lAF6) or short afadin (sAF6) (Mandai et al. 1997; Saito et al. 1998; Takai et al. 2008; Yates et al. 2015) (Figure 1b). lAF6 and sAF6 have a molecular weight of 205 and 190kDa, respectively (Mandai et al. 1997). While the sAF6 isoform is found throughout the

body, it has been mainly described in neurons and remains less well studied relative to lAF6 (Kobayashi et al. 2014; Mandai et al. 2013). The long isoform is also expressed in different sites in the body (Mandai et al. 1997; Mandai et al. 2013). The main difference between the isoforms is that the lAF6 has a carboxy-terminal F-actin binding (FAB) domain, which contains a third proline rich region, while the sAF6 does not (Mandai et al. 1997; Mandai et al. 2013) (Figure 1b). The lAF6 isoform is typically localized proximal to the plasma membrane within the cell cytoplasm, while sAF6 can be found in the cytoplasm or nucleus. In the nucleus, the sAF6 clusters into nuclear foci (Buchert et al. 2007). It was found that the lAF6, and not the sAF6 isoform, is required in puncta adherentia junction formation (Maruo et al. 2018). Relevant afadin isoforms are mentioned when indicated in the studies that are referenced in this text.

1.4.2. Domain structure and interacting partners: Afadin is a multi-domain protein with numerous binding partners that can modulate various signaling pathways (Figure 1b, Table 1). Both afadin isoforms contain two Ras-association (RA) domains, two proline rich regions (PRR1/2), a PSD95/Dlg1/ZO1 (PDZ) domain, a dilute (DIL) domain, and a forkhead-associated (FHA) domain. Typically, the lAF6 binds to the C-terminus of nectins and links it to the F-actin cytoskeleton in AJs (Asakura et al. 1999; Takahashi et al. 1999). It has been shown to regulate many pathways including migration, cell signaling, and leading edge formation (Fukumoto et al. 2011; Lorger and Moelling 2006; Mandai et al. 2013; Nakata et al. 2007). The sAF6 isoform likely also interacts with nectin via the PDZ domain (Kobayashi et al. 2014). Numerous proteins have been identified as interacting partners of afadin, as described in Table 1. While afadin has many interacting partners, we will focus our review on those that are more relevant to cancer progression.

1.4.3. Ras–association domains (RA1/RA2): The RA domains of afadin are particularly important as they interact with activated Ras and are involved in suppressing downstream Ras signaling (Fournier et al. 2011; Kuriyama et al. 1996; Linnemann et al. 1999; Manara et al. 2014; Yamamoto et al. 1999; Yamamoto et al. 2015; Zhang et al. 2018). Afadin interacts with numerous small GTPases, including different members of the Ras family (H-Ras, N-Ras, K-Ras), the R-Ras subfamily, (R-Ras, TC21, M-Ras) (Boettner et al. 2000; Iwasawa et al. 2012; Kuriyama et al. 1996; Linnemann et al. 1999; Quilliam et al. 1999) and the Rap subfamily (Rap1a, Rap2a) (Boettner et al. 2000; Linnemann et al. 1999; Miyata et al. 2009a; Su et al. 2003).

Table 1: Afadin interacting proteins

Afadin Domain	Interacting Protein	References
Ras-Association Domain	Afadin	Liedtke *et al.*, 2010; Smith *et al.,* 2017
	Rap1	Boettner *et al.*, 2000; Miyata *et al.*, 2009a; Su *et al.*, 2003
	Rap1A	Boettner *et al.*, 2000; Linneman *et al.*, 1999
	Rap2A	Linneman *et al.*, 1999
	Ras	Linneman *et al.*, 1999;Kuriyama *et al.*, 1996; Yamamoto *et al.,* 1997
	H-Ras	Kuriyama *et al.*, 1996 ; Quilliam *et al.,* 1999
	N-Ras, K-Ras, M-Ras	Boettner *et al.*, 2000; Quilliam *et al.,* 1999
	R-Ras	Iwasawa *et al.*, 2012; Linneman *et al.*, 1999
	TC21	Linneman *et al.*, 1999
Forkhead-associated Domain	Scribble	Goudreault et al. 2022
Dilute Domain	ADIP	Asada *et al.*, 2003; Fukumoto *et al.*, 2011
PDZ Domain	Nectins 1-4	Takahashi *et al.*, 1999; Reymond *et al.,* 2000; Reymond *et al.,* 2001
	EphA6-7; EphB2-3, 5-6	Buchert *et al.*, 1999; Hock *et al.*, 1998
	JAM-A	Severson *et al.*, 2009; Ebnet *et al.,* 2000
	Jagged-1	Popovic *et al.*, 2011; Hock *et al.*, 1998
	Neurexin 1-3	Hock *et al.*, 1998
	Connexin 36	Li *et al.*, 2012
	SPA-1	Su *et al.*, 2003
	Bcr	Radziwill *et al.*, 2003
	c-Src	Radziwill *et al.*, 2007
	CFTR	Sun *et al.*, 2014
	Ryk	Halford et al. 2000
Proline-Rich Regions (1,2,3)	ZO-1	Ooshio *et al.*, 2010
	Ponsin	Mandai *et al.*, 1999
	Drebrin	Rehm *et al.*, 2013
F-actin Binding Domain	F-actin	Mandai *et al.*, 1997
	LMO7	Ooshio *et al.*, 2004
Miscellaneous	Profilin	Boettner *et al.*, 2000
	Fam	Taya *et al.*, 1998
	LMO2	Begay-Muller *et al.*, 2002
	SHP-2	Nakata *et al.*, 2007
	nArgBP2	Kawabe *et al.*, 1999
	α-catenin	Pokutta *et al.*, 2002; Tachibana *et al.*, 2000
	Cingulin	Cordenosi *et al.*, 1999
	14-3-3	Jin *et al.*, 2004
	Rap1GAP	Su *et al.*, 2003
	Rit	Shao *et al.*, 1999
	Rin	Shao *et al.*, 1999
	PLEKHA7	Kurita *et al.*, 2013
	ZO-3	Wittchen *et al.*, 2003
	HDAC6	Lundh *et al.*, 2019
	ArhGAP29	Tagashira *et al.*, 2018
	LGN	Carminati *et al.*, 2016
	EphA2	Perez *et al.*, 2017
	Claudin-2	Tabariès *et al.*, 2019
	Claudin-6	Yang *et al.*, 2018
	ZO-2	Monterio *et al.*, 2013
	ADAM10	Shah *et al.*, 2018
	Dvl2	Xu *et al.,* 2015
	FOXE1	Xu *et al.,* 2015
	p85	Kanzaki et al. 2008

The RA1 domain also allows for self-association of the afadin portion portion of the Mixed Lineage Leukemia (MLL)-AF6 fusion protein (Liedtke et al. 2010; Smith et al. 2017).

1.4.4. PDZ domain: PDZ domains typically bind to specific amino acid motifs at the C-terminus of target proteins; however, they may also interact with embedded motifs (Lee and Zheng 2010). This afadin domain is involved in a variety of cellular functions including signal transduction and AJ formation and maintenance (Lee and Zheng 2010; Mandai et al. 2013). Binding to the PDZ domain may be disrupted by post-translational modifications such as phosphorylation or disulfide bond formation (Lee and Zheng 2010). Afadin binds to ephs-1-4 via its PDZ domain in AJs (Reymond et al. 2000; Reymond et al. 2001; Satoh-Horikawa et al. 2000; Takahashi et al. 1999). Afadin also interacts with a variety of proteins in the brain via this domain, including Eph receptors, connexin36, and neurexin 1-3 (Buchert et al. 1999; Hock et al. 1998; Li et al. 2012).

1.4.5. Additional Afadin domains (Forkhead-associated domain (FHA), Proline-rich-regions (PRR), Dilute domain (DIL), C-terminal F-actin-binding (FAB) domain): Afadin binds scribble via the FHA domain and impacts cell motility upon EGF stimulation (Goudreault et al. 2022). The PRR domain is important for AJ and TJ formation. Afadin transiently interacts with ZO-1 via this domain, which is required for TJ formation in MDCK cells (Ooshio et al. 2010). lAF6 also binds to ponsin via the PRRs, which may help to link nectin-afadin to the cadherin-catenin complex within AJs (Mandai et al. 1999). The only known interacting partner that binds lAF6 through the DIL domain is afadin dilute domain-interacting protein (ADIP) (Asada et al. 2003; Fukumoto et al. 2011). ADIP is also thought to link nectin-afadin and cadherin-catenin systems via α-actinin (Asada et al. 2003; Fukumoto et al. 2011). The FAB domain of afadin is important as it binds actin, thus creating a link between nectin and the actin cytoskeleton (Mandai et al. 1997). LMO7 also binds to afadin within AJs via the FAB domain (Ooshio et al. 2004).

1.4.6. Additional Afadin-interacting proteins: A variety of other proteins have also been shown to interact with afadin; however, the exact domains within afadin that mediate these interactions are unknown (Table 1). lAF6 has been shown to interact with PLEKHA7 and α-catenin and be involved in AJ and/or TJ formation (Cordenonsi et al. 1999; Kurita et al. 2013; Tachibana et al. 2000). Afadin also associates with Profilin, Fam, claudin-2, and HDAC6 (Boettner et al. 2000; Lundh et al. 2019; Tabariès et al. 2019; Taya et al. 1998).

1.5. Afadin exerts various functions in normal cells

Identifying the functions of afadin in normal cells may help to highlight potential pathways that afadin regulates, which could be involved in cancer progression.

1.5.1. Lessons from Afadin knockout mice: Global deletion of afadin in mouse embryos causes developmental defects in the ectoderm and mesoderm, resulting in embryonic lethality at E10.5 (Ikeda et al. 1999; Zhadanov et al. 1999). Deletion of afadin leads to decreased polarization and disorganization of cell-cell adhesions in the ectoderm, as well as disrupted ZO-1 localization (Ikeda et al. 1999; Zhadanov et al. 1999). Conditional loss of afadin in the embryonic central nervous system resulted in hydrocephalus and death (Yamamoto et al. 2013). This afadin knockout also resulted in loss of AJs in radial glial and ependymal cells in the midbrain (Yamamoto et al. 2013). Tissue-specific afadin loss within the intestinal epithelium of mice resulted in the mis-localization and deformation of Paneth cells in the small intestine (Tanaka-Okamoto et al. 2014). Afadin deletion also impaired proper formation of AJs and TJs in the base of the crypts of the small intestine, thus further demonstrating the importance of afadin in AJ and TJ formation (Tanaka-Okamoto et al. 2014). A reduction in Rap1 and EphB3 expression was also observed upon afadin knockout, hindering Paneth cell movement toward the top of villi and maintains their adhesion to neighboring crypt cells (Tanaka-Okamoto et al. 2014). Knockout of afadin in the intestinal epithelia of mice after birth inhibited localization of nectin-2 and 3 at apical junctions. However, it did not disrupt localization of other cell-cell adhesion proteins such as ZO-1 and E-cadherin. Loss of afadin was also associated with disruption of epithelial permeability in the intestines (Tanaka-Okamoto et al. 2011). Finally, afadin is required for proper lumen formation in the nephron of the kidney. In this instance, afadin is also important for nectin clustering at the cell surface (Yang et al. 2013). Afadin therefore plays vital roles in development.

1.5.2. Junctional formation/maintenance: Loss of afadin inhibits AJ and TJ formation (Ooshio et al. 2010). It has been reported that the PRR1/2 domains of afadin are recognized and bound by the SH3 domain of ZO-1 in HEK293 cells (Ooshio et al. 2010). This afadin and ZO-1 interaction is required for the formation of TJs in MDCK cells; however, it is dispensable for AJ formation (Figure 1a) (Ooshio et al. 2010). Afadin interacts with the N-terminal portion of cingulin *in vitro*, where cingulin is likely associated with the protein complex surrounding TJs in epithelial cells (Figure 1a) (Cordenonsi et al. 1999).

Afadin helps to maintain the complex of proteins that are found at AJs. Indeed, lAF6 binds to ADIP in small intestine absorptive epithelial cells, and then binds to α-actinin which, in turn, binds α-catenin (Asada et al. 2003). This interaction creates a link between the afadin-nectin and catenin-cadherin complex in AJs (Asada et al. 2003). LMO7 is also found in AJs, where it binds to afadin and connects it to E-cadherin via α-actinin in HEK293 cells, in a manner similar to ADIP (Ooshio et al. 2004). ADIP and LMO7 may then be important in maintaining junctional integrity by linking the components of AJs (Asada et al. 2003; Ooshio et al. 2004). lAF6 can bind to αE-catenin via its putative coiled coil region in HEK293 cells (Maruo et al. 2018). This interaction is important as α-catenin facilitates colocalization of nectin-afadin and E-cadherin-catenin at AJs (Pokutta et al. 2002; Tachibana et al. 2000). lAF6 co-localizes with ponsin at cell-cell AJs in rat 3Y1 fibroblasts. lAF6 binds to the ponsin SH2/3 domain via its PRR3 domain in COS7 cells (Mandai et al. 1999). Vinculin also binds to ponsin; however, vinculin and afadin bind to ponsin in a competitive manner (Mandai et al. 1999). The interaction between afadin and ponsin is likely important in formation and maintenance of AJ integrity (Asakura et al. 1999; Mandai et al. 1999). Similarly to ponsin, nArgBP2 binds to lAF6 and vinculin in COS cells (Kawabe et al. 1999). 14-3-3 also associates with afadin; however, the physiological consequences of this interaction are unknown (Jin et al. 2004). Afadin binds with Rap1A, Rap2A, and TC21; however, it has the greatest affinity for Rap1A (Linnemann et al. 1999).

After AJ formation, afadin plays an important role in the organization of actomyosin, where afadin interacts with αE-catenin in Eph4 cells (Sakakibara et al. 2020). Profilin, a protein involved in actin assembly, is also an afadin binding partner in MDCK cells (Boettner et al. 2000). Profilin is involved in the activation of actin subunits and cortical actin assembly (Boettner et al. 2000). The interaction between afadin and profilin potentially implicates afadin in regulating actin modelling (Boettner et al. 2000).

1.5.3. Cell adhesion: Afadin binds to Rap1 and to SPA-1 (a Rap1GTPase-activating protein), via its PDZ domain in 293T cells and thymocytes (Su et al. 2003). Afadin, SPA-1 and Rap1 also co-localized at sites of cell-cell adhesion upon induction in HeLa cells. Afadin facilitates interaction between SPA-1 and Rap1, thus promoting SPA-1 mediated inactivation of Rap1, which results in a decrease in β1-integrin mediated cell adhesion (Su et al. 2003). In contrast, loss of afadin resulted in formation of a cleft palate in mouse embryos that was attributed to impaired formation of cell-cell adhesions, which are necessary for palate closure (Lough et al. 2020). Indeed,

afadin has been shown to play important roles in maintaining and promoting formation of cell-cell adhesions, such as AJs and TJs (Mandai et al. 2013).

1.5.4. Survival/proliferation: Afadin inhibits apoptosis following Fas-ligand stimulation or serum starvation (Kanzaki et al. 2008). Mechanistically, afadin promotes cell survival by regulating platelet-derived growth factor (PDGF)-induced PI3K-Akt signaling in NIH3T3 fibroblasts. Indeed, the PDGF receptor binds nectin-3, which interacts with the PDZ domain of afadin. Finally, afadin interacts with p85 (PI3K subunit), activating PI3K-Akt signaling (Kanzaki et al. 2008). Thus, afadin is important for activation of the PI3K-Akt signaling pathway, which regulates cell proliferation and survival (Hemmings and Restuccia 2012; Kanzaki et al. 2008).

Afadin can also inhibit proliferation by binding Bcr (Radziwill et al. 2003). The Bcr kinase can phosphorylate and bind to the PDZ domain of afadin in HEK293 epithelial cells, which increases the efficiency of active Ras binding via the RA domains of afadin (Radziwill et al. 2003). The resulting three protein complex inhibits activation of the Raf/MEK/ERK signaling pathway (Radziwill et al. 2003).

1.5.5. Cell migration/invasion: Junctional adhesion molecule A (JAM-A) dimerizes and interacts with afadin and PDZ-guanine nucleotide exchange factor 2 (PDZ-GEF2) in SKCO-15 epithelial cells (Ebnet et al. 2000; Severson et al. 2009). PDZ-GEF2 activates Rap1A, which stabilizes β_1-integrin levels to induce cell migration. Loss of afadin in this model was associated with diminished β_1-integrin expression and decreased epithelial cell migration (Severson et al. 2009).

Afadin can be bound to nectin at cell-cell adhesions or found at the leading edge where it helps regulate directional cell movement and leading edge formation (Miyata et al. 2009a). Afadin binds to active Rap1 and localizes at the leading edge. This recruits SHP-2, a tyrosine phosphatase, which controls the activation of the PDGF receptor (Miyata et al. 2009a). Upon PDGF stimulation, afadin is required for recruitment of Necl-5, PDGF receptor, and $\alpha_v\beta_3$ integrin at the leading edge, promoting its formation (Miyata et al. 2009a). Afadin can also help coordinate leading edge formation and cell-movement by stimulating Rap1 and Rac1 and inactivating RhoA in NIH3T3 fibroblasts (Miyata et al. 2009b). These Rho family G proteins are activated and inactivated in a cyclical manner by afadin via SPA-1 and ARAP1 (Miyata et al. 2009b). ADIP and afadin are also important for leading edge formation upon PDGF stimulation in NIH3T3 fibroblasts (Fukumoto

et al. 2011). Here, ADIP and afadin co-localize at the leading edge and regulate cell movement by stimulating Rac activation via Vav2 (Fukumoto et al. 2011).

Afadin also binds to the C-terminus of ZO-3 and may help to regulate migration in MDCK cells (Wittchen et al. 2003). Low lAF6 levels in breast epithelial cells were also shown to promote cell migration by improving the directionality of cell migration in a wound closure assay (Lorger and Moelling 2006). These findings were supported by other work, where knockdown of afadin in ErbB2-expressing MCF10A cells resulted in an increased cell migration and invasion (Chatterjee et al. 2012).

1.5.6. Polarity: Knockdown of afadin in the MCF10A breast epithelial cell line results in disruption of basolateral polarity (Xu et al. 2015). Loss of polarity is consistent with the observation that afadin impairment prevented recruitment of nectins to the apical surface, therefore affecting polarity and AJ formation (Yang et al. 2013). Disrupted polarity is associated with cancer progression and may present a mechanism for afadin action as a tumor suppressor (Lee and Vasioukhin 2008).

1.5.7. Oriented Cell Division: Phosphorylation of afadin by S6K1 impairs cell-cell adhesion formation, which alters oriented cell division. This phenomenon is important in tuberous sclerosis complex mutant kidney cells, as mTOR and S6K1 stimulation of these cells promotes the formation of kidney cysts via afadin mediated oriented cell division. (Bonucci et al. 2020).

1.5.8. Signaling: Afadin has been implicated in a variety of signaling pathways (Table 1). However, we will focus on two of the more relevant signaling intermediates that are modulated by afadin; namely Ras and Src.

Afadin is involved in the regulation of Ras signaling. Indeed, afadin can bind to active Ras where it has a suppressive effect on Ras/Raf/MEK/ERK signaling (Radziwill et al. 2003). Mutations in components of the pathway or upstream events that activate the Ras/Raf/MEK/ERK signaling pathway occur in many cancers (Li et al. 2016), which impacts cellular processes that include proliferation, cell survival, and cell migration (De Luca et al. 2012; McCubrey et al. 2007). Thus, in normal cells or cancer cells that have not constitutively activated the Ras pathway, afadin functions to negatively regulate Ras/Raf/MEK/ERK signaling. In this situation, loss of afadin could promote tumorigenesis and enhance tumor growth through activation of this pathway (Table 3 and 4).

Afadin has also been associated with c-Src kinase activity (a non-receptor tyrosine kinase), serving as both a c-Src substrate and a modulator of c-Src activity (Nakata et al. 2007; Radziwill et al. 2007). PDGF stimulation promotes phosphorylation of afadin by c-Src, which facilitates binding to and increased SHP-2 phosphatase activity (Nakata et al. 2007). This results in a SHP-2-mediated reduction in PDGF receptor phosphorylation and a decrease in Ras-ERK activity in NIH3T3 (Nakata et al. 2007). Knockdown of afadin increased ERK activity and also affects cellular morphology, thus demonstrating a second mechanism through which afadin can modulate ERK activity (Nakata et al. 2007). Afadin can also recruit c-Src to cell-cell adhesions in MCF10A cells via an interaction with the PDZ domain of afadin (Radziwill et al. 2007). Phosphorylation of c-Src at Tyr527 inhibits binding to afadin (Radziwill et al. 2007). When bound to afadin, c-Src phosphorylation at Tyr416 and its kinase activity is impaired (Radziwill et al. 2007). c-Src is activated in multiple cancers where it promotes tumor growth and metastatic progression (Wheeler et al. 2009). The ability of afadin to suppress c-Src activation may contribute to the tumor suppressor activities of this scaffold protein in non-transformed cells.

