

# Piezo ion channels in cancer cell mechanotransduction

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A la mare i al pare, per ser tots dos una exageració.  
(les dice el hijo a los padres)

A la Gemma,  
*now that I can dance.*



Siempre hay un Lector.

*El día del Watusi, Francisco Casavella*





## Agraïments

*Les persones són la seva acció i les seves condicions materials de vida.*  
Karl Marx, en traducció lliure.

Una tesi és un treball col·lectiu i, com a tal, ha de ser agraït a més gent de la recordable per les meves limitades memòria i capacitat expressiva.

En el colmo de la originalidad, empezaré agradeciendo esta tesis a su director: Miguel A. Valverde. Sin ti no hay veranos de segundo (2009) y tercero (2010) de carrera en el lab, verano de cuarto en Valparaíso (2011 y una de las experiencias que más ha marcado mi vida), proyectos finales ni de Licenciatura (2012, y lo bien que fue), ni Máster (qué alegría recibir aquel correo del editor de PNAS el 23 de abril de 2013), ni FPI (2014 y hasta ahora), ni Baltimore (2015), ni Biophysics, ni Curie, ni CNIC (2016), ni llamar a tu puerta cuando sea para comentar la última pesca en Pubmed, ni descubrir el mundo de los canales de cloruro, ni *cambia una cosa cada vez*, ni *keep it simple*, ni *we have not gone too far, but we are much better informed*, ni *vale, pruébalo a ver qué pasa* ni *mails Ok, Ciao*. Que en el verano de 2010 me propusieses quedarme en el lab a coger carrerilla científica (!7 años!) me hizo afrontar cuarto de carrera de una manera muy distinta. Y fue el mejor curso de la Licenciatura (dentro y fuera de clase), así que gracias también por eso. Y por dejarme leer y montarme mis ideas sobre cómo narices hacer tal experimento o responder tal pregunta o encajar el cubo en el círculo. Sé que como estudiantes se espera que hagamos más experimentos (y tengamos resultados para cuando se nos piden). En esto yo no siempre he cumplido, pero jamás me lo has recriminado. Por último, y a la luz de diversos casos que conozco bien, debo mostrar mi gratitud por el final de la

presente tesis. No es infrecuente<sup>1</sup> que director y estudiante acaben a malas o, al menos, tengan intensos roces. En el momento de escribir estas líneas, esto no ha pasado. Es más, diría que a medida que se ha ido acercando el final, hemos acumulado una inercia que nos ha llevado a buen puerto aun cuando las (mis) fuerzas flaqueaban. Brindo por todo ello, por Sir Isaac, por Miles, por ti y por mí. Nos vemos.

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<sup>1</sup> Esto, me temo, cuenta como anglicismo

ser elevado sin ser engreído, elegante sin ser elitista y entusiasta manteniendo el escepticismo sin caer en el cinismo. Coltrane y Casavella aspiraban a ello. Tú lo bordas. Yo lo intentaré toda mi vida.

El ritme de funcionament d'un laboratori és un tot-terreny en una carretera nevada, de nit, i qui arriba nou és l'animalet que mira fatalment la llum dels fars, que s'acosta amenaçant...fins que l'animal deixa de veure res. Als *departed but not forgotten*, gràcies per fer-me fàcil la incorporació al lab reduint la velocitat i deixant-me pujar a la part del darrere. Aquí voldria aprofitar per recordar especialment la Gemma. El teu escandalós riure encara ressona quan el lab queda buit als vespres.

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<sup>2</sup> Ale, ya está, ya lo he dicho. ¡Estarás contenta!

a la intemperie, darte espacio para que desarrolles tu propio gusto y proyecto científicos y delegar en ti faenas de mi proyecto. ¡Al cielo con ella!

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## Coda

No sé si això toca aquí, però per una vegada que puc agrair en llista l'existència de la idea del bé<sup>3</sup>...*in ordine sparso*:

Això de la 3a de Beethoven



Curie, Cala Morell, Diane Keaton a *Annie Hall*, Coleman Hawkins, Jennifer Lippincott-Schwartz, *Esperando nada*, Muddy Waters, Buenos Aires, Gian Lorenzo Bernini, el combo Earth Wind & Fire + KC & The Sunshine Band + Kool & The Gang + Chic, el gintònic, el Ctrl+Z, Bessie Smith, el fet de moure'ns per l'Univers a tota velocitat i ni adonar-nos-en, Valparaíso, les camises de lli, riure de David Carabén mentre em quedo embobad amb el que escriu, Chet Baker tocant sense dents, Alexandra Elbakyan, aquest acudit:

- A: *Oye, que mi gato ha matado a tu perro.*
- B: *Pero ¿cómo va a haberlo matado un gato si mi perro es un rottweiler?*
- A: *Ya, pero es que mi gato es hidráulico.*