1.6. Afadin can suppress tumor growth and metastasis

Afadin has been shown to have both pro-tumorigenic and tumor suppressive functions. These findings seem to vary depending on various factors including cancer type, stage or model system. However, considerable literature supports a tumor suppressor role for afadin in many cancer types including breast, endometrial, pancreatic, colon cancer and osteosarcoma (Table 2). The specific context in which afadin has a tumor suppressive role will be further explored, including the molecular mechanisms that may contribute to tumor growth and metastasis following afadin loss.

1.6.1. Afadin expression and clinical correlations with patient prognosis: Afadin is located in a chromosomal region commonly deleted in ovarian cancer, an observation that is supportive of a tumor suppressor function (Saito et al. 1996). This tumor suppressor role has been seen in many cancer types including osteosarcoma, endometrial, colon, breast, and pancreatic cancer (Table 2) (Letessier et al. 2007; Sun et al. 2014; Xu et al. 2015; Yamamoto et al. 2015; Zhang et al. 2018). Specifically, immunohistochemical staining of patient endometrial tumors found that there is an inverse association between low afadin levels and high histological grade (Yamamoto et al. 2015). Low afadin expression in pancreatic, breast, and colon cancer patients was associated with

Table 2: Afadin expression in clinical samples

Cancer Type	Method	Number of Samples	Expression Change	Clinical Association	Suppressor/ Promoter	References
Colon	RT-qPCR	74	Reduced AF6 in CRC samples vs normal colonic epithelium	Low AF6 expression associated with poor overall survival	Suppressor	Sun *et al.*, 2014
Endometrial	IHC	90	Reduced AF6 in high grade samples	Low AF6 expression associated with higher Myometrial invasion	Suppressor	Yamamoto *et al.*, 2015
Pancreatic	IHC	83	Reduced AF6 in PCa samples vs normal pancreatic epithelium	Low AF6 expression associated with poor overall survival and increased tumor size and grade	Suppressor	Xu *et al.*, 2015
Osteosarcoma	IHC	27	Reduced AF6 in OS samples vs normal bone	Low AF6 expression associated with lung metastasis	Suppressor	Zhang *et al.*, 2018
Breast	IHC	352	Reduced AF6 in a subset of BCa samples vs normal breast epithelium	Low AF6 expression associated with lower metastasis-free survival	Suppressor	Letessier *et al.*, 2007
Breast	IHC	1,111	Elevated AF6 in a subset of BCa samples	High AF6 expression associated with lower disease-free survival	Promoter	Charpin *et al.*, 2012
Breast	IHC	206	Elevated AF6 in a subset of BCa samples	High AF6 expression associated with lower disease-free, relapse-free survival and lung metastasis-free survival	Promoter	Tabariès *et al.*, 2019

CRC: colorectal cancer; Pca: pancreatic cancer; OS: osteosarcoma; BCa: breast cancer

decreased survival (Letessier et al. 2007; Sun et al. 2014; Xu et al. 2015). Specifically, in breast cancer, 81.8% of patients with afadin-positive tumors exhibited 5-year metastasis free survival rates, while only 67.8% of patients with afadin-negative tumors had 5-year metastasis free survival (Letessier et al. 2007). The majority of the patients in this cohort were diagnosed with triple positive breast cancer (ER+, PR+, HER2+) (Letessier et al. 2007). In pancreatic cancer patients, those who had low afadin expression in their primary tumor had a median survival of 10 months, whereas those with high afadin expression had a median survival of 23.5 months (Xu et al. 2015). In osteosarcoma and pancreatic adenocarcinoma patients, afadin expression was lower in the tumor relative to the healthy tissue (Xu et al. 2015; Zhang et al. 2018). Finally, low afadin expression in osteosarcoma patients was also associated with low claudin-2 levels and increased pulmonary metastasis (Table 2) (Zhang et al. 2018). Thus, loss of afadin is associated with poor prognosis in multiple cancer types.

1.6.2. Induction of EMT: In pancreatic cancer it was found that afadin can negatively regulate snail expression (Xu et al. 2015). Snail is a transcription factor that can induce an EMT program, an important event in promoting cancer progression (Xu et al. 2015). Afadin was found to interact with both FOXE1 and Dvl2 (Xu et al. 2015). Reduced afadin levels resulted in the upregulation Dvl2, which then binds and increases FOXE1 activation of snail transcription, promoting cell proliferation and tumor progression (Xu et al. 2015). This elucidates a potential mechanism where afadin knockdown can engage a snail-induced EMT program, thus promoting cancer progression (Table 3) (Xu et al. 2015). A similar effect is seen upon gastric infection with *Helicobacter pylori* (Marques et al. 2018). Infection causes a decrease of afadin levels *in vitro* and a reduction of afadin at cell-cell contacts *in vivo*, as well as disruption of TJs and AJs (Marques et al. 2018). This results in an increase in snail expression that promotes EMT and development of gastric cancer (Marques et al. 2018).

1.6.3. Cell migration/invasion: Migration and invasion are fundamental processes required for metastatic cancer progression (Friedl and Wolf 2003). In osteosarcoma, endometrial cancer or breast cancer, afadin knockdown resulted in increased invasion and/or migration (Table 3) (Fournier et al. 2011; Yamamoto et al. 2015; Zhang et al. 2018). Loss of afadin resulted in activation of ERK in cell lines of all three cancer types (Fournier et al. 2011; Yamamoto et al. 2015; Zhang et al. 2018). Activation of ERK signaling is associated with increased cell

Table 3: *In vitro* studies describing afadin functions in cancer

Cancer Type	Cell Line	Subtype	Morphology	Presence of TJ/AJ	Signaling mutations[A]	Afadin Expression	Associated Phenotype	References
Breast	T47D	Luminal A	Epithelial	Yes	PIK3CA mutation	Induced Expression	Decreased collective cell migration	Fournier *et al.*, 2011
Breast	SK-BR-3	HER2	Epithelial	Yes	No mutations	Knockdown	Increased collective cell migration, Increased individual cell migration, Increased invasion and anchorage-independent growth, Increased phosphorylation of ERK1/2 and Src activation	Fournier *et al.*, 2011
Breast	MDA-MB-231	TNBC	Mesenchymal	No	KRAS and BRAF mutations	Knockdown	Increased collective cell migration	Fournier *et al.*, 2011
Breast	MCF7	Luminal A	Epithelial	Yes	ErbB4 and PI3KCA mutation	Knockdown	Increased collective cell migration	Fournier *et al.*, 2011
Breast (mouse)	2776	TNBC	Spindle-shaped	No	Unknown	Normal (High)	Promotes colony formation in soft agar	Tabariès *et al.*, 2019
Pancreatic	BxPC-3	N/A	Epithelial	Yes	ErbB3[B]	Knockdown	Formation of larger spheroid-like structure, Increased invasion and proliferation	Xu *et al.*, 2015
Pancreatic	HPAC	N/A	Epithelial	Yes	KRAS mutation	Knockdown	Increased invasion and proliferation	Xu *et al.*, 2015
Endometrial	Ishikawa	Type 1	Epithelial	Yes	PI3KR1 and PTEN mutation	Knockdown	Increased invasion, Increased phosphorylation of ERK1/2 and Src kinases	Yamamoto *et al.*, 2015
Endometrial	HEC1A	Type 2	Epithelial	Yes	KRAS and BRAF mutation	Normal (Low)	Increased invasion	Yamamoto *et al.*, 2015
Osteosarcoma	U2OS	N/A	Epithelial	Yes	No mutations	Knockdown[C]	Increased migration and invasion, Increased pERK/pMEK	Zhang *et al.*, 2018
Colon	HRT-18	N/A	Epithelial	Yes	KRAS mutation	Knockdown[D]	Decreased epithelial tightness, Increased migration and invasion, Increased pERK	Sun *et al.*, 2014

Mutated cells lines likely have highly active RAS-MAPK, PI3K/AKT and/or Src kinase activity while cell lines with no mutations are assumed to have low/regular signaling activity. [A]Mutation status was obtained from the Sanger COSMIC database and only mutations in RAS, RAF, EGFR, PI3K, PTEN and Src kinase are described as afadin has been shown to play a role in their associated pathways; [B]Unknown significance; [C]after Claudin-2 overexpression (which also increased afadin expression); [D]as a result of CFTR knockdown.

proliferation, survival, and increased migration (Deschênes-Simard et al. 2014; Tanimura and Takeda 2017). In the breast and endometrial cancer models, reduced afadin expression also resulted in activation of Src kinase (Table 3) (Fournier et al. 2011). In the breast models, reduced afadin levels were associated with increased lamellipodia formation, enhanced anchorage-independent growth, greater invasion, and enhanced individual or collective cell migration (Fournier et al. 2011). These phenotypes were enhanced after HRG-β1 stimulation of SK-BR-3 breast cancer cells in which afadin levels were stably reduced (Fournier et al. 2011). Interestingly, two of the three breast cancer models used in this study (MCF7, SK-BR-3) are characterized by intact TJs/AJs and afadin loss resulted in activation of Ras/Raf/MEK/ERK signaling (Table 3). Therefore, loss of afadin can promote cell migration or invasion and stimulate ERK signaling under certain conditions.

Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) and afadin interact with one another at AJs in colon cancer cells (Sun et al. 2014). Suppression of CFTR in HRT-18 cells resulted in degradation of afadin and the loosening of epithelial cell junctions, which ultimately resulted in increased migration and invasion (Table 3) (Sun et al. 2014). These cellular responses were attributed to activation of ERK, which were reversed upon afadin overexpression (Sun et al. 2014). These findings further confirm that reduction of afadin results in 1) activation of ERK signaling and 2) increased migration and invasion following diminished junctional integrity.

1.6.4. Drug resistance: Afadin expression has been associated with drug resistance in endometrial cancer. Reduction of afadin in the Ishikawa cell line, which expresses high levels of afadin, conferred varying levels of resistance to doxorubicin, paclitaxel and cisplatin (Yamamoto et al. 2015). Afadin knockdown in HEC1A cells, which contain very low levels of afadin, failed to promote chemoresistance (Yamamoto et al. 2015).

1.6.5. Metastasis: In vivo studies revealed that primary tumor growth and spontaneous lung metastases are both increased after mammary fat pad (MFP) injection of SK-BR-3 breast cancer cell lines lacking afadin, when compared to afadin-proficient controls (Fournier et al. 2011). Similarly, pancreatic cancer models lacking afadin exhibited enhanced primary tumor growth and

increased spontaneous micro-metastases in the liver (Xu et al. 2015) (Table 4). Together, these studies highlight the tumor suppressive role played by afadin in cancer metastasis *in vivo.*

1.7. Afadin promotes tumorigenesis and metastasis

The potential role of afadin as a tumor promoter was first described in acute myeloid leukemia. However, afadin can positively modulate several cellular processes such as cell survival and migration that can promote cancer progression to metastasis. Furthermore, it has been demonstrated that afadin is required for efficient metastasis under certain conditions.

1.7.1. Acute Myeloid Leukemia: Afadin was first identified as a fusion partner in the MLL-AF6 translocation t(6:11)(q27;q23), which is found in acute myeloid leukemia and associated with poor outcome (Manara et al. 2014; Prasad et al. 1993). The MLL gene is now known as Lysine [K]-specific Methyl Transferase 2A (Winters and Bernt 2017). MLL is a member of the SET1 family of histone H3 lysine 4 methyltransferases that regulate chromatin modification and gene expression (Cosgrove and Patel 2010; Winters and Bernt 2017). The MLL-AF6 fusion comprises the N-terminus of MLL and nearly the entire afadin protein (missing only a small region of the N-terminus), which can oligomerize (Smith et al. 2017). The MLL-AF6 fusion is thought to promote leukemogenesis in two ways.

The first mechanism is dependent on localization of afadin and its impact on Ras signaling and cancer progression. Here, afadin is typically found in the cytoplasm and is associated with low Ras activity (Figure 2) (Manara et al. 2014). However, cells expressing the MLL-AF6 fusion protein reveal a predominantly nuclear localization for afadin (Joh et al. 1997; Manara et al. 2014). This in turn increases Ras-GTP levels in the cytoplasm and increases phosphorylation of ERK, which may promote cancer progression (Manara et al. 2014). Thus, cytoplasmic afadin suppresses Ras activity in normal cells; however, this suppression is removed upon nuclear localization of the MLL-AF6 fusion protein, which leads to increased Ras signaling (Figure 2) (Manara et al. 2014). Indeed, it has been demonstrated that afadin can bind to active Ras in HEK293 cells, an interaction that is promoted by phosphorylation and binding of afadin by Bcr (Radziwill et al. 2003). These interactions then inhibit downstream signaling and ERK activation (Radziwill et al. 2003). Therefore, the nuclear localization of afadin (as seen in MLL-AF6) plays an important regulatory role in promoting cancer progression. However, it remains to be seen if re-localization of afadin may play a similar role in other cancer types.

Table 4: *In vivo* studies describing afadin functions in cancer

Cancer Type	Cell Line	Subtype	Morphology	Presence of TJ/AJ	Signaling mutations[A]	Afadin Expression	Associated Phenotype	References
Breast	SK-BR-3	HER2	Epithelial	Yes	No mutations	Knockdown	Increased lung metastasis, Increased primary tumor growth	Fournier *et al.*, 2011
Breast	MDA-MB-231	TNBC	Mesenchymal	No	KRAS and BRAF mutations	Normal (High)	Promotes liver and lung metastasis	Tabariès *et al.*, 2019
Breast (mouse)	2776	TNBC	Spindle-shaped	No	Unknown	Normal (High)	Promotes liver metastasis	Tabariès *et al.*, 2019
Pancreatic	BxPC-3	N/A	Epithelial	Yes	ErbB3[B]	Knockdown	Increased primary tumor growth, Increased liver micro-metastases	Xu *et al.*, 2015

Mutated cells lines likely have highly active RAS-MAPK, PI3K/AKT and/or Src kinase activity while cell lines with no mutations are assumed to have low/regular signaling activity. [A]Mutation status was obtained from the Sanger COSMIC database and only mutations in RAS, RAF, EGFR, PI3K, PTEN and Src kinase are described as afadin has been shown to play a role in their associated pathways; [B]unknown significance

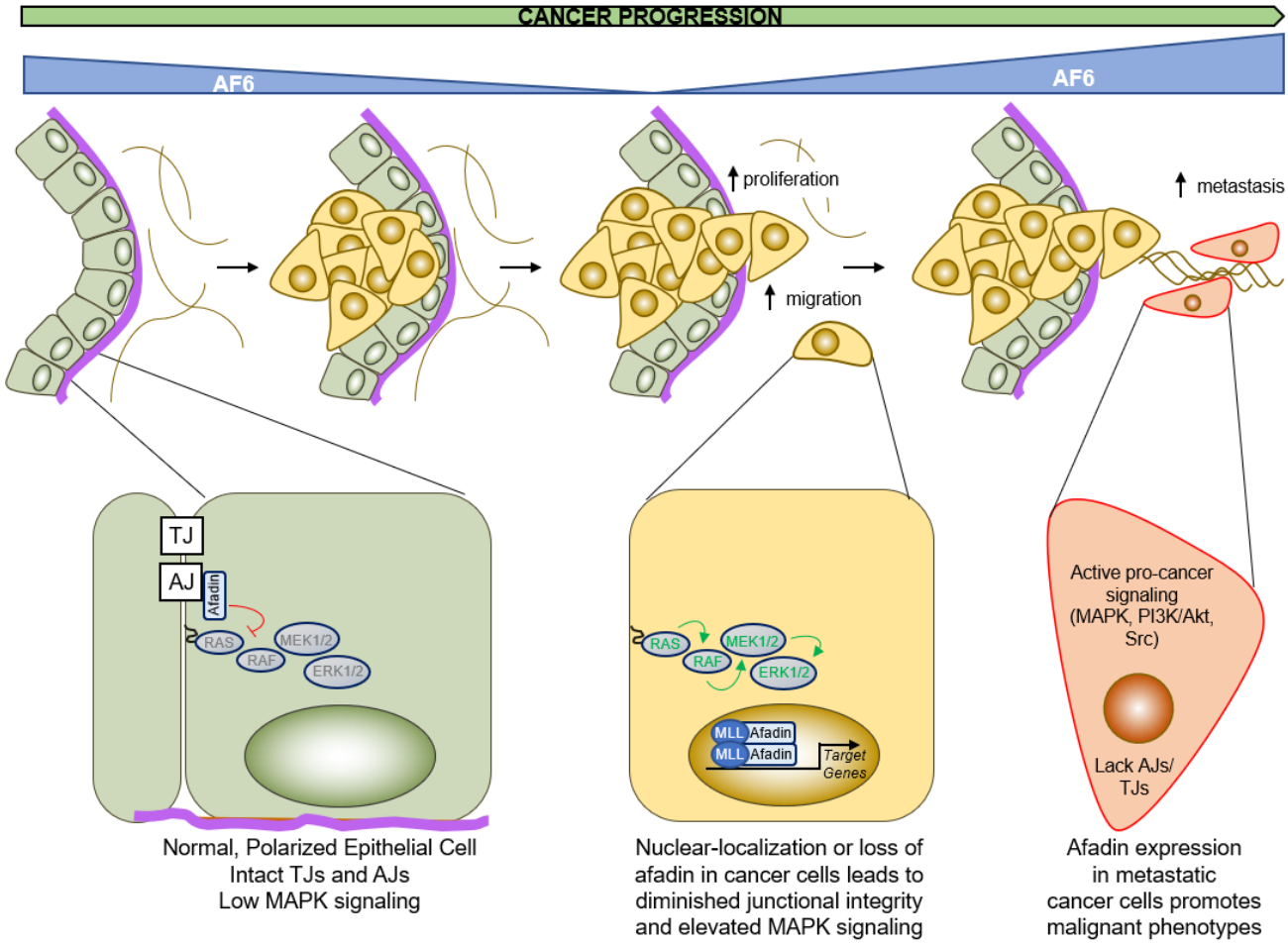


Figure 2. Model of potential afadin roles during cancer progression. In early stages, afadin maintains AJ/TJ integrity and inhibits MAPK signaling. Under certain conditions, such as MLL-AF6 driven leukemias, afadin is re-localized to the nucleus, an event that relieves repression of Ras and promotes MAPK signalling. The MLL-AF6 fusion protein also exhibits novel functions that promotes leukemogenesis. In certain contexts, decreased afadin expression can result in AJ/TJ disruption and enhance signaling and migration/invasion, contributing to cancer progression. In metastatic cancer cells, which already lack AJ/TJ, and may have increased tumorigenic signaling, gain of afadin expression results in malignant phenotypes.

The second leukemogenic mechanism is dependent on the oligomerization of the MLL-AF6 fusion. The RA1 domain of afadin, within the MLL-AF6 fusion protein, is required for immortalization of myeloid progenitor cells and induction of acute myeloid leukemia *in vivo* (Liedtke et al. 2010). Specifically, fusion of MLL and AF6 causes a change in the RA1 domain of afadin, which enables self-association and dimerization of the MLL-AF6 protein via RA1 (Liedtke et al. 2010; Smith et al. 2017). This dimerization of MLL-AF6 is required for leukemogenesis,

thus demonstrating the importance of the RA1 domain in dimerization and cancer progression (Smith et al. 2017). Additionally, it has been demonstrated that the dimerization of other MLL fusions can promote leukemogenesis (Dobson et al. 2000; So et al. 2003). Furthermore, oligomerization of these fusion proteins may promote transcriptional activity of MLL, which is critical for leukemias to form (Smith et al. 2017). It is conceivable that the MLL-AF6 fusion functions in a similar manner. RA1-mediated dimerization may then promote MLL transcriptional activity which leads to leukemogenesis. MLL-AF6 can also promote leukemogenesis through DOT1L, a histone methyltransferase (H3K79) (Deshpande et al. 2013). Indeed, in leukemia cells which harbor MLL-AF6 there is an increase in H3K79 dimethylation at MLL-fusion target genes. This DOT1L activity is required for transformation (Deshpande et al. 2013). These observations argue for gain-of-function effects of the MLL-AF6 fusion event that are critical for leukemogenesis.

Afadin binds to LMO2 (Table 1), a bridging protein that connects components of hematopoietic gene regulatory protein complexes (Begay-Muller et al. 2002). This is of interest because the MLL-AF6 translocation has been found in some leukemias (Manara et al. 2014). The interaction between LMO2 and afadin or the MLL-AF6 fusion protein may elucidate another potential mechanism for promoting leukemogenesis.

1.7.2. Breast Cancer: More recently, afadin has been shown to have a metastasis promoting role in breast cancer (Table 2) (Charpin et al. 2012; Tabariès et al. 2019). In one study, immunohistochemistry for afadin was performed on primary breast carcinoma and correlated with 8-year patient outcomes. This revealed that high afadin levels in the primary tumor are associated with reduced disease free survival (Charpin et al. 2012). Similarly, a high degree of afadin staining in an independent cohort of primary breast tumors was prognostic of reduced breast cancer specific survival, relapse free, survival and lung metastasis free survival (Tabariès et al. 2019). These observations are contrary to previously reported data in which low afadin expression was associated with decreased survival and afadin-negative tumors exhibited worse 5-year metastasis free survival rates (Letessier et al. 2007). The discrepancy between these studies may arise from the nature of the patients selected in each cohort. Indeed, the samples in the Charpin and Tabariès studies were of a higher grade, indicating aggressive disease, when compared to the Letessier cohort (Charpin et al. 2012; Tabariès et al. 2019). Furthermore, in the Tabariès study, 100% of patients had metastases (Tabariès et al. 2019). These findings may present a hypothesis where high

afadin expression is an important promotor in higher grade tumors and more advanced disease. Alternatively, afadin may cooperate with other proteins to influence patient outcome, such as the tight junctional protein claudin-2 (Tabariès et al. 2019). In agreement, the prognostic accuracy of afadin was found to improve when afadin expression levels were combined with other markers such as PI3K and pAkt (Charpin et al. 2012). This demonstrates that afadin may function alongside other factors to influence and predict patient survival.

It has been demonstrated that claudin-2 is a driver of liver metastasis (Tabariès et al. 2011). Indeed, in breast cancer, claudin-2 enhances the ability of breast cancer cells to attach to specific extracellular matrix components and promotes cancer cell interactions with hepatocytes, thus promoting liver metastasis (Tabariès et al. 2011; Tabariès et al. 2012). Afadin was recently found to cooperate with claudin-2 to efficiently promote liver metastasis, demonstrating that afadin may act as a driver in soft tissue metastasis (Tabariès et al. 2019). In agreement with these pre-clinical studies, metastatic breast cancer patients with high afadin expression in their primary tumor were also found to have reduced breast cancer specific survival, relapse free survival, and lung metastasis free survival (Table 2) (Tabariès et al. 2019). Patients who have high afadin and high claudin-2 expression in their primary breast tumor have even more severe outcomes (decreased breast cancer specific survival, relapse free survival, and liver and lung-metastatic free survival) (Tabariès et al. 2019).

1.7.3. Cell migration/invasion: Localization of afadin was also found to impact cell migration. Thus, phosphorylation of afadin by Akt promotes re-localization of afadin from the membrane to the nucleus, which disrupts AJs and results in increased cell migration in breast cancer (Elloul et al. 2014; Zhai et al. 2018). Nuclear localization of afadin, which was associated with increased migration, is greater in invasive breast cancer (Elloul et al. 2014). This was also demonstrated in endothelial cells present within glioblastomas, where a decrease in membrane-localized afadin and an increase of afadin within the nucleus was observed (Zhai et al. 2018). Inhibition of this Akt-mediated phosphorylation event results in reduced endothelial cell migration and may also affect permeability and angiogenesis (Zhai et al. 2018).

Afadin can also promote cancer cell migration and invasion through its role in cancer associated fibroblasts (Labernadie et al. 2017). Indeed, nectin/afadin complexes are required for repolarization of these fibroblasts, which enhances their leader cell function and promotes

cooperative cancer cell invasion. Afadin also helps in the patterning of leader and follower cancer cells during collective cell migration (Labernadie et al. 2017).

1.7.4. *Maintained expression:* The pro-tumorigenic functions of afadin in breast cancer may also be mediated by a cROBO1/KLF5/FUS positive feedback loop, which was found to promote breast cancer liver metastasis (Wang et al. 2022). Indeed, this feedback loop can suppress autophagy-mediated degradation of afadin via BECN1, thus maintaining afadin expression, which may promote cancer progression (Wang et al. 2022).

1.7.5. Drug resistance: Afadin has been associated with resistance to Adriamycin in MDA-MB-231 breast cancer cells (Yang et al. 2018). Claudin-6 promoted afadin expression and subsequently formed a claudin-6/afadin complex that hampered ERK signaling (Yang et al. 2018). E-cadherin levels were elevated, and vimentin levels were reduced in breast cancer cells expressing afadin, while numerous stem cell markers were increased. Finally, engagement of claudin-6/afadin resulted in MDA-MB-231 cells becoming resistant to Adriamycin treatment (Yang et al. 2018).

1.7.6. Metastasis: In vivo, loss of afadin in the HT29 CRC model resulted in reduced liver metastasis (unpublished findings from the Siegel lab). Knockout of afadin in MDA-MB-231 breast cancer cells also resulted in reduced liver metastasis following splenic injection and diminished lung metastasis following tail vein injection (Tabariès et al. 2019). These findings demonstrate that afadin may also be required for efficient metastasis under certain conditions (Table 4). Interestingly, there is little change in primary mammary tumor growth following afadin knockout in breast cancer. The role of the sAF6 and lAF6 isoforms was also investigated, revealing that both isoforms were capable of partially rescuing the metastasis defect exhibited by afadin deficient breast cancer cells (Tabariès et al. 2019). This same study found that afadin can interact with the tight junctional protein claudin-2. Interestingly, claudin-2 has similar pro-tumorigenic functions as afadin in the context of breast and CRC cancer metastasis. Indeed, loss of claudin-2 inhibits liver and lung metastasis *in vivo* (Tabariès et al. 2019). Based on these findings, afadin and claudin-2 may interact to promote breast cancer progression. However, further work is required to determine through what mechanism this may occur.

1.8. Claudin-2 is a tight junction protein

Claudin-2 is a member of the claudin family of proteins, which are tetra-span transmembrane proteins, found in tight junctions of both epithelial and endothelial cells (Lal-Nag and Morin 2009; Tabariès and Siegel 2017). In this capacity, claudins help maintain cell-cell adhesions and polarity, as well as regulate ion and solute movement between adjacent cells (Lal-Nag and Morin 2009; Tabariès and Siegel 2017). Claudins have an intracellular N-terminus, while the C-terminus is found in the cytoplasm and contains a PDZ BD (Tabariès and Siegel 2017). Claudins also have two extracellular loops which allow it to interact with other claudins on the surface of neighboring cells, as well as control what ions can pass between them (Colegio et al. 2002; Piontek et al. 2008). Specifically, claudin-2 has a molecular weight of 24.5kDa and is found in the TJs of leaky epithelia but has also been shown to play a role in other cellular functions such as migration and proliferation (Furuse et al. 2001; Venugopal et al. 2019). The PDZ BD of claudin-2 is notable as it facilitates interaction with a variety of proteins within the cell (Tabariès and Siegel 2017). Some proteins of particular interest that can interact with this domain include ZO-1 (a membrane-associated guanylate kinase) and afadin (Itoh et al. 1999; Tabariès et al. 2019; Venugopal et al. 2019).

1.9. Tumor suppressor function of Claudin-2

1.9.1. Claudin-2 expression and clinical correlations with patient prognosis: Claudin-2 exerts tumor suppressive functions in certain cancer types. In a group of primary triple negative breast cancer patients, those with lower claudin-2 expression had poorer overall survival and relapse-free survival (Ma et al. 2014). Two additional studies found that there was a decrease in claudin-2 expression in primary tumors relative to normal breast tissue (Jia et al. 2019; Kim et al. 2008). Low claudin-2 expression was also associated with increased risk of metastases, decreased survival, and increased lymph node metastasis (Jia et al. 2019; Kim et al. 2008). Low claudin-2 expression has also been associated with poorer survival in renal cell carcinoma (Kumar et al. 2021).

1.9.2. In vitro phenotypes associated with cancer progression: Claudin-2 has also been shown to possess tumor suppressive functions *in vitro*. Indeed, reduced claudin-2 expression promoted EMT in human kidney cells. In accordance, overexpression of claudin-2 in renal clear cell carcinoma cells inhibited EMT, an important step in the metastatic cascade (Kumar et al.

2021). These effects may be regulated through claudin-2 and YAP association via the PDZ BD of claudin-2, where claudin-2 can regulate activation and localization of YAP (Kumar et al. 2021). In lung adenocarcinoma, MEK/ERK, PI3K/Akt, and Jak/Stat3 are able to upregulate claudin-2 expression (Eguchi et al. 2021).

1.10. Pro-tumorigenic functions of claudin-2

1.10.1. Claudin-2 expression and clinical correlations with patient prognosis: Claudin-2 has been shown to have pro-tumorigenic functions in many cancer types including breast, colorectal, endometrial, lung, and gastric cancer (Dhawan et al. 2011; Kimbung et al. 2014; Okada et al. 2020; Paquet-Fifield et al. 2018; Tabariès et al. 2021; Tabariès et al. 2011; Tabariès et al. 2019; Wang et al. 2018; Wei et al. 2021). Claudin-2 expression levels in the primary tumor of metastatic breast cancer patients were able to predict patient outcome. Indeed, patients with elevated claudin-2 expression levels had poorer outcomes (breast cancer-specific survival, relapse free survival, lung, and liver-metastatic free survival) (Tabariès et al. 2019). In agreement, a previous study found that patients with high claudin-2 expression in the primary breast tumor had an increased risk of developing liver metastases and a decrease in relapse-free survival (Kimbung et al. 2014). Claudin-2 was also found to be upregulated in the liver metastases of these patients (Kimbung et al. 2014). Similar findings are seen in CRC, where patients with higher claudin-2 expression had poorer overall survival or increased risk of developing liver metastases (Tabariès et al. 2021; Wei et al. 2021). Claudin-2 expression has also been shown to be elevated in primary tumors relative to healthy tissue in CRC, lung, and endometrial cancer (Dhawan et al. 2011; Maruhashi et al. 2018; Okada et al. 2020; Wang et al. 2018). High claudin-2 expression in two different groups of stage II/III CRC patients that were treated with chemotherapy was associated with reduced relapse free survival or cancer specific survival (Paquet-Fifield et al. 2018). These findings may be attributed to the enhanced ability for CRC self-renewal in cells expressing claudin-2 (Paquet-Fifield et al. 2018). These findings suggest that claudin-2 is clinically relevant and may have pro-tumorigenic functions in certain cancers.

1.10.2. Metastasis: Claudin-2 has been identified as a promoter of breast cancer metastasis. Indeed, claudin-2 was found to be upregulated in a liver-metastatic subset of the 4T1 breast cancer cells (2776). Knockdown of claudin-2 in this model was shown to inhibit liver metastasis (Tabariès et al. 2011; Tabariès et al. 2012; Tabariès et al. 2019; Tabariès et al. 2015b). Interestingly,

expression of a mutant claudin-2, which lacks the C-terminal PDZ BD, also inhibited liver metastasis (Tabariès et al. 2019). Loss of claudin-2 is also able to inhibit lung metastasis in a the human breast cancer cell line MDA-MB-231 (Tabariès et al. 2019). These findings are supported by a CRC model (using HT29 cells) where loss of claudin-2 and/or the PDZ binding domain of claudin-2 are able to inhibit liver and lung metastasis (Tabariès et al. 2021). Two different mechanisms have been elucidated that help explain the pro-metastatic functions of claudin-2. First, claudin-2 is able to promote liver metastasis by increasing expression of $\alpha_2\beta_1$- and $\alpha_5\beta_1$- integrins at the cell surface, which allows the cells to better adhere to fibronectin and type IV collagen (components of the extracellular matrix) (Tabariès et al. 2011). Furthermore, claudin-2 promotes breast and CRC liver metastasis by claudin-2-claudin-2 trans homotypic interactions between tumor cells and hepatocytes, which may allow for survival in this new metastatic site (Tabariès et al. 2021; Tabariès et al. 2012). These findings help to explain why elevated claudin-2 expression in breast and CRC patients may be associated with poorer outcomes.

1.10.3. In vitro phenotypes associated with cancer progression: Elevated claudin-2 expression has been associated with increased cancer cell migration and invasion, two *in vitro* phenotypes important for metastatic ability. Indeed, in an endometrial adenocarcinoma model, knockdown of claudin-2 resulted in a decrease in migration and invasion (Okada et al. 2020). Complementary findings were seen in gastric adenocarcinoma, where claudin-2 overexpression was able to promote migration and invasion (Mima et al. 2008). In the CRC cell line Caco-2, overexpression of claudin-2 caused an increase in migration and a decrease in transepithelial resistance, suggesting a reduction in TJ function (Takehara et al. 2009). Overexpression of claudin-2 in the SW480 and HCT116 CRC cell lines also enhanced their tumorigenic ability. Specifically, claudin-2 expressing cancer cells exhibited increased anchorage-independent growth and proliferation *in vitro*. They also possessed faster tumor growth *in vivo* after subcutaneous injection (Dhawan et al. 2011). In agreement, loss of claudin-2 expression in HT29 CRC cells inhibited migration and invasion (Wei et al. 2021). One mechanism explaining these findings involves the formation of a complex between claudin-2, ZO-1, and ZONAB at the plasma membrane, which inhibits NDRG1 expression. However, loss of claudin-2 allows ZONAB nuclear translocation where it may promote NDRG1 expression; thereby, inhibiting cancer progression (Wei et al. 2021). These findings further support a potential pro-tumorigenic function for claudin-2.

1.10.4. Drug resistance: Claudin-2 expression in lung cancer has been associated with drug resistance (Maruhashi et al. 2018). Indeed, claudin-2 is elevated in lung adenocarcinoma relative to healthy tissue (Maruhashi et al. 2018). Stable knockdown of claudin-2 in the lung cancer cells A549 resulted in an increase of cytotoxicity following treatment with many anticancer drugs including; cisplatin, gefitinib, doxorubicin, carboplatin, and SN-38 (Maruhashi et al. 2018). Decreased claudin-2 expression was associated with a decrease in expression of the multidrug resistance-associated protein ABCC2 (a drug efflux transporter), resulting in the accumulation of doxorubicin in A549 cells and a spheroid model (Maruhashi et al. 2018). Claudin-2 overexpression in CRC cells provided protection during 5-FU treatment, where the claudin-2 high cells exhibited increased proliferation and less apoptosis relative to controls (Dhawan et al. 2011). Increased claudin-2 expression in some CRC cell lines was associated with EGF stimulation via EGFR/ERK1/2 signaling (Dhawan et al. 2011). The findings from these two studies suggest that claudin-2 may be associated with increased drug resistance in certain cancers.

1.10.5. Targeting Claudin-2 to impair cancer progression: Claudin-2 has pro-tumorigenic and pro-metastatic functions; therefore, it represents a good drug target to restrict cancer progression. The feasibility of targeting claudin-2, with respect to potential unwanted side effects in normal tissues, has been revealed by claudin-2 knockout mouse models, which were found to be normal with the exception of some impaired transepithelial reabsorption of Na^+ and Cl^- in the renal proximal tubules (Muto et al. 2010). Additionally, induction of kidney stress by administering a 2% NaCl solution resulted in an increase in Na^+ and Cl^- loss in the urine (Muto et al. 2010). The effects of inhibiting claudin-2 in cancer was demonstrated using non-steroidal anti-inflammatory drugs, which were able to inhibit claudin-2 expression in the gastric adenocarcinoma cell lines AGS, KATO-III, and T-84, and the lung adenocarcinoma cell line A549 (Mima et al. 2008). Drug-induced reductions in claudin-2 levels resulted in reduced invasive ability of the AGS cell line. In agreement, overexpression of claudin-2 in AGS promoted invasion and migration (Mima et al. 2008). Another example was shown in colon cancer. Typically, claudin-2 is found in the TJs of leaky epithelia and is associated with reduced TJ integrity. In order to improve TJ barrier function, a monoclonal antibody targeting the first extracellular loop of claudin-2 was generated. This antibody was able to improve TJ integrity, measured using a transepithelial electrical resistance model, in the colon cancer cell line Caco-2 (Takigawa et al. 2017). The antibody was also able to minimize TJ integrity disruption in a TNF-α induced TJ dysfunction model.

Furthermore, treatment of Caco-2 cells with the claudin-2 monoclonal and infliximab or adalimumab after TNF-α insult, further improved the TJ barrier function (Takigawa et al. 2017). These finding, suggest that claudin-2 is a viable drug target to restrict cancer progression.

1.11. Claudin-2 interacting partners

The importance of the PDZ binding domain within claudin-2 for promoting breast and CRC liver and/or lung metastasis has been previously demonstrated (Tabariès et al. 2021; Tabariès et al. 2019). To identify potential interacting partners of the PDZ binding domain of claudin-2 immunoprecipitation was performed, followed by silver staining and mass spectrometry (Tabariès et al. 2019). Proteins that contained a PDZ domain, which could theoretically interact with the PDZ BD of claudin-2, were prioritized. Seven potential interacting proteins were identified including; afadin, Arhgap21, Pdlim2, Pdlim7, Rims2, Scrib, and ZO-1 (Tabariès et al. 2019). Each candidate was then individually knocked down in the 2776 liver-aggressive cell line (4T1 derived) and their effect on anchorage independent growth and/or liver metastasis was assessed. Afadin and Pdlim7 were found to inhibit anchorage independent growth and liver metastasis. However, afadin was ultimately pursued as commercial reagents targeting afadin were more readily available (Tabariès et al. 2019).

Co-immunoprecipitation experiments revealed that claudin-2 and afadin interact via the PDZ BD of claudin-2 (Tabariès et al. 2019). However, the exact mechanism through which these two proteins interact remains to be elucidated. Two potential mechanisms are possible. Claudin-2 and afadin may interact directly via the PDZ BD of claudin-2 and the PDZ domain of afadin to exert the pro-metastatic functions of these proteins. Alternatively, claudin-2 may interact indirectly with afadin via another protein such as ZO-1. Claudin-2 and ZO-1 have been shown to interact via the PDZ binding domain (Itoh et al. 1999; Ooshio et al. 2010). Afadin can also interact with ZO-1 via the PRR1/2 domain of afadin and the SH3 domain of ZO-1 (Ooshio et al. 2010). Such an interaction between ZO-1 and afadin occurs in Madin-Darby canine kidney cells during the formation of TJs (Ooshio et al. 2010). Therefore, these three proteins may be able to form a complex that can regulate cancer progression (Tabariès et al. 2019). Given the data supporting the importance of claudin-2 and/or afadin in cancer progression, improved understanding of how these proteins interact, be it directly, indirectly, or through parallel signaling pathways, is therefore of interest.

1.12. Rationale.

Previous research from the Siegel lab found that claudin-2 expression in breast cancer is both necessary and sufficient to promote liver and lung metastasis (Tabariès et al. 2019). Importantly, the carboxy-terminal PDZ-BD of claudin-2 was shown to be required for liver metastasis promoting activity of claudin-2 (Tabariès et al. 2019). Afadin is a PDZ domain containing protein that is able to interact with claudin-2 and has also been shown to promote liver and lung metastasis (Tabariès et al. 2019). Afadin and claudin-2 are therefore important mediators of metastasis, yet the precise molecular mechanisms through which they interact to enhance metastasis remains unclear. Whether claudin-2 and afadin interact directly, via a mediator protein or function through parallel pathways will be studied. Additionally, the importance of specific functional domains of afadin will also be explored.

We hypothesize that claudin-2 and afadin promote metastasis, either by cooperating together within the same complex or functioning independently in distinct pathways in breast cancer. Furthermore, specific functional domains within afadin will contribute to its metastatic functions. The main objective of this project is to better understand how claudin-2 and afadin interact and the roles they play in breast cancer metastasis. This is then divided into two sub-objectives.

i. Determine if claudin-2 and afadin interact directly via the PDZ domain, through a mediator protein such as ZO-1 or function in parallel signaling pathways.
ii. Improve the understanding of the afadin domains and their role in downstream signaling that promote metastasis.

CHAPTER 2: MATERIALS AND METHODS

2.1. Cell culture: MDA-MB-231TR cells (luciferase-tagged triple negative breast cancer cells) were obtained from Dr. Joan Massagué (Minn et al. 2005). They were maintained in high glucose Dulbecco's Modified Eagle Medium (DMEM; Cat. No.: 319-005-CL, Wisent Bioproducts) supplemented with 10% fetal bovine serum (FBS; Cat. No.: 10082-147, Thermo Fisher Scientific), and 1.0 mM MEM non-essential amino acids (Cat. No.: 321-011-EL, Wisent Bioproducts), 0.2% amphotericin B (Cat. No.: 450-105-QL, Wisent Bioproducts), and 0.05 mg/mL gentamicin sulfate (Cat. No.: 450-135-EL, Wisent Bioproducts). HT-29 cells were obtained from the American Type Culture Collection and were incubated in the same media, modified to contain 100 mM sodium pyruvate (Cat. No.: 600-110-EL, Wisent Bioproducts) instead of non-essential amino acids. All afadin or claudin-2 knockout cell lines were supplemented with 2 µg/mL of puromycin (Cat. Code: ant-pr, Invivogen). After retroviral infection, MDA-MB-231TR cells were cultured in media containing 5 mg/mL blasticidin (ant-bl, Invivogen), Cell containing the lAF6 also contained 0.4mg/ml hygromycin B (Cat. No.: 450-14+-XL, Multicell). All cells were incubated at 37°C with 5% CO_2.