Maradona en plan barrilete cósmico, La Oreja de Van Gogh als karaokes i només als karaokes, Ben Lehner, les Fender Telecaster, Jack Vettriano, els pins, tancar bars i seguir, *El quadern gris*, Rita Haywoth jugant amb uns guants, conèixer Jim Lovell, *The Big Lebowski*, Roma, Eduardo Mendoza, el (*What's the Story*) *Morning Glory* d'Oasis, *La Gozadera*, Albert Camus i Jacques Monod sabotejant els Nazis, Coyoacán, Françoise Hardy amb un tres quarts, el record del meu avi rient desbocadament i picant de peus a terra com un nen petit, Norma Jean, Jay Kay ballant, les vaques, les Gretsch Electromatic, *Love and Death*, The Temptations, Jep Gambardella, *Danza Kuduro*, Els Surfing Sirles, Matthieu Piel, *Aterriza como puedas*, Bill Evans, Manhattan, Justin Bieber, la cursa espacial, Lester Freamon, la llista *R&B indie 2017* de l'Spotify de mon pare, Billie Holliday, Pier Paolo Pasolini, la BSO i l'escena de *Love is Strange* de *Dirty dancing*...bé, tot aquest peliculón mereix una plaça a cada poble. Deixem-ho aquí.

*Agradecida y emocionada, solamente puedo decir gracias por venir.*



## Abstract

The mechanical dependence of transformation and metastasis is an emerging field, but the role of mechanosensitive channels has been largely omitted. This thesis focuses on the roles played by the mechanosensitive ion channels Piezo1 and Piezo2 in the transduction of mechanical stimuli (confinement, adhesion, substrate rigidity, adhesive ligand concentration) by cancer cells.

In a first chapter, we show that confinement triggers Piezo1-mediated calcium entry. This activates phosphodiesterase 1, reducing cAMP levels and, consequently, PKA $\rightarrow$ Rac1 activity, relieving Myosin II from its inhibition. We also find a parallel, direct activation of Myosin II by confinement. As a combined result, cells stiffen and optimize their adhesion-free migration mode, usually responsible for *in vivo* migration during metastatic invasion. Piezo1 knockdown suppresses confinement-induced calcium entry and impairs the underlying circuitry in ovarian epithelial (CHO) or melanoma (A375) cells. As a result, *siPiezo1* cells show reduced migratory capacity under confinement.

In the second chapter, we discover an essential role for Piezo2 as a transducer of environmental mechanical cues into RhoA activation to modulate the mechanobiological responses of MDA-MB-231-BrM2 brain metastatic breast cancer cells. Piezo2 KD disturbs stress fibre formation, adhesion orientation, force transmission and nuclear accumulation of the malignant co-transcriptional activator YAP, and this is phenocopied by extracellular calcium suppression. Promoting Actin polymerization with jasplakinolide or by over-expressing constitutively active forms of Rho or mDia1 restores stress fibres and

nuclear YAP accumulation in Piezo2-KD cells. In addition, Piezo2 knockdown disrupts several pro-metastatic functions: cell proliferation, migration, invadopodia formation, extracellular matrix degradation, and secretion of SERPINB2, a protein needed for protecting invasive cells from brain parenchymal defence mechanisms.

The works presented in this thesis unveil important roles for Piezo channels as a first line of mechanical input detectors in distinct cells. These discoveries are relevant for several fields, e.g. cancer research, and highlight the importance of ion channels as transducers of environmental stimuli.

## Resum

La dependència mecànica de la transformació i la metastasi és un camp d'estudi / de recerca emergent, però el paper que hi juguen els canals iònics mecanosensibles s'ha omès fins ara. Aquesta tesi se centra en els rols dels canals Piezo1 i Piezo2 en la transducció d'estímuls mecànics per cèl·lules canceroses, com ara confinament, adhesió, rigidesa del substrat, concentració de lligands adhesius.

En un primer capítol, mostrem que el confinament dispara l'entrada de calci per mitjà de Piezo1. Això activa la fosfodiesterasa 1, que redueix els nivells d'AMPC i, en conseqüència, l'activitat PKA → Rac1, que deixen d'inhibir Miosina II. També trobem una activació paral·lela de Miosina II directament per confinament. Com a resultat final, les cèl·lules guanyen rigidesa i optimitzen el seu mode migratori independent d'adhesions, que és el preponderant *in vivo* durant la invasió metastàtica. Reduir els nivells de Piezo1 suprimeix l'entrada de calci induïda per confinament i desactiva el circuit subjacent en cèl·lules ovàriques epitelials (CHO) i de melanoma (A375). Això minva la capacitat migratòria de les cèl·lules *siPiezo1*.