2.2. Transfection and Viral Infection: For retrovirus production, 293VSV cells were expanded in DMEM + 10% heat inactivated FBS (Cat. No.: 10082-147, Thermo Fisher Scientific) supplemented with 5 µg/mL tetracycline. An Effectene kit (Cat. No.: 301427, Qiagen) was used to transfect the cells using 2 µg DNA of interest and 0.5 µg pVpack-VSV-G following the manufacturer's protocol (Cat. No.: 301427, Qiagen). At 24 hours post-transfection, the media was changed, and tetracycline removed to permit viral production. The media containing viral particles was subsequently collected every day for 5 days. The virus was then spun down at 1,200 rpm for 4 minutes and filtered through a 0.45 µm filter. For retroviral infection, 200,000 cells (MDA-MB-231TR or HT-29) were plated in a 60 mm dish the day before infection. The following day, a 1:1 ratio of media to retrovirus was added to each dish, with the addition of polybrene to a final concentration of 8 µg/mL. The media was changed 24 hours later. At 48 hours post-infection, the cells were expanded and placed under selection. For lentiviral infection with ZsGreen-luc, 150,000 cells were plated in a 6-well plate. The following day, a 1:10 ratio of media to virus was added to each dish, with the addition of polybrene to a final concentration of 8 µg/mL. The day after infection the media was changed. At 2 days post infection, cells were expanded and subsequently subjected to fluorescence-activated cell sorting using GFP as the marker.

2.3. Luciferase assay: Luciferase assay were performed using the luciferase assay systems from Promega (Cat. No.: E1500), following manufacturer's protocol.

2.4. Immunoblot analysis: Cells were lysed using TNE (0.05 M pH8 Tris-HCL, 0.15 M NaCl, 1% NP40, 0.002 M pH8 EDTA in dH_2O) or RIPA lysis buffer (0.01 M $NaPO_4$, 1% Triton X-100, 0.1% SDS, 0.01 g/mL Sodium deoxycholate, 0.15 M NaCl, EDTA 0.0002 M pH8 in dH_20) supplemented with 0.0025 M Na pyrophosphate, 0.01 M NaF, 0.01 mg/mL aprotinin and leupeptin, 0.001 M Na_3VO_4 and 0.1% B-glycerophosphate. Cell lysates were then rotated at 4°C for 20 minutes and centrifuged at 15,000 rpm for 10 minutes at 4°C. Protein concentrations were determined using a Bradford assay using the Protein assay dye reagent concentrate (Cat. No.: 5000006, Biorad). Protein lysates were separated in a 6% or 12% polyacrylamide gel and transferred to immobilion-P polyvinylidene difluoride (PVDF, Cat. No.: IPVH00010, Milipore Sigma) or immobilion-FL PVDF (Cat. No.: IPFL00010, Milipore Sigma) membranes and blocked for 1 hour in 2% milk in TBST-T or Intercept blocking buffer (Cat. No.: 927-60001, Li-Cor) (used for HA only). Membranes were then incubated overnight at 4°C with the appropriate primary antibody. The following primary antibodies were used; afadin (1:5000; Cat. No.: 610732, BD Transduction,), Flag (1:5000; Cat. No.: F1804-1MG, Sigma-Aldrich), adaptin (1:5000; BD biosciences, 610502), claudin-2 (1:5000; Cat. No.: 325600, Thermo Fisher), ZO-1 (1:5000; Cat.No.: 617300, Invitrogen) and α-tubulin (1:10000; Sigma, T9026). Membranes were then washed in TBS-T and incubated in the corresponding horseradish peroxidase (HRP)-conjugated anti-IgG secondary antibody (1:10,000, Jackson ImmunoResearch Laboratories, Inc.) for 1 hour. IRdye secondary antibody from Li-Cor was used for the HA blots. The membranes were then washed in TBS-T and visualized using SuperSignal West Pico PLUS chemiluminescent substrate (Cat. No.: 34578, Thermo Fisher Scientific) or using an Li-Cor Odyssey Infrared imaging system (Cat. No.: 12391-12870-14160-14161).

2.5. Plasmid construction: lAF6 with blasticidin resistance was expressed in the pQCXIB retroviral expression vector as previously described, to generate the plasmid named pMD8 (Tabariès et al. 2019). To epitope tag afadin, 2x Flag oligos were synthetized, annealed (Table below) and introduced into lAF6 (pMD8) using *Eag*I/*Not*I to make the plasmid called pST78. The

2x Flag-tagged lAF6 cDNA was then cloned into pMSCV hygro (clontech) using *Not*I/*Bam*HI to generate a hygromycin resistant 2x-Flag-lAF6 construct that was labeled pJH14.

The lAF6 vector (pMD8) was also utilized to generate the panel of afadin mutants. For the FHA mutant, the N-terminus region of lAF6 (pMD8) was digested with *Bam*HI/*Not*I and first shuttled into pBluescript II KS(+) vector (Stratagene) to generate the plasmid named pBL2. The pBL2 vector was then PCR amplified to delete the FHA domain using the primers found in Table below, to create plasmid pBL7. The mutant domain was then shuttled back into the lAF6 vector (pMD8) using *Bam*HI/*Not*I to create the vector named pBL10. A 2x Flag tag was then added to the FHA mutant vector (pBL10) from pST78 using *Not*I/*Eag*I to create the plasmid pJH4. The same procedure was used for the DIL mutant; however, a *Bspe*I/NotI digest was used to transfer the mutated DIL domain back into the lAF6 (pMD8), to generate the vector named pNT3. For the RA1 and RA2 mutants, following PCR amplification to delete each domain (using primers found in table below), the mutant RA1 or RA2 domains were transferred back into the lAF6 vector (pMD8) using *Bspe*I/*Not*I, making the pNT1 and pBL11 plasmids, respectively. The mutant RA1 or RA2 domains were then transferred into pBluescript using *Eco*RI/*Not*I, generating pJH6 and pJH7. A 2x Flag-tag from pST78 was then added to the RA1 and/or RA2 mutant pBluescript vector using *Not*I/*Eag*I to make the pJH10 and pJH11 constructs. Finally, the flagged RA1 and RA2 mutants were transferred back into the lAF6 vector (pMD8), generating pJH12 and pJH13 plasmids, respectively. For the PDZ mutant, a central region of lAF6 (pMD8) was first digested with *Bam*HI/*Ale*I and shuttled into pBluescript to generate the pBL1 construct. The pBL1 vector was then PCR amplified to delete the PDZ domain using the primers found in Table below, generating the pBL4 construct. The mutant PDZ domain was then transferred back into the lAF6 vector (pMD8) and a 2x Flag-tag was added using the same enzymes and vector as described for the FHA mutant, creating the pJH3 construct. For the PRR1/2 mutant, the C-terminus region of lAF6 (pMD8) was cut with *Nhe*I/*Bam*HI and shuttled into pSL301 to generate the pJH1 plasmid. Oligos of the mutated PRR1/2 domain were PCR amplified and then swapped into pJH1 using *Ale*I/*Fspa*I to create the pJH8 construct. The mutant PRR domain was the cloned into pST78 using *Ale*I/*Rsr*II, generating a Flag-tagged PRR mutant construct called pJH9. The sAF6 vector was described in (Tabariès et al. 2019), to which a 2x Flag-tag was added from pST78 using *Not*I/*Eag*I making the pJH5 construct. All plasmids were sequence-verified and found to be in-frame and contain no unexpected mutations.

Mutant claudin-2 plasmids (EV, WT, WT-HA, ΔPDZ BD-HA) were generated as previously described (Tabariès at l. 2019).

Mutant Domain	Primers used
RA1	5'-atat**gctagc**GAACTCCAAATCCTCGGT-3'
	5'-atat**gctagc**AATGACGCCATTCCTCCTAA-3'
RA2	5-'atat**gctagc**TGAATCAGGCCGCCCATC-3'
	5'-atat**gctagc**CCAAAGAAAACCAAGAAACACTTGG-3'
FHA	5'-atat**gctagc**CAACTGGATAGAGTTGTCATCC-3'
	5'-atat**gctagc**CTTGCAAAAAGATCTGTGGATGG-3'
DIL	5'-atat**gctagc**GATAATCGCACCCCAGTAA-3'
	5'-atat**gctagc**GGAAGGGAAGTGCAGTTG-3'
PDZ	5'-atat**gctagc**TTCAGGTTCTTTCCTCAGAGG-3'
	5'-atat**gctagc**GCCATCTACCACGGTCTG-3'
PRR	5'-cccttgaacctcctcgttcgacCCACCCCTGTGGCCGTCTCCCAGCCA ATCCGAACAGACCTG**catatg**CAGATAGGGCTGCCGTCTGCGCA GGTGGCTGCTGCTGAACGGAGAAAGAGAGAAGAACATCAGCG TTGGTATGAGAAGGAGAAGGCCCGCCTGGAGGAGGAGCGGGA GAGGAAGCGGAGAGAGCAGGACAGGAAGTTGGGCCAGATGC GCACgacaaatggaagtagcacgtctc-3'
2xFlag	5'-ataTGCGGCCGCGCCACCatggattacaaagacgatgacgataaggattac aaagacgatgacgataagatggcctcggcgggcggccgtgacga-3'
	5'-tcgtcacggccgcccgccgaggccatcttatcgtcatcgtctttgtaatccttatcgtcatc gtctttgtaatccaTGGTGGCGCGGCCGCAtat-3'

Letters in red correspond to a new restriction site, capital letter are homologous to the plasmid used.

2.6. Animal work: To assess primary mammary tumor growth, 1.0 x 10^5 MDA-MB-231TR cells were injected into the fourth mammary gland of 4- to 6-weeks old female NSG mice as previously described (Tabariès et al. 2019). Primary tumor growth was monitored by caliper measurement and individual mammary tumors were resected at a volume of approximately 600mm^3 (4.5-6.5 weeks after injection). The mice were then followed using in *vivo* bioluminescent imaging (IVIS) for an additional 2 weeks post-resection to permit formation of spontaneous metastases in the lungs and liver. Lung and liver metastatic burden was quantified following hematoxylin and eosin (H&E) staining, using Imagescope software (Aperio), by measuring metastatic lesion area/lung (or liver) area as previously described (Tabariès et al. 2011, Rose et al., 2007). The mice were housed in facilities managed by the McGill University Animal Resources

Centre and all animal experiments were conducted under a McGill University approved Animal Use Protocol in accordance with guidelines established by the Canadian Council on Animal Care.

2.7. Statistical analysis: Statistical significance (P value) was measured using unpaired Student's t-test in Graphpad Prism 9. Experimental variability bars for primary tumor growth curves expressed as standard deviation. Bars in lung and liver metastatic burden represent mean.

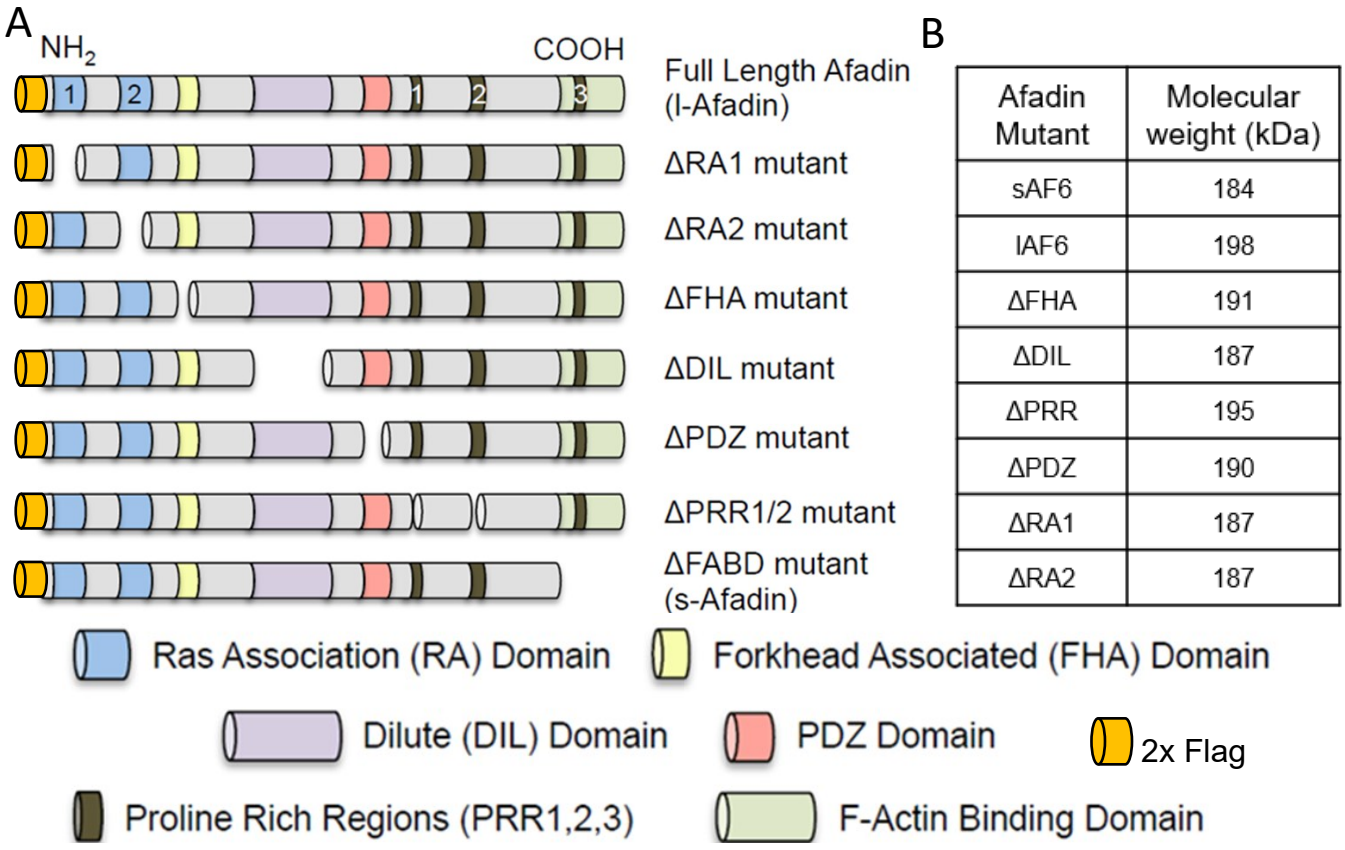
CHAPTER 3: RESULTS

3.1. Generation of a panel of afadin isoforms

Claudin-2 is an important driver of breast cancer liver metastasis and was shown to interact with afadin via its PDZ binding domain (Tabariès et al. 2019). Afadin was also found to be an important regulator of breast cancer metastasis, as knockout of afadin inhibited liver and lung metastasis. In order to better understand the various roles that afadin plays in cancer progression, afadin was previously knocked out in the triple negative breast cancer cell line MDA-MB-231TR ($231TR^{AF6CR}$) (Tabariès et al. 2019). The $231TR^{AF6CR}$ cells also contain a triple-fusion protein reporter construct, which encodes herpes simplex virus thymidine kinase 1, green fluorescent protein (GFP) and firefly luciferase (Minn et al. 2005). This allows these cells to be tracked using IVIS. To assess the importance of the long and short afadin isoforms to the formation of lung and liver metastases, a 2xFlagged vector was generated, which harbored either the short or long isoform of afadin. These isoforms were then reconstituted in the $231TR^{AF6CR}$ breast cancer cells, thus generating cells that express either 1) Flag-sAF6 alone, 2) Flag-lAF6 alone or 3) co-express Flag-sAF6+Flag-lAF6 (Figure 3A and 4A). An empty vector was used as control. Individual clones expressing each afadin isoform or harboring an empty vector construct were then picked and afadin levels assessed by immunoblot, and the luciferase activity was confirmed using the luciferase assay system (Figure 4 (B-G) and Table 5). Based on these findings, pools of three independent clones were generated for each cell line, except for lAF6 where only two clones were pooled (Figure 5A). The afadin expression levels and luciferase activity was confirmed a final time prior to injection (Figure 5A and B).

3.2. Re-constitution of afadin isoforms rescues metastatic phenotype

The primary tumor growth of the $231TR^{AF6CR}$ afadin flagged isoforms was monitored by caliper measurement (Figure 5C). A slight delay in mammary tumor growth was observed with breast cancer cells reconstituted with $231TR^{AF6CR}$, an empty vector, sAF6 alone, lAF6 alone and sAF6 + lAF6, relative to the parental control; however, these differences were not significant (Figure 5C). Following mammary tumor resection, mice were followed for 2 weeks by IVIS imaging to track the development of breast cancer metastases (to the lung or liver) (Figure S1). Metastases developed in the lungs of mice injected with breast cancer cells expressing sAF6 alone and sAF6+lAF6. Mice bearing the $231TR^{AF6CR}$ and EV populations exhibited a significant reduction in lung-metastatic burden relative to the parental control (Figure 5D and S2).



B

Afadin Mutant	Molecular weight (kDa)
sAF6	184
lAF6	198
ΔFHA	191
ΔDIL	187
ΔPRR	195
ΔPDZ	190
ΔRA1	187
ΔRA2	187

Figure 3: Panel of afadin mutants. (A) Cartoon of afadin protein with deletion of individual domains. (B) Estimated molecular weight of afadin mutants. Deletion of individual afadin domains, as seen in mutants, results in a change in the size of the afadin protein.

Re-constitution with sAF6 or sAF6+lAF6 was able to rescue the lung-metastatic deficit observed in the knockout populations (Figure 5D). Indeed, both of these cell populations had a greater lung-metastatic burden when compared to the parental population, while the sAF6+lAF6 had the greatest lung-metastatic burden. Unexpectedly, the lAF6 was unable to rescue the lung-metastatic deficit, and instead had a similar burden to the 231TR^{AF6CR} and EV populations. This data demonstrates that afadin is required for spontaneous lung metastasis and that expression of sAF6 or sAF6+lAF6 is able to rescue this deficit.

The liver metastatic burden was also assessed following H&E staining. The parental 231TR cells had the greatest liver-metastatic burden (Figure 5E and S3). As expected, loss of afadin expression (231TR^{AF6CR}) resulted in significant impairment of liver metastasis. Similar findings were seen in the EV population however the reduction in liver metastatic ability did not reach significance. Re-constitution of sAF6 alone or sAF6+lAF6 partially rescued the formation of liver

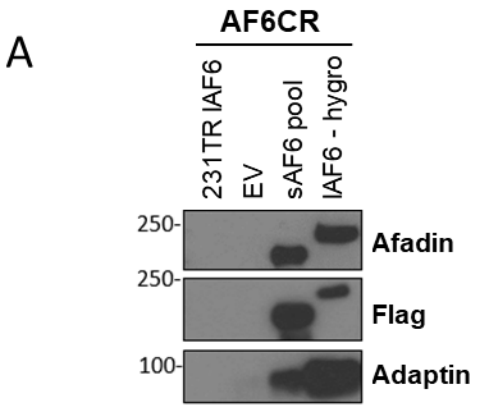
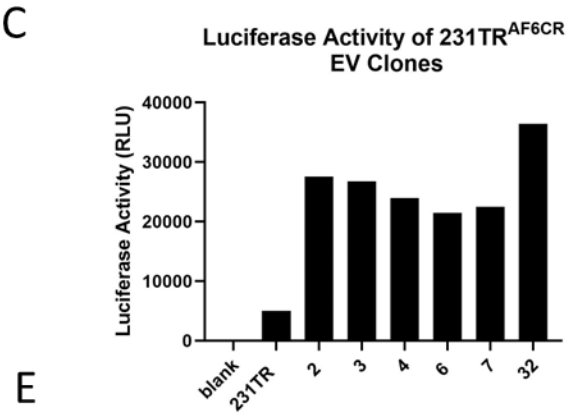
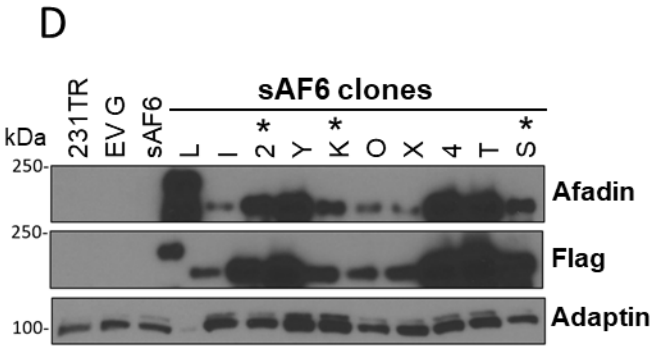
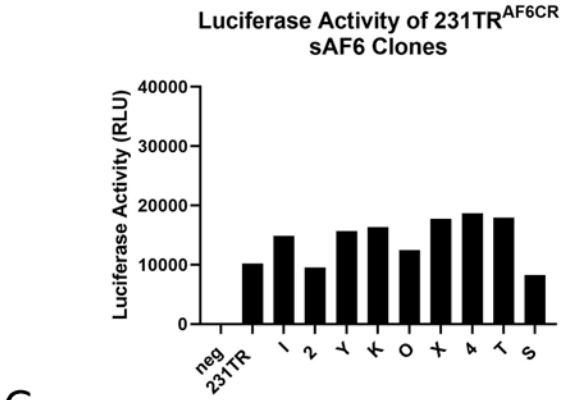
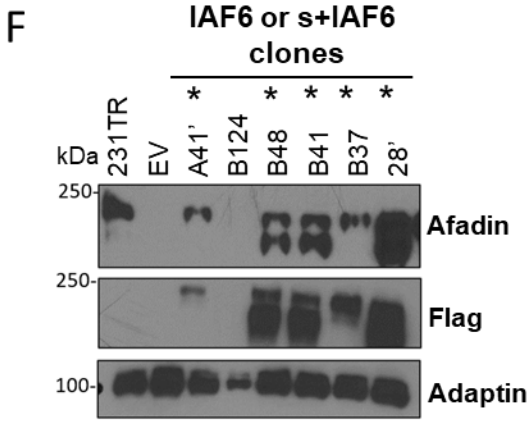


Figure 4. Screening of afadin isoform clones to generate a pool. (A) Immunoblot of hygromycin resistant lAF6, α-adaptin was used as a loading control. (B, D, F) Immunoblot analysis of MDA-MB-231TR^{AF6CR} harboring flagged EV, sAF6, lAF6 or sAF6 + lAF6 vectors, α-adaptin was used as a loading control. *clones used to generate pool. (C, E, G) Luciferase activity of afadin isoform clones confirmed using luciferase assay system. 231TR lAF6 positive control did not work in panel A, however all other bands appear as expected. Variability in 231TR luciferase activity likely due to repeated freeze/thaw.









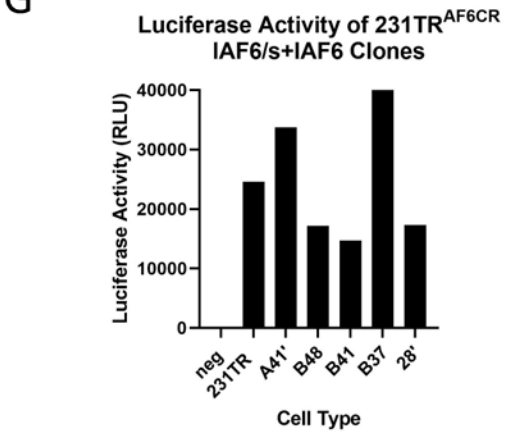


Table 5: Summary of MDA-MB-231TR^{AF6CR} mutant clones screened

Mutants	# Clones	# Screened	Positive
EV	18	N/A	N/A
sAF6	9	9	9
lAF6	83	83	2
s+lAF6	179	179	3
ΔPDZ	7	7	6
ΔPRR	10	10	6
ΔFHA	10	10	8
ΔDIL	18	18	7
ΔRA1	14	14	6
ΔRA2	20	20	6
Total	368	350	54

metastases compared to afadin-deficient breast cancer cells where the rescue of sAF6+lAF6 population had a slightly greater rescue and reached significance (Figure 5E). Once again, the lAF6 was not able to rescue the liver-metastatic deficit and had a similar burden to the 231TR^{AF6CR} or EV populations. In agreement with our previous findings, afadin is required for spontaneous liver metastasis, where the sAF6 or sAF6+lAF6 populations were able to partially rescue the metastatic burden seen after loss of afadin.

3.3. Generation of a panel of afadin mutants

To evaluate the importance of each afadin domain, a panel of afadin mutant vectors were generated using the Flagged-lAF6 isoform, where each individual afadin domain was deleted (ΔRA1, ΔRA2, ΔFHA, ΔDIL, ΔPDZ, ΔPRR) (Figure 3). These mutant afadin vectors were then re-expressed in 231TR^{AF6CR} and expression confirmed using immunoblot (Figure 6A). Individual clones for each afadin mutant cell line were then picked and afadin expression confirmed using immunoblot, while the luciferase activity was confirmed using the luciferase assay system (Figure 6 (B-G), 7 (A-F) and Table 5). Based on these findings, clones expressing relatively equal amounts of afadin as the 231TR parental cell line were then used to generate pools of three independent clones for each cell line (Figure 8A). The afadin expression levels and luciferase activity was confirmed a final time prior to injection (Figure 8A and B).

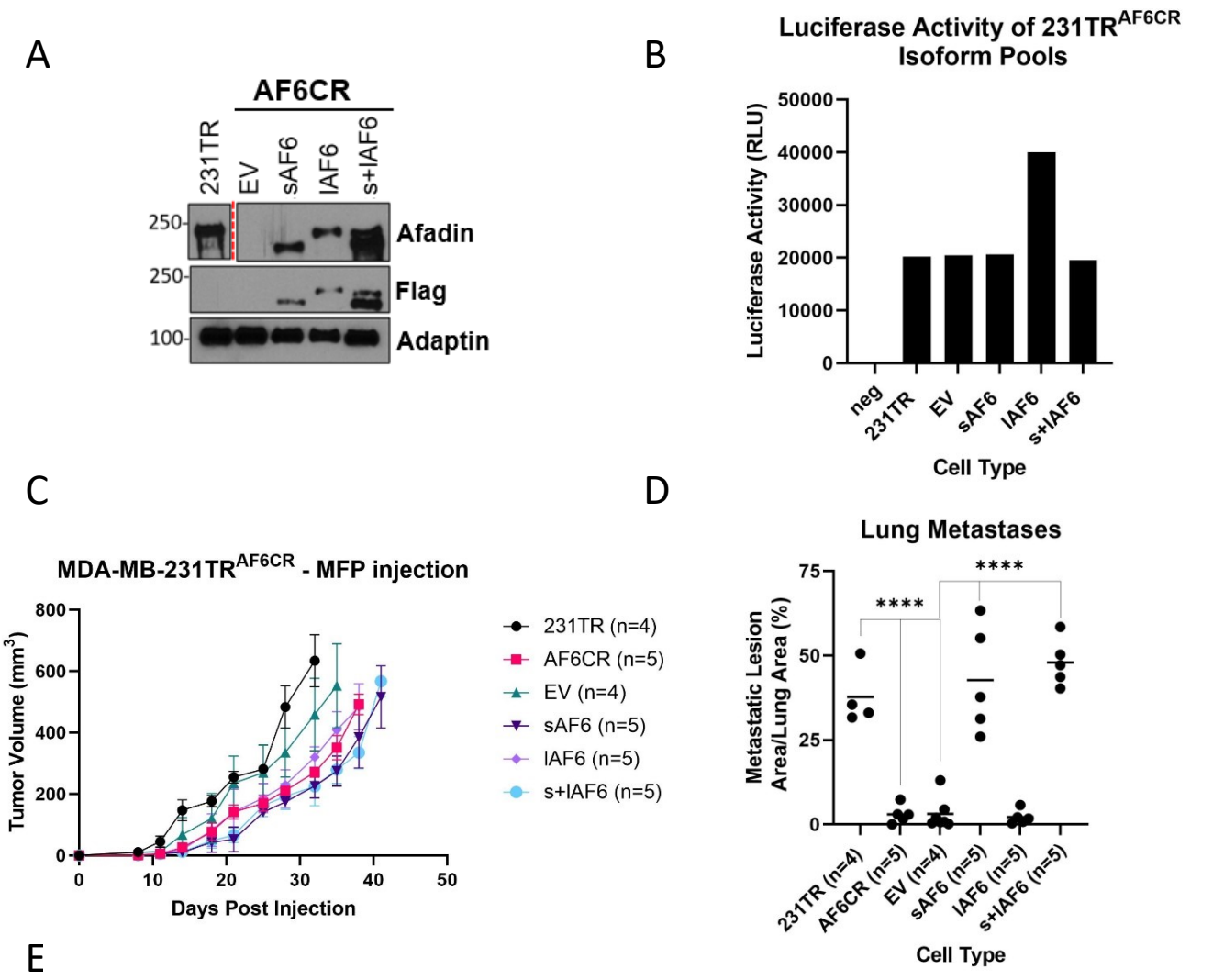
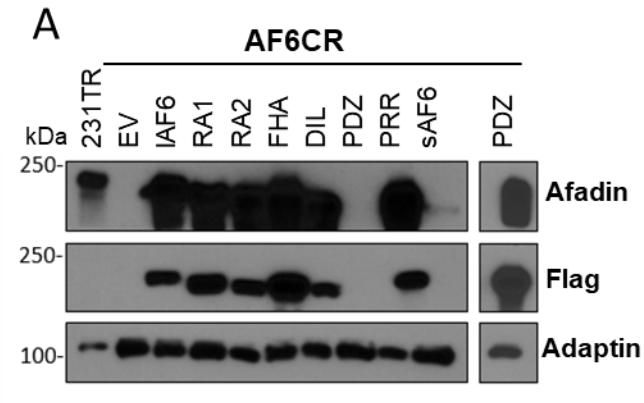
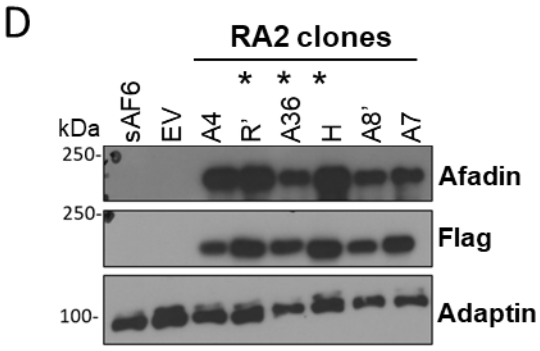


Figure 5. Afadin isoforms rescue metastatic deficit seen after afadin knockout. (A) Immunoblot analysis of MDA-MB-231TR^{AF6CR} pools (n=3, except lAF6 where n=2) re-expressing flagged afadin isoforms, α-adaptin was used as a loading control. (B) Luciferase activity of mutant afadin pools confirmed using luciferase assay system. (C) Primary tumor growth in the mammary fat pad was measured until a size of ~600mm^3 was reached, it was then resected. n= number of mice per group. Experimental variability expressed as SD (D) Lung metastatic burden (tumor area per tissue area) of afadin isoforms. (E) Liver metastatic burden (tumor area per tissue area) of afadin isoforms. (*) P<0.05, (****) P<0.0001.





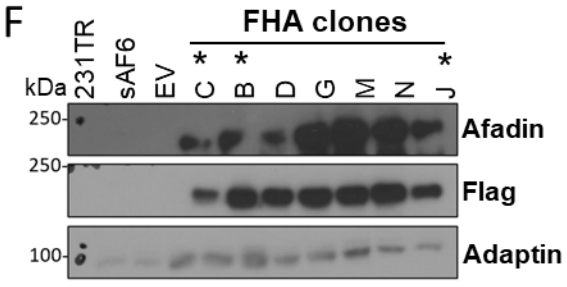
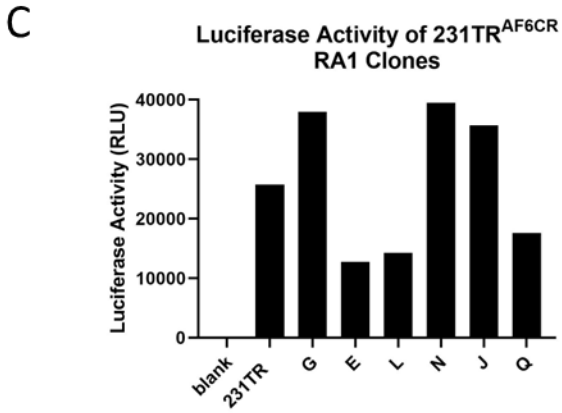
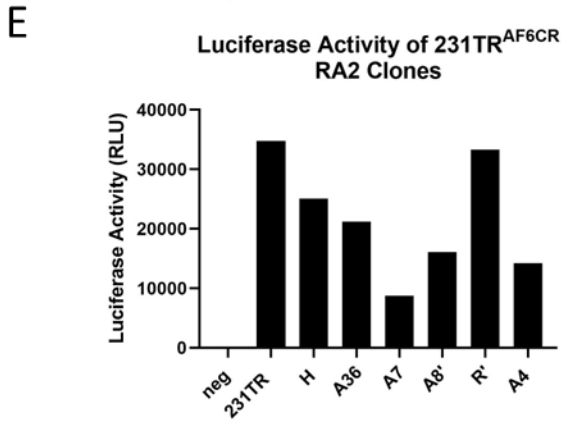
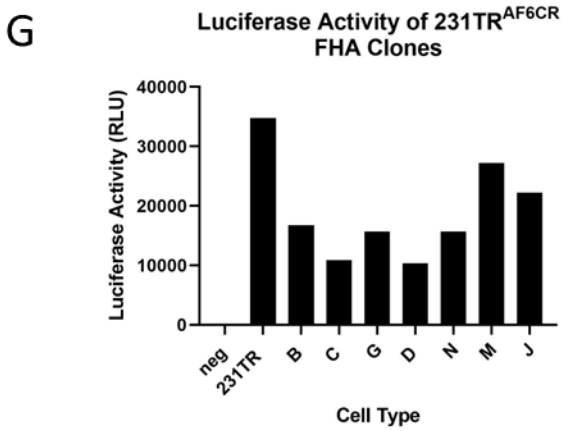


Figure 6. Screening of mutant afadin clones to generate a pool. (A) Immunoblot analysis of MDA-MB-231TR^{AF6CR} harboring various flagged mutant forms of afadin (B, D, F) Immunoblot analysis of MDA-MB-231TR^{AF6CR} harboring flagged RA1, RA2, FHA vectors, α-adaptin was used as a loading control. *clones used to generate pool. (C, E, G) Luciferase activity of afadin mutant clones confirmed using luciferase assay system. Variability in 231TR luciferase activity likely due to repeated freeze/thaw. Note: problems with some loading controls, however all clones appear as expected.







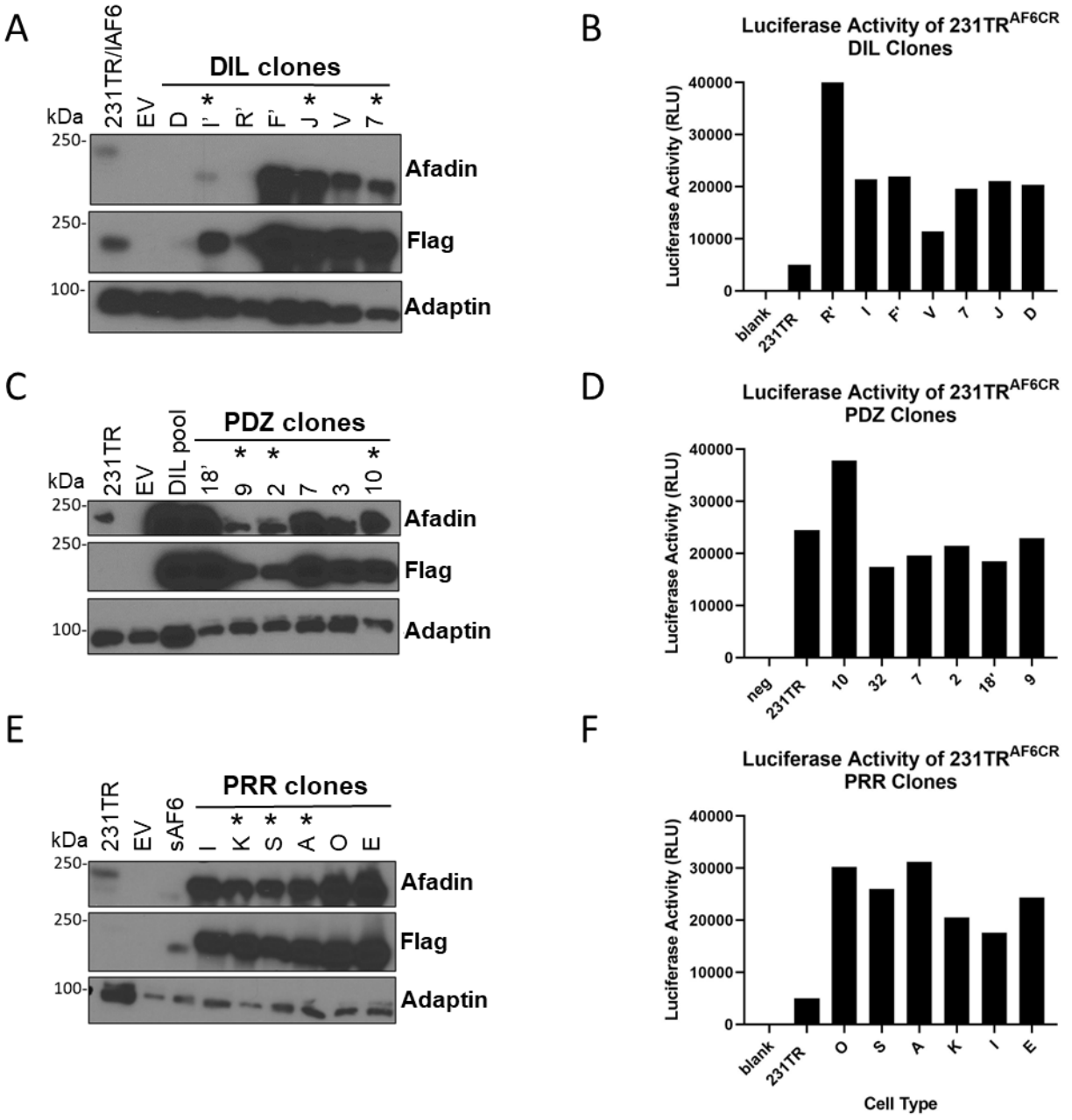


Figure 7. Screening of mutant afadin clones to generate a pool. (A, C, E) Immunoblot analysis of MDA-MB-231TR^{AF6CR} harboring flagged DIL, PDZ, PRR vectors, α-adaptin was used as a loading control. *clones used to generate pool. (B, D, F) Luciferase activity of afadin mutant clones confirmed using luciferase assay system. Variability in 231TR luciferase activity likely due to repeated freeze/thaw.

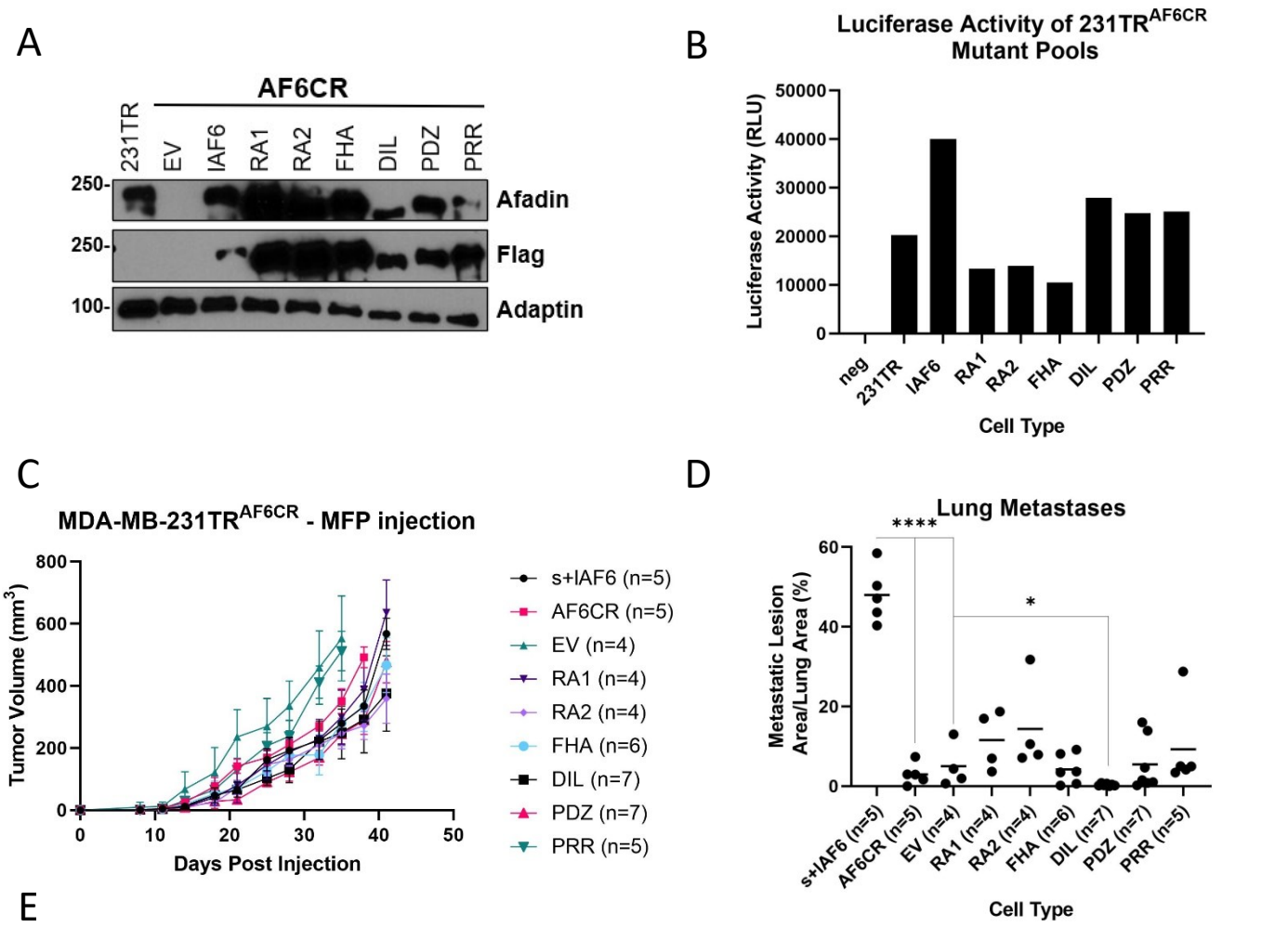


Figure 8. Mutation of afadin domains impairs metastatic ability. isoforms rescue metastatic deficit seen after afadin knockout. (A) Immunoblot analysis of MDA-MB-231TR^{AF6CR} pools (n=3) re-expressing flagged afadin mutants, α-adaptin was used as a loading control. (B) Luciferase activity of mutant afadin pools confirmed using luciferase assay system. (C) Primary tumor growth in the mammary fat pad was measured until a size of ~600mm^3 was reached, it was then resected. n= number of mice per group. Experimental variability expressed as SD (D) Lung metastatic burden (tumor area per tissue area) of afadin mutants. (E) Liver metastatic burden (tumor area per tissue area) of afadin mutants. (*) P<0.05, (**) P<0.005, (****) P<0.0001.

3.4. Expression of afadin mutants affects metastatic ability

Mammary tumor growth was measured for the panel of 231TR breast cancer populations (231TR^{AF6CR}) reconstituted with the various afadin mutants. The growth rate of all 231TR populations expressing the afadin mutants was similar (Figure 8C). Since the lAF6 control cohort unexpectedly lost its lung-metastatic ability, the burden of lung metastases associated with each mutant was compared to the sAF6+lAF6 control and 231TR^{AF6CR} and EV (Figure 8D, S4 and S5). Deletion of the RA1, RA2 or PRR resulted in a slight rescue of lung-metastatic burden relative to the afadin knockout populations. While the FHA and PDZ domains had a burden similar to the afadin knockouts. The DIL mutant had the lowest lung-metastatic burden. In summary, deletion of each afadin functional domain resulted in a reduced lung-metastatic burden relative to the sAF6+lAF6 control.

Assessment of the liver-metastatic potential revealed that the sAF6+lAF6 had the greatest liver burden, while loss of afadin, as seen in the 231TR^{AF6CR} and EV populations significantly inhibited liver metastases (Figure 8E and S6). Only the RA2 population was able to produce a slight rescue of liver-metastatic burden relative to the afadin knockout controls. The remaining mutants (ie. RA1, FHA, DIL, PDZ and PRR) were not able to rescue the liver-metastatic burden and had a similar burden to the afadin knockout populations (231TR^{AF6CR} and EV). Relative to the sAF6+lAF6 population, deletion of each afadin functional domain was associated with an impaired liver-metastatic ability. These findings suggest that all afadin domains play a role in promoting liver and lung metastasis.

3.5. Loss of ZO-1 inhibits breast cancer lung metastasis

Previous observations suggest that afadin, claudin-2 and ZO-1 could potentially interact in regulating breast cancer metastasis (Tabariès et al. 2019). Indeed, previous literature has suggested that ZO-1 could potentially bridge an interaction between claudin-2 and afadin (Itoh et al. 1999; Ooshio et al. 2010). To determine if ZO-1 may also play a role breast cancer metastasis, a MDA-MB-231TR ZO-1 knockout cell line (231TR^{ZO1CR}) was generated, and two pools of three clones were reconstituted (Figure 9A). Immunoblotting was performed to confirm that afadin and claudin-2 were still expressed in the 231TR^{ZO1CR} populations (Figure 9A). Unexpectedly, pool 1 lost claudin-2 expression, while pool 2 maintained expression of both afadin and claudin-2 (Figure 9A). As a result, further analysis was restricted to 231TR^{ZO1CR} pool #2. These cells were then

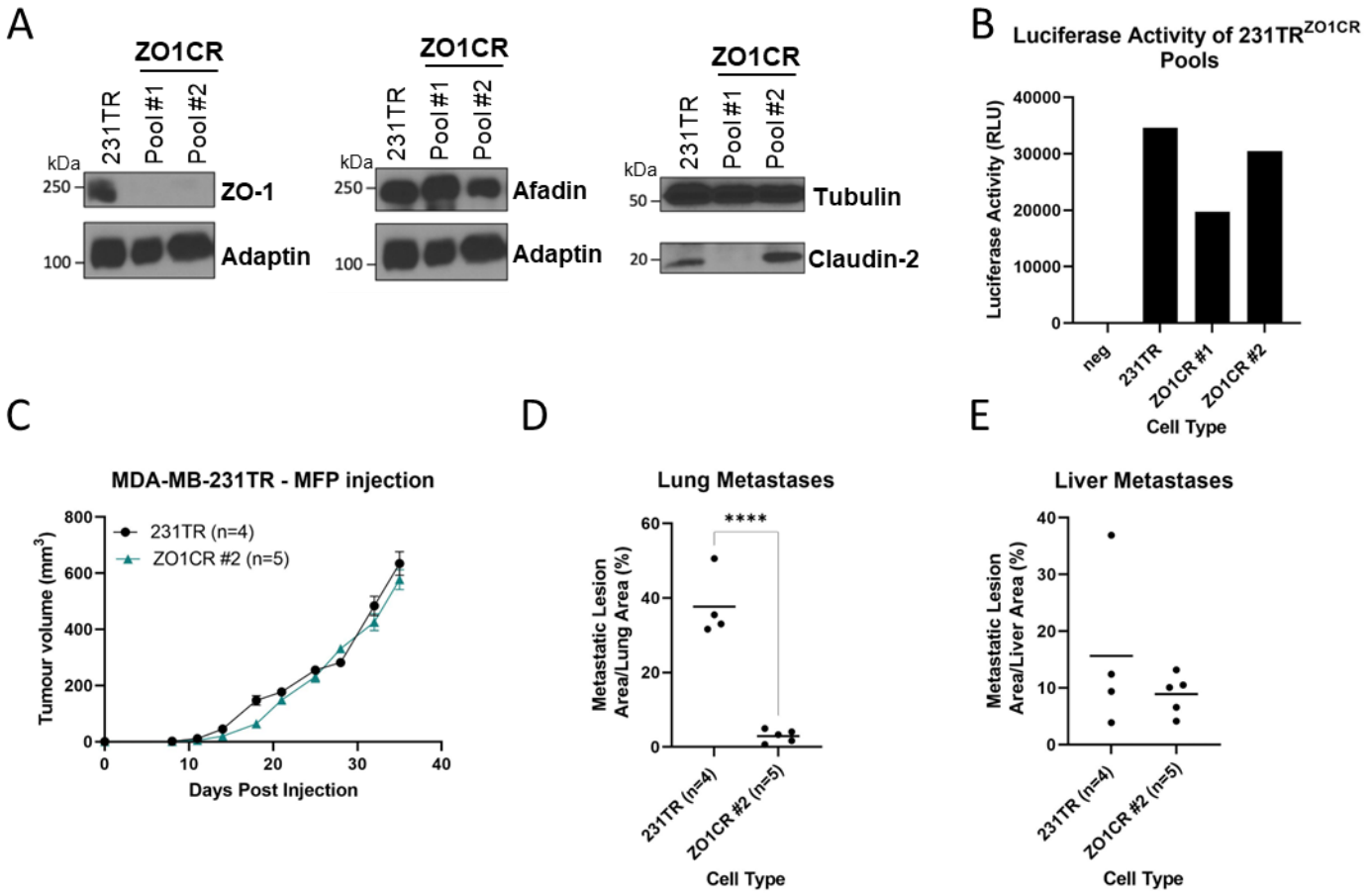


Figure 9. Loss of ZO-1 has no effect on metastasis.(A) Immunoblot analysis of MDA-MB-231TR^{ZO1CR} pools for ZO-1, afadin and claudin-2 expression (n=3), α-adaptin or tubulin was used as a loading control. (B) Luciferase activity of mutant afadin pools confirmed using luciferase assay system. (C) Primary tumor growth in the mammary fat pad was measured until a size of ~600mm^3 was reached, it was then resected. n= number of mice per group. (D) Lung metastatic burden (tumor area per tissue area) of ZO-1 knockout pool (E) Liver metastatic burden (tumor area per tissue area) of ZO-1 knockout pool. Experimental variability is expressed as s.e.m. (****) P<0.0001.

injected into the mammary fat pad of mice and primary tumor growth was monitored. There was no significant difference in the growth rate of the primary tumors following ZO-1 knockout. However, loss of ZO-1 significantly inhibited the lung-metastatic ability of the population relative to the parental 231TR (Figure 9D-E and S7-8). No significant difference in liver-metastatic burden was observed following ZO-1 knockout. Together, these observations suggest that ZO-1 is required for lung metastasis.

CHAPTER 4: DISCUSSION

4.1. Different afadin isoforms involved in breast cancer metastasis

Afadin has a long and short isoform, which share six domains, and differ only in the addition of an F-actin binding domain found in the long isoform (Mandai et al. 2013). A panel of cells were generated which re-constituted EV, sAF6 alone, lAF6 alone or sAF6+lAF6 in MDA-MB-231TR cells lacking endogenous afadin ($231TR^{AF6CR}$). Modest non-significant delays in primary tumor growth were observed with afadin loss, as well as those with reconstituted afadin expression (sAF6, lAF6 or sAF6+lAF6), relative to the parental control. These results are in agreement with previous results that indicated afadin was dispensable for primary mammary tumor growth (Tabariès et al. 2019). Knockout of afadin significantly inhibited the lung-metastatic ability of the $231TR^{AF6CR}$ and EV cells relative to the 231TR control. This suggests that afadin may have a pro-tumorigenic function in the context of lung metastasis, where afadin expression is required for lung metastasis formation. These findings are in agreement with previous work where loss of afadin also inhibited lung-metastatic ability following tail vein injection (Tabariès et al. 2019).

Breast cancer cells expressing sAF6 alone or sAF6+lAF6 formed lung metastases to a greater extent than observed with afadin knockout cells (Figure 5D). Indeed, these populations had a significantly greater metastatic burden relative to the parental cells. However, this increased metastatic burden may be in part attributed to the longer time that the primary tumor remained in the animal (approximately 1 week more). Indeed, this would allow more time to the cancer cells to spread to distant organs resulting in a greater metastatic burden. Additionally, these findings may be due to the fact that the cell line generated expressed afadin at a level that was higher than what was expressed endogenously in the parental 231TR cells. Therefore, increased afadin expression may have led to enhanced metastasis. Interestingly, the sAF6+lAF6 had the greatest lung-metastatic burden. This result makes sense given that this cell line re-expresses even more afadin compared to the other cell lines. Therefore, any pro-tumorigenic function being exerted by afadin would likely be increased even further.

To our surprise, the lAF6 population was unable to rescue the lung-metastatic defect seen upon loss of afadin (ie. AF6CR or EV) (Figure 5D). These findings were unexpected as the sAF6 and sAF6+lAF6 were able to rescue this deficit. One would assume that lAF6 would act similarly especially considering that the rescue observed with sAF6+lAF6 is greater than with the sAF6 alone, suggesting some contribution from the lAF6 counterpart. Indeed, these isoforms may have an additive effect in promoting lung metastasis. Alternatively, given that the difference in lung-

metastatic burden is not significantly different between the sAF6 and sAF6+lAF6, it is possible that the lAF6 is having no effect at all. The sAF6 may instead be driving the increased metastasis. This may be attributed to the greater sAF6 expression in this sAF6+lAF6 population, as seen in the immunoblot in Figure 5A. A potential explanation for the inability of lAF6 to rescue the metastatic defect may be due to differences in the clone selection used to generate the pools. Indeed, it was very difficult to isolate lAF6 positive clones (two positive clones found out of 83 screened for lAF6 or three positive clones out of 179 screened for the sAF6+lAF6 co-expressing cells (Table 5). Such difficulty in generating this pool may have led to selection of clones that would have unusual phenotypes, which do not exert the same pro-metastatic effect as expected. The lAF6 pool only contains 2 clones, thus increasing the impact of any potential off target effects on the population and in the experiment. Another possible explanation stems from anecdotal *in vitro* findings, where the lAF6 pool had the slowest growth rate of all the pools generated. These findings could extend to the *in vivo* results and explain why there was so little metastasis formation, given the equivalent time allowed for metastasis monitoring (two weeks). Independently, compared to all other constructs, lAF6 was the only population generated using a different vector backbone, using a hygromycin resistance marker instead of blasticidin. This was done to enable co-infection and co-selection (hygromycin plus blasticidin) of sAF6 and lAF6 in the sAF6+lAF6 cell population. Indeed, we needed to use different selection markers to ensure dual infection. At the same time, we decided to generate a new lAF6 population harboring this new vector to try and improve our efficiency at picking positive clones. Nonetheless, the use of a different vector with a different promoter and antibiotic resistance marker may have in turn affected the resulting populations when compared to all other constructs. However, it is unlikely that this alone is having such a robust effect, as the hygromycin resistant lAF6 vector was used in the sAF6+lAF6 population as well and one would assume that any issues in the lAF6 metastatic ability would extend to the s+lAF6 long as well. To resolve these discrepancies, it will be prudent to generate a new lAF6 pool using the same expression vector employed for all other constructs, which will be the most optimal control. Alternatively, one could generate the panel of afadin mutants using the sAF6 construct as the backbone, which may make interpreting findings easier given that the sAF6 behaved as expected.

Loss of afadin also inhibited the liver-metastatic ability (Figure 5E). Indeed, the $231TR^{AF6CR}$ and EV populations had a greatly reduced number of surface liver lesions relative to

the parental control, demonstrating a pro-metastatic function in the liver (where the AF6CR population reached a significant reduction). Reconstitution of sAF6 alone or sAF6+lAF6 were able to partially rescue the formation of liver metastases. Mice injected with breast cancer cells co-expressing sAF6+lAF6 had the greatest liver-metastatic burden, followed by mice bearing sAF6-expressing cells and finally lAF6-expressing cells did not rescue the liver-metastatic deficit at all. Previous work has investigated the role of afadin isoforms in liver metastasis using a direct metastatic assay (splenic injections), instead of the spontaneous model used in these experiments. Despite this difference in methodology, in this previous work, re-expression of the sAF6 had this same partial rescue effect (Tabariès et al. 2019). Expression of lAF6 was also found to rescue the liver-metastatic deficit, but to a greater extent than the sAF6 (Tabariès et al. 2019). The difference in the ability of lAF6 to rescue the ability of MDA-MB-231TR to form liver metastases suggests that the rare cell populations we identified that express lAF6 have selected additional phenotypes that impact metastatic fitness. In light of these results, an alternative lAF6 population needs to be generated to be able to confidently interpret the findings presented in this report. The addition of more mice per cohort (currently have 4-7 mice per cell population) will also help to further stratify any findings.

4.2. Afadin mutants inhibit breast cancer metastasis

Mutation of the various afadin domains had no significant effect on primary tumor growth (Figure 8C). Relative to the sAF6+lAF6, individual loss of all the afadin functional domains (RA1, RA2, FHA, DIL, PDZ, PRR), when reconstituted in MDA-MB-231TR cells lacking endogenous afadin, had a negative impact on the formation of lung metastases when compared to cells co-expressing sAF6+lAF6 (Figure 8D). Furthermore, the RA1, RA2 and PRR mutants were able to partially rescue the lung-metastatic deficit seen upon afadin loss. Furthermore, the FHA, DIL and PDZ mutants had the lowest lung metastatic burden. Of note, all of the afadin mutants were generated from the lAF6 backbone; thus, lAF6 is the appropriate control when trying to determine if deletion of an afadin domain affects primary tumor growth or metastatic ability. However, re-constitution of lAF6 failed to rescue the lung and liver-metastatic ability of MDA-MB-231TR cells, which behaved similarly to the $231TR^{AF6CR}$ or EV populations. While not ideal, MDA-MB-231TR cells reconstituted with sAF6+lAF6 have been used as a point of reference when comparing the degree of rescue for each of the additional afadin single domain mutants, until a more

appropriate control can be generated. All findings will need to be re-analyzed once a functional lAF6 control has been generated to assess the real importance of all domain mutants.

Only the RA2 afadin domain mutant was able to partially rescue the liver-metastatic defect seen upon loss of afadin (Figure 8E). However, relative to the sAF6+lAF6 control, loss of all individual afadin functional domains greatly inhibited liver metastasis. Indeed, deletion of the RA1, FHA, DIL, PDZ and PRR domains resulted in a great negative impact on liver metastasis, where these populations had a liver-metastatic burden that was similar to the afadin knockout populations. Together, these results suggest that each afadin domain contributes, to a greater or lesser extent, to the formation of breast cancer metastases, where the RA2 domain has the least effect.

The observation that each afadin domain negatively impacts metastatic ability is also a cause for concern with the current analysis. In addition, the first pass for the panel of afadin mutants involved the deletion of large domains of afadin. It is possible that these deletions may have disrupted the secondary structure of the protein and effected its functionality. In order to assess whether all domains are of real importance it may be prudent to make point mutations within the domains of interest. This should help to assess the importance of any domain, allowing us to evaluate any downstream mechanism involved, without having too robust an effect on afadin's functionality.

Assuming that loss of each afadin domain does actually impact liver and lung metastasis, it will be important to conduct follow-up experiments that look specifically at metastasis to certain sites to try and confirm and possibly amplify these results. To do this, splenic or tail vein injections may be done with these mutants so that one can get a clearer idea regarding the importance of each domain with respect to site specific metastasis. These additional experiments will also help to narrow down the domains that are most relevant for a specific metastatic site.

4.3. Role of afadin domains in breast cancer metastasis

Loss of all domains of afadin were found to inhibit the lung and liver-metastatic ability of the cell population (Figure 8). Therefore, identification of proteins which are known to interact with these domains may help to elucidate the mechanism through which afadin exerts its pro-tumorigenic effect. While an extensive list of proteins that interact with afadin is described in Table

1, only proteins that have been shown to directly interact with a specific domains will be investigated.

4.3.1. RA1/2 domains: Afadin is known for its ability to bind active Ras via its RA1/2 domains (Radziwill et al. 2003). In doing so it has a suppressive effect on Ras/Raf/MEK/ERK signaling, which can inhibit cancer progression. This program is likely not of significant interest in our model system as the MDA-MB-231TR have KRAS and BRAF mutations, which results in a highly active Ras/Raf/MEK/ERK signaling pathway (COSMIC 2020). Therefore, changes in the ability of afadin to bind Ras likely has limited effects on signaling via the MAPK pathway in MDA-MB-231 cells. Whether the RA1 and RA2 domains within afadin contribute to the metastatic phenotypes via mechanisms other that modulating MAPK signaling remains to be determined.

Afadin can promote leading edge formation and directional movement by interacting with active Rap1 via the RA domains in NIH3T3 fibroblasts (Miyata et al. 2009a). Indeed, afadin binds Rap1 and localizes at the leading edge alongside the PDGF receptor, Necl-5 and $\alpha_v\beta_3$ integrin following PDGF stimulation. Afadin and Rap1 interaction also recruits SHP-2 (tyrosine phosphatase), where SHP-2 can control PDGF receptor activation. This promotes leading edge formation via PDGF-induced ERK activation. Interestingly, upon PDGF stimulation, expression of afadin that lacked the RA domains inhibited the leading edge formation and directional cell movement (Miyata et al. 2009a). The development of the leading edge promotes cell migration, while directional cell movement helps ensure efficient migration and is important in metastasis (Suraneni et al. 2015; Yuan et al. 2017). These *in vitro* phenotypes may suggest that the RA domains of afadin could potentially improve metastatic ability *in vivo* (Yuan et al. 2017). Therefore, these findings help to explain how loss of RA1/2 may impact the metastatic ability of our cell populations. Indeed, it supports our work that demonstrated that the RA1/2 domains play an important role in breast cancer liver and lung metastasis.

4.3.2. FHA: domain: Afadin can bind scribble via the FHA domain (Goudreault et al. 2022). Furthermore, interaction of afadin with KRAS can actually promote binding of scribble and afadin. Activation of RAS signalling via EGF promoted cell motility in MCF7 cells, however this motility was lost upon knockout of afadin or scribble. This motility deficit was attributed to a reduction in adhesion and polarity. Afadin and scribble may have a similar pro-motile effect in our model system. However, this is unlikely given that the MDA-MB-231TR already have highly-

active RAS signaling and lack intact AJ/TJ. Therefore, stimulation of RAS signaling or loss of afadin are unlikely to have significant effects on adhesions and polarity in our system (Goudreault et al. 2022).

4.3.3. DIL domain: The only protein that afadin has been shown to interact with through the DIL domain is ADIP (Asada et al. 2003; Fukumoto et al. 2011). Indeed, ADIP binds afadin and links to α-actinin; thus, connecting the nectin-afadin and catenin-cadherin complexes of the AJ to the actin cytoskeleton. ADIP therefore plays a supportive role and helps to maintain AJ integrity (Asada et al. 2003; Fukumoto et al. 2011). Loss of the DIL domain may therefore inhibit interaction between afadin and ADIP, thus disrupting the AJ, facilitating EMT (Friedl et al. 2012). However, our experiments utilize the MDA-MB-231TR cell line, which lacks functional AJ/TJ, therefore this mechanism is unlikely to be playing a significant role in inhibiting breast cancer metastasis (Tabariès et al. 2011). Alternatively, afadin and ADIP have been involved in regulating leading edge formation and migration upon PDGF stimulation in NIH3T3 fibroblasts (Fukumoto et al. 2011). The formation of the leading edge is an important step in promoting cell migration (Ridley et al. 2003) . Indeed, ADIP and afadin were shown to regulate cell movement and leading-edge formation by stimulating Rac activation via Vav2, where Rac is involved in the formation of lamellipodial cell protrusions. Loss of the afadin binding domain within ADIP inhibited migration and leading-edge formation (Fukumoto et al. 2011). The same findings were seen upon loss of the afadin DIL domain (through which it binds ADIP). These results demonstrate the importance of the interaction of these two proteins in promoting cell movement upon PDGF stimulation (Fukumoto et al. 2011). The reduced lung and liver-metastatic ability shown in our results may be explained in part by a similar mechanism. Indeed, loss of the afadin DIL domain may inhibit interaction with ADIP, thus preventing efficient leading-edge formation and migration. Such defects could result in a diminished cellular migration, thus resulting in decreased metastasis formation. These findings demonstrate a second mechanism through which afadin is involved in leading edge formation (via RA and DIL domains) and supports afadin's pro-metastatic functions through these domains.

4.3.4. PDZ domain: Afadin interacts with a variety of proteins through its PDZ domain. Interestingly, afadin has been shown to interact with JAM-A via its PDZ domain, where JAM-A will dimerize and bind to afadin and PDZ-GEF2 (Severson et al. 2009). This interaction causes Rap1A activation via PDZ-GEF2, which stabilizes β_1-intergin levels and induces cell migration.

Loss of JAM-A or afadin was able to inhibit cell migration, in a scratch wound assay (Severson et al. 2009). As such, deletion of the PDZ binding domain, which would inhibit interaction of these proteins may have a similar effect. Indeed, migratory ability is an important step in the metastatic process (Friedl and Wolf 2003). These findings provide a potential mechanism and supports our findings that deletion of the PDZ domain of afadin inhibits its lung and liver-metastatic abilities, seeing as migration is an important part of the metastatic process.

Afadin may also interact with claudin-2 via the PDZ domain, as interaction between these proteins is mediated via the PDZ BD of claudin-2, which likely binds the PDZ domain of afadin (Tabariès et al. 2019). Similarly, to afadin, loss of claudin-2 is able to inhibit breast cancer lung and liver metastasis (Tabariès et al. 2019). Claudin-2 allows cancer cells to better adhere to extracellular matrix components by increasing $\alpha_2\beta_1$- and $\alpha_5\beta_1$- integrins expression at the cell surface (Tabariès et al. 2011). Additionally, claudin-2 has been shown to promote metastasis through claudin-2-claudin-2 interactions between cancer cells and hepatocytes, which facilitates seeding of the liver (Tabariès et al. 2012). Afadin may therefore promote metastasis by supporting and cooperating with claudin-2 during these processes.

Afadin may also promotes cell survival via PDGF induced PI3K-Akt signaling (Kanzaki et al. 2008). Indeed, afadin helps inhibit apoptosis upon Fas-ligand stimulation or serum starvation. Afadin is able to regulate PDGF-induced PI3K-Akt signaling. This occurs when the PDGF receptor binds nectin-3, which binds afadin via its PDZ domain in NIH3T3 fibroblasts. Afadin in turn binds the PI3K subunit, p85, and activates PI3K-Akt signaling, which helps to regulate cell survival and proliferation (Hemmings and Restuccia 2012; Kanzaki et al. 2008). Afadin may therefore utilize the PI3K-Akt signaling pathway to promote metastasis.

4.3.5. PRR domains: Afadin has been shown to interact with ZO-1, Ponsin and Drebrin through its PRR1/2 domain. All of these proteins are involved in cell-cell adhesion maintenance and formation. Indeed, afadin and ZO-1 interact prior to TJ formation, and is required for TJ formation in MDCK cells (Ooshio et al. 2010). Deletion of the PRR domain of afadin was sufficient to inhibit this interaction, and the formation of TJ. This interaction would be most relevant in the context of intact TJs, where inhibition of TJ formation could promote EMT and lead to cancer progression (Friedl et al. 2012). It is unlikely that this interaction is playing an important role in our model system, as MDA-MB-231TR cells lack functional AJ/TJs (Tabariès et al. 2011). Ponsin and drebrin are also involved in supporting cell-cell adhesions. Ponsin and afadin

have been shown to interact at AJ/TJs, where ponsin likely plays a supportive role to the adhesion (Yokoyama et al. 2001). The importance of this interaction needs to be further elucidated. Drebrin, was found to be important in nectin stabilization and AJs formation in endothelial cells (Rehm et al. 2013). Inhibition of afadin and drebrin interaction in the endothelial cells is relevant for intravasation and extravasation of cells during the metastatic cascade, as loss of this contact likely disrupts AJ integrity. However, changes in the ability of these proteins to interact within the cancer cell likely has no effect on the metastatic ability of the cell as afadin is not lost within the endothelial cells in our *in vivo* system.

Based on these findings, the RA, PDZ and PRR should be a priority for future research. Indeed, the RA domains have been shown to bind many proteins that are involved in regulating Ras/Raf/MEK/ERK a pathway that plays critical roles in cancer progression. Despite the highly active status of our model system, the RA domains and any associated pathways should be further investigated. The PDZ domain is also of interest as it has been shown to mediate protein-protein interaction that is important for migration, metastasis, and cell survival (Kanzaki et al. 2008; Severson et al. 2009; Tabariès et al. 2012). Further research is still to explain the mechanism and importance of the various afadin domains in cancer metastasis. To do this BioID may be preformed using the 231TR^{AF6CR} cells expressing lAF6 along side each mutant. Differences in interacting partners may help to highlight potential pathways through which the domains are required for breast cancer lung and liver metastasis.

4.4. Claudin-2 and afadin may interact to promote metastasis

Work presented in Tabariès et al.'s 2019 paper found that claudin-2 and afadin both have a pro-tumorigenic function in the context of breast cancer metastasis (ie. lung and liver). It was found that loss of the PDZ binding domain of claudin-2 inhibited the liver-metastatic ability of the population and that the PDZ binding domain is required for the pro-tumorigenic function of claudin-2 (Tabariès et al. 2019). It was further found that afadin and claudin-2 interact through the PDZ binding domain of claudin-2 (which may bind to the PDZ domain of afadin). Follow-up experiments in this report found that loss of the PDZ domain of afadin in the 231TR^{AF6CR} also inhibited the spontaneous liver and lung-metastatic ability of the cells (Figure 8). Based on these findings, these proteins likely interact directly via the PDZ binding domain of claudin-2 and the PDZ domain of afadin, to exert their pro-tumorigenic function for breast cancer liver and lung

metastasis. To confirm this, immunoprecipitation experiments were conducted to see if loss of the PDZ domain of afadin would inhibit this interaction, however experimental difficulties have inhibited the collection of this data. Further troubleshooting of this immunoprecipitation protocol is ongoing and will need to be resolved before we can conclusively say that these two proteins work together to promote metastasis.

4.5. Effects of ZO-1 on soft tissue metastasis

ZO-1 was identified as a potential interacting partner of claudin-2 and afadin (Itoh et al. 1999; Ooshio et al. 2010). Its role in breast cancer metastasis in our model system was therefore investigated (Itoh et al. 1999; Ooshio et al. 2010). To do this, ZO-1 was knocked out in the MDA-MB-231TR breast cancer cell line using CRISPR/Cas9. A $231TR^{ZO1CR}$ pool was generated and the loss of ZO-1 on cancer progression *in vivo* were assessed. Knockout of ZO-1 did not result in a change in the liver-metastatic ability of the cells, relative to the parental control (Figure 9D and E). However, loss of ZO-1 did significantly inhibit the lung metastatic ability. Given these results, it is possible that ZO-1, claudin-2 and afadin may form a complex to promote lung metastasis. While, it is unlikely that these proteins interact in promoting liver metastasis. This will be further validated through immunoprecipitation experiments with afadin and claudin-2 using our $231TR^{ZO1CR}$ cell line.

4.6. Conclusions and future directions

The role of afadin in cancer progression is clearly complex and context dependent. Our work tries to elucidate how afadin may exert a pro-tumorigenic function in the context of breast cancer metastasis. Early findings suggest that all afadin domains are important in regulating metastasis as deletion of any domain was able to inhibit the lung and liver-metastatic ability of the populations. The RA and PDZ domains are of particular interest given the role that afadin plays in regulating Ras/Raf/MEK/ERK signaling and the probable interaction between afadin and claudin-2. Furthermore, the role of the FHA, DIL and PDZ domains are of interest for lung metastasis, while all domains except the RA2 domain are of interest for their role in promoting liver metastasis. Increasing the number of replicates will help to further stratify our findings and identify if certain domains play a larger role than others in promoting cancer progression. Follow up experiments include performing direct metastatic assays such as, tail vein and splenic injections to

get more clean metastatic systems and see if any domain effects will be amplified. Generation of point mutations within any domains of interest may also help to elucidate our findings. BioID experiments with the different domain mutants may also help to identify protein interacting partners and the mechanism through which afadin is exerting its pro-tumorigenic effects. Furthermore, checking the activation status of various cancer promoting pathways, such as Ras/Raf/MEK/ERK, Src or PI3K/Akt may help to explain our results. Given that loss of ZO-1 inhibits lung metastasis, it is possible that afadin and claudin-2 interact via ZO-1 to promote lung metastasis. Afadin and claudin-2 likely do not interact via ZO-1 to promote liver metastasis. Alternatively, afadin and claudin-2 may still interact via another protein or simply function in parallel signaling pathways to effect soft tissue metastasis. Immunoprecipitation experiments to validate the direct interaction of afadin and claudin-2 hypothesis were attempted. However, these experiments have not been successful thus far. Continued efforts towards this should be done as this will provide further clarity to our findings in demonstrating a pro-tumorigenic interaction and function for afadin and claudin-2.

In conclusion, this thesis has made progress towards understanding how afadin and its various domains function to regulate cancer progression. Further work is still required to understand the mechanism of action through which afadin exerts its effects.

CHAPTER 5: REFERENCES

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CHAPTER 6: APPENDIX

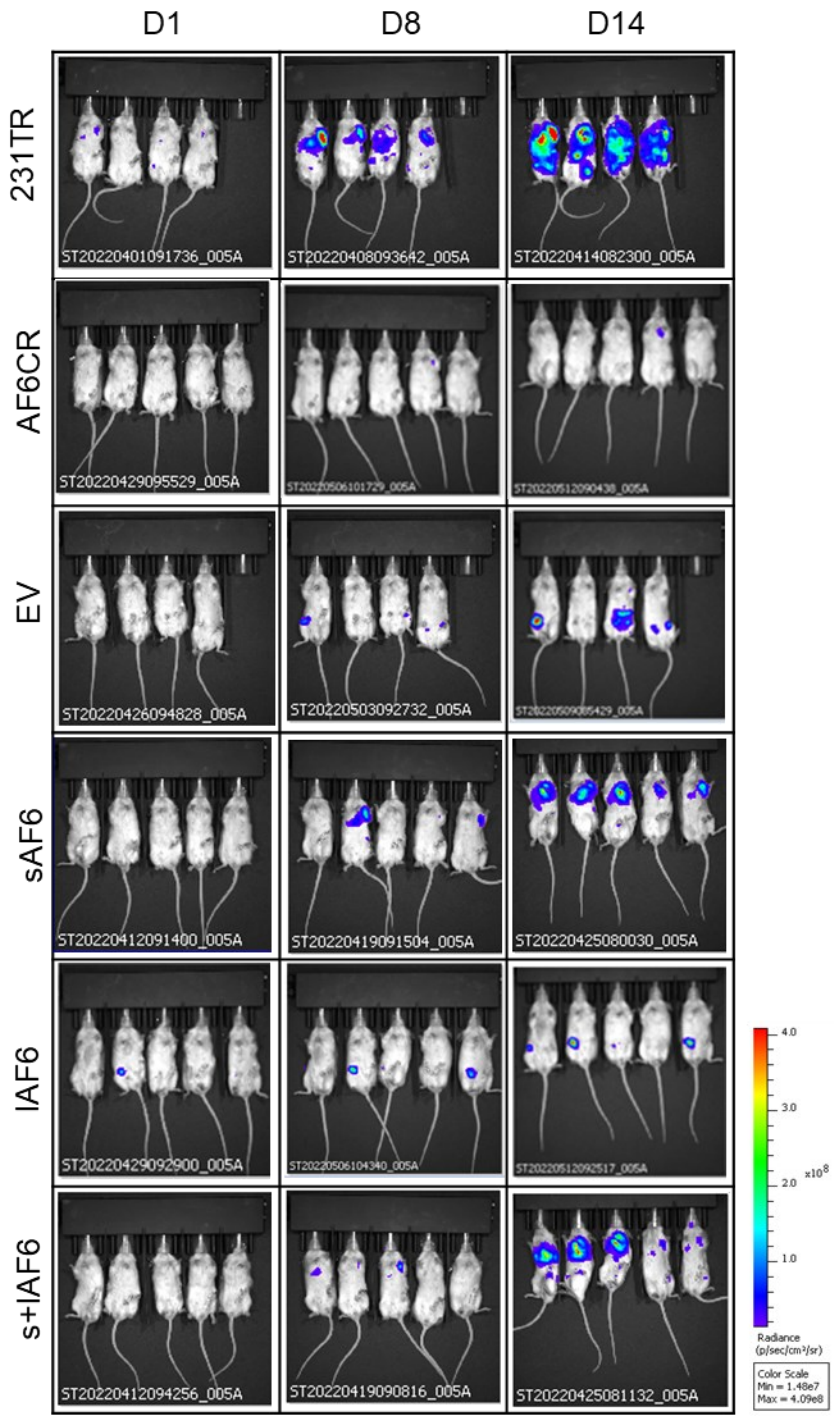

Figure S1. Progression of metastases over time in 231TR^{AF6CR} isoform panel using IVIS. Animals injected with luciferin are displayed at days 1, 8 and 14. Upper torso region of mouse was used to measure lung metastatic signal while mid-section immediately below lung region was used to measure liver metastatic signal.

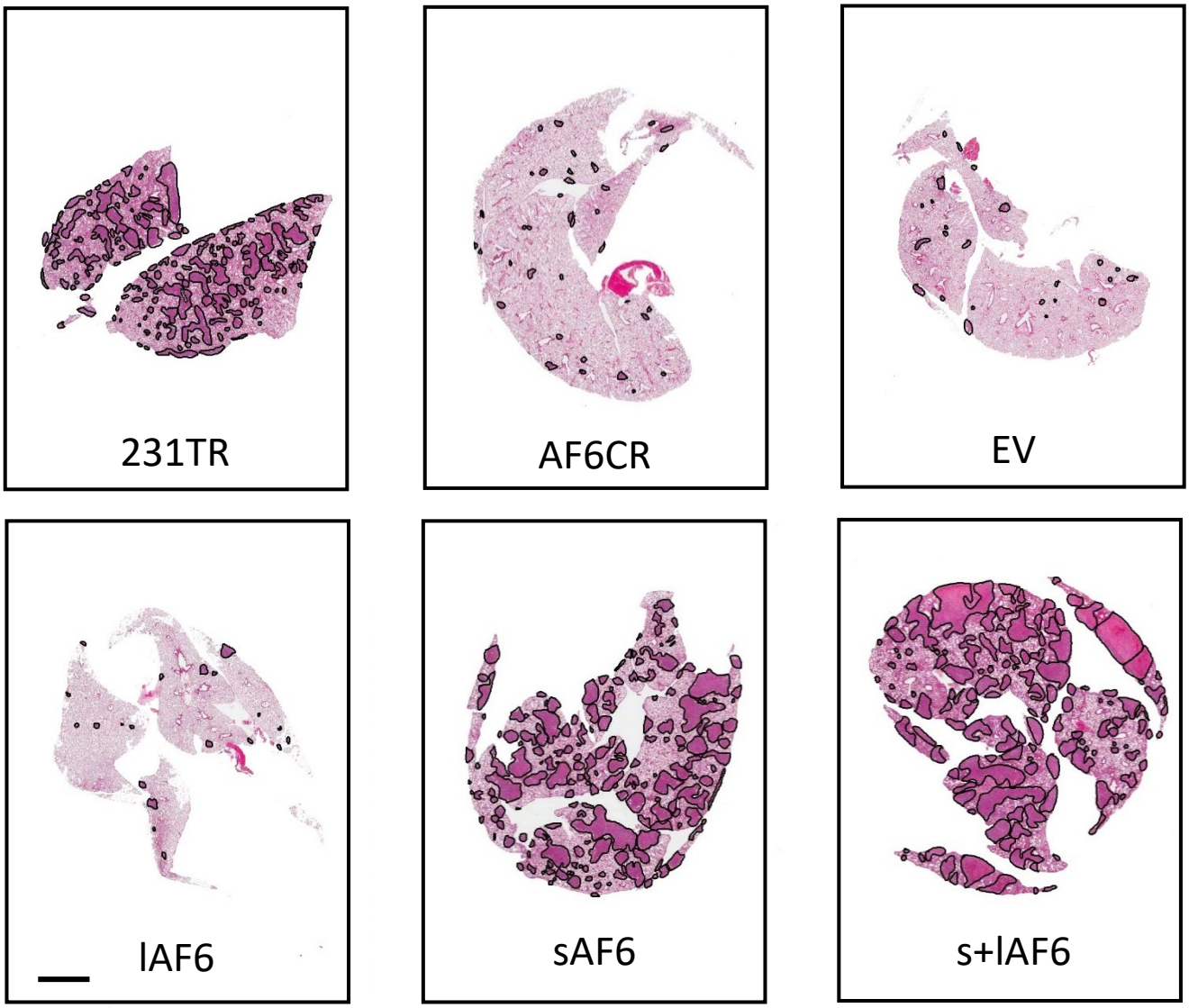

Figure S2. Representative images of lung metastatic burden in 231TR^{AF6CR} isoform panel. Metastatic lesions are indicated by dotted line. Bar, 2 mm.

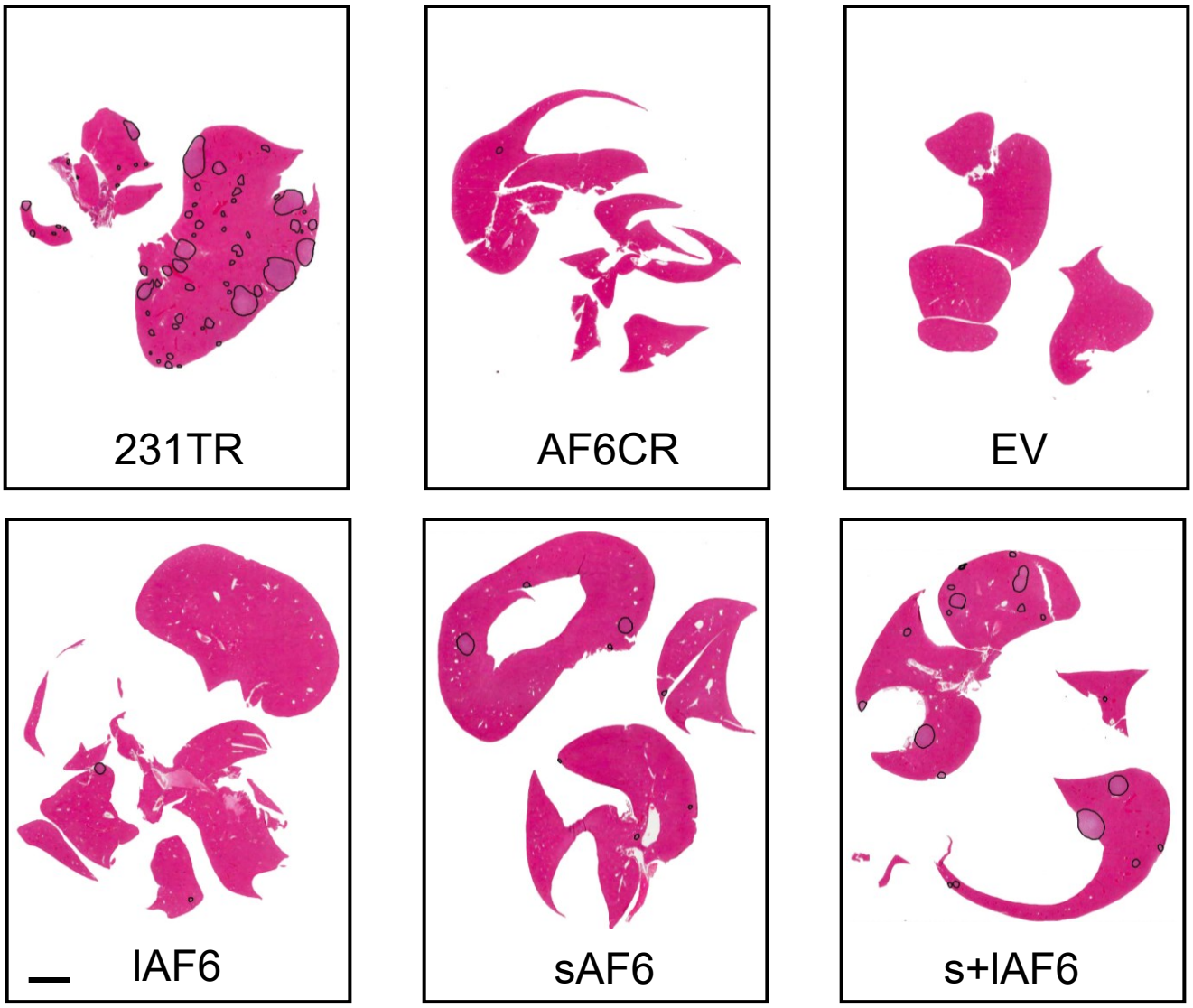

Figure S3. Representative images of liver metastatic burden in 231TR^{AF6CR} isoform panel. Metastatic lesions are indicated by dotted line. Bar, 2 mm.

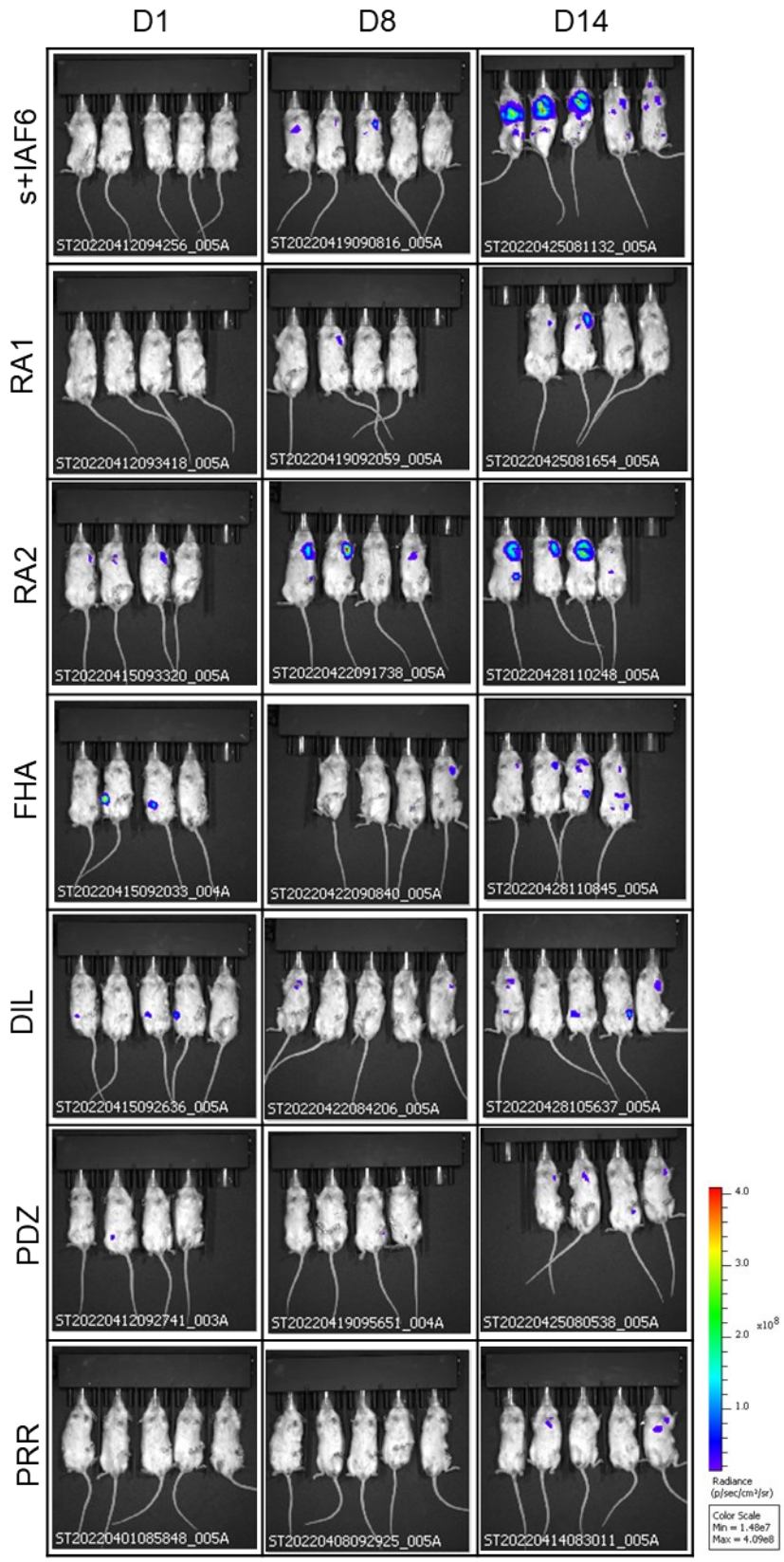

Figure S4. Progression of metastases over time in 231TR^{AF6CR} mutant panel using IVIS. Animals injected with luciferin are displayed at days 1, 8 and 14. Upper torso region of mouse was used to measure lung metastatic signal while mid-section immediately below lung region was used to measure liver metastatic signal.

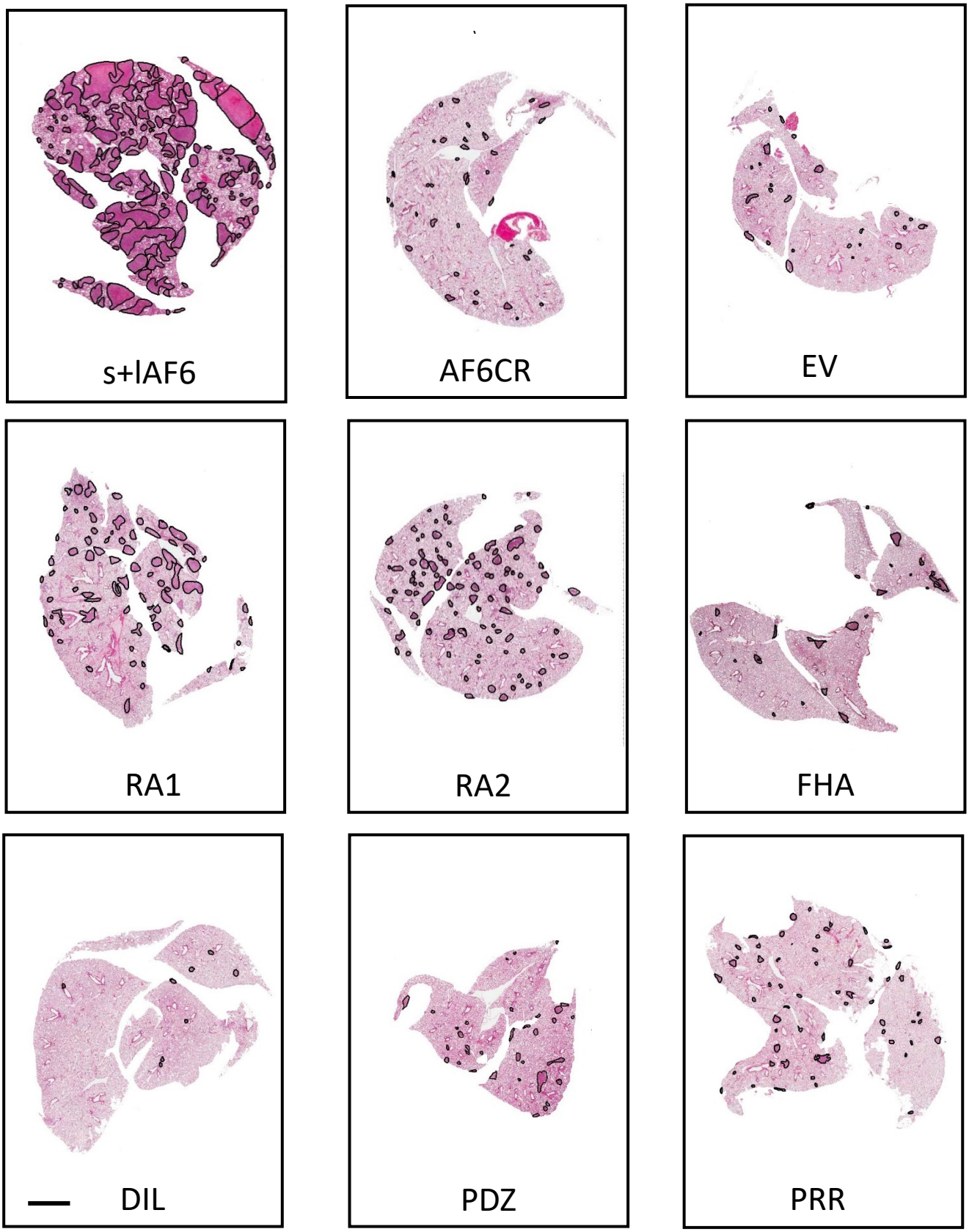

Figure S5. Representative images of lung metastatic burden in 231TR^{AF6CR} mutant panel. Metastatic lesions are indicated by dotted line. Bar, 2mm.

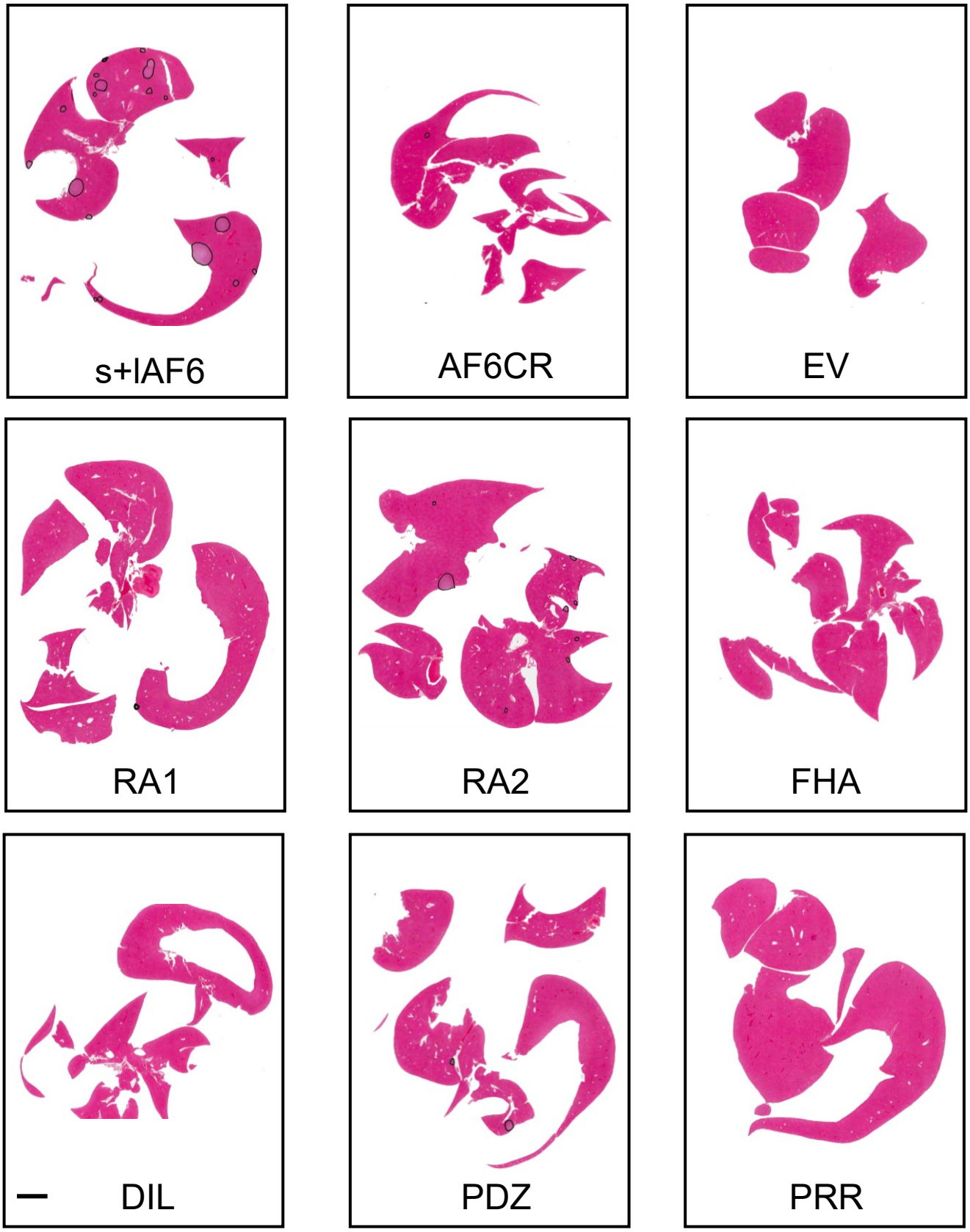

Figure S6. Representative images of liver metastatic burden in 231TR^{AF6CR} mutant panel. Metastatic lesions are indicated by dotted line. Bar, 2mm.

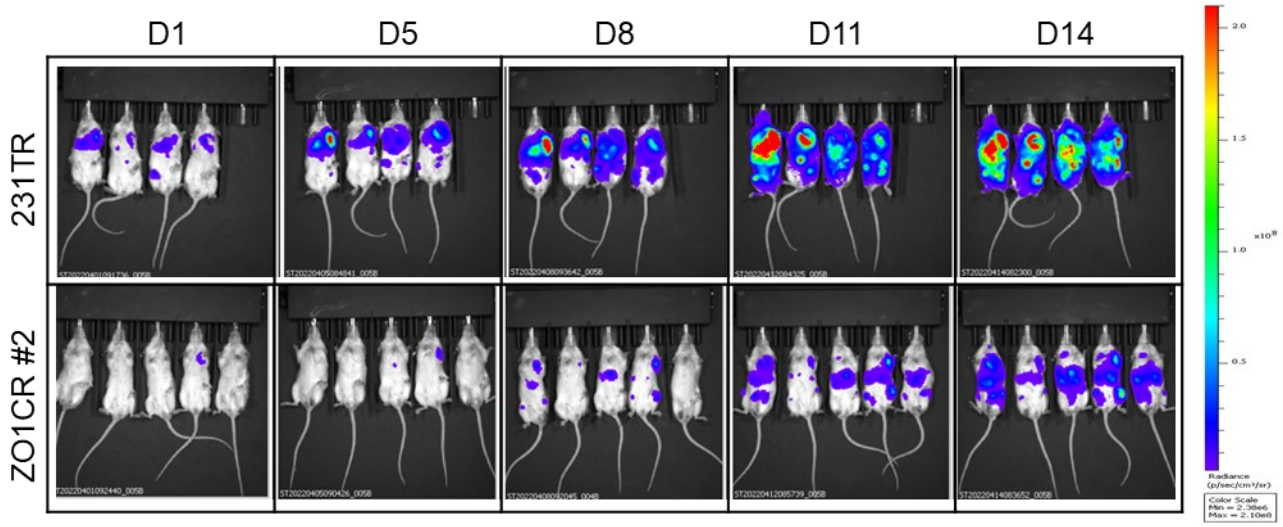

Figure S7. Progression of metastases over time in 231TR^{ZO1CR} pool using IVIS. Animals injected with luciferin are displayed at days 1, 5, 8, 11 and 14**.**

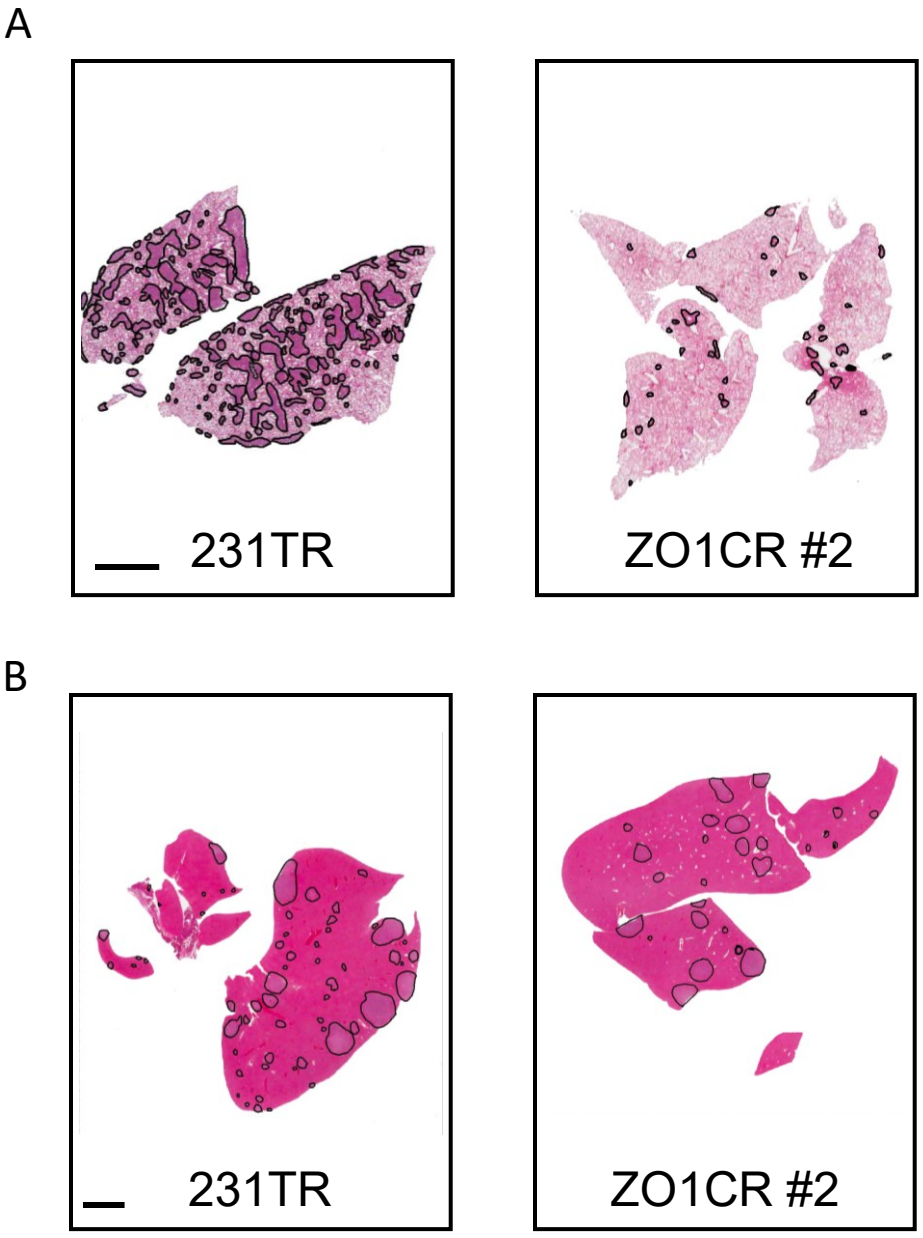

Figure S8. Representative images of lung and liver metastatic burden in 231TR^{ZO1CR}. (A) Lung metastatic lesions. (B) Liver metastatic lesions. Metastatic lesions are indicated by black line. Bar, 2mm.