En un segon capítol, descobrim un rol essencial per a Piezo2 com a activador de RhoA en resposta a estímuls mecànics. Això modula les respostes mecanobiològiques de les cèl·lules MDA-MB-231-BrM2, de càncer de mama metastàtic a cervell. La reducció dels nivells de Piezo2 destorba la formació de fibres d'estrès, l'orientació de les adhesions, la transmissió de forces i l'acumulació nuclear del regulador transcripcional prometastàtic YAP. Suprimir el calci extracel·lular fenocòpia aquests resultats. Promoure la polimerització d'Actina amb jasplaquinolida o mer mitjà de la sobreexpressió de formes

constitutivament actives de RhoA o mDia1 restableix les fibres d'estrès i l'acumulació nuclear de YAP. A més, la reducció de Piezo2 suspèn diverses funcions prometastàtiques: proliferació cel·lular, migració, formació d'invadopodis, degradació de la matriu extracel·lular i secreció de SERPINB2, una proteïna necessària per protegir les cèl·lules invasores dels mecanismes de defensa del parènquima cerebral.

Els treballs presentats en aquesta tesi desvelen rols importants pels canals Piezo com a una primera línia de detectors d'estímuls mecànics en diferents tipus cel·lulars. Aquests descobriments són rellevants per a diversos àmbits, com ara la recerca en càncer, i remarquen la importància dels canals iònics com a transductors d'estímuls ambientals.

## Resumen

La dependencia mecánica de la transformación y la metástasis es un campo emergente, pero el papel que juegan en ellas los canales iónicos mecanosensibles ha sido omitido. Esta tesis se centra en los roles de los canales Piezo1 y Piezo2 en la transducción de estímulos mecánicos por parte de células cancerosas, como ahora confinamiento, adhesión, rigidez del sustrato, concentración de ligandos adhesivos.

En un primer capítulo, mostramos que el confinamiento dispara la entrada de calcio mediante Piezo1. Esto activa la fosfodiesterasa 1, que reduce los niveles de AMPc y, en consecuencia, la actividad  $PKA \rightarrow Rac1$ , que dejan de inhibir Miosina II. También encontramos una activación paralela de Miosina II directamente por confinamiento. Como resultado final, las células aumentan su rigidez y optimizan su modo migratorio independiente de adhesiones, que es el preponderante *in vivo* durante la invasión metastática. Reducir a los niveles de Piezo1 suprime la entrada de calcio inducida por confinamiento y desactiva el circuito subyacente en células ováricas epiteliales (CHO) y de melanoma (A375). Esto merma la capacidad migratoria de las células siPiezo1.

En un segundo capítulo, descubrimos un papel esencial para Piezo2 como activador de RhoA en respuesta a estímulos mecánicos. Esto modula las respuestas mecanobiológicas de las células MDA-MB-231-BrM2, de cáncer de mama que metastatiza a cerebro. La reducción de los niveles de Piezo2 perturba la formación de fibras de estrés, la orientación de las adhesiones, la transmisión de fuerzas y la acumulación nuclear del regulador transcripcional prometastático YAP. La supresión del calcio extracelular fenocopia estos resultados. Promover la

polimerización de Actina con jasplaquinolida o mediante la sobreexpresión de formas constitutivamente activas de RhoA o mDia1 reestablece las fibras de estrés y la acumulación nuclear de YAP. Además, la reducción de Piezo2 suprime varias funciones prometastáticas: proliferación celular, migración, formación de invadopodios, degradación de la matriz extracelular y secreción de SERPINB2, una proteína necesaria para proteger a las células invasoras de los mecanismos de defensa del parénquima cerebral.

Los trabajos presentados en esta tesis desvelan roles importantes de los canales Piezo como una primera línea de detectores de estímulos mecánicos en diversos tipos celulares. Estos descubrimientos son relevantes para varios ámbitos, por ejemplo, la investigación en cáncer, y remarcan la importancia de los canales iónicos como transductores de estímulos ambientales.



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# 1.Introduction



*Let's get physical...*

*Physical*, Olivia Newton-John, 1981

For many years, cell biology has focused its attention on biochemical pathways triggered by specific interactions between ligands and their receptors. This is also the perspective still adopted in many cell biology courses. Yet, advances in what we could call mechanobiology<sup>4</sup> make necessary to widen this approach at cell, systemic and organic scales.

Let us take humans as an example. Even with our eyes closed we know whether we are in close contact with anything or anyone. We also know whether what we are touching is rough or smooth, pointy or blunt. This is possible because our skin contains, among many structures, mechanoreceptors, i.e. sensory neurons and accessory cell types that transmit action potentials in response to different types of mechanical stimulation (gentle touch, pressure, slow or fast vibrations). This is what we normally call the sense of touch, but it is not only the mechanical perception we can experience. Closing our eyes again, we still can discern whether we are laying, sitting or standing. Even more, we can distinguish between standing still or moving, provided that there is a change in velocity, i.e. acceleration. This is also due to mechanosensitive structures that detect joint and muscle tension concertedly working

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<sup>4</sup> *Mechanobiology* is defined as the study of mechanical forces in a biological context: how cells sense mechanical forces and how do they respond to them, which are the (patho)physiological consequences of these processes, etc. According to Pubmed, this discipline emerged in the mid 80s (1500 to 3500 annual records, 2.3 increase from 1986 to 1988), stalled in the 90s (5000-6000) and has experienced a recent surge (doubling from 5000 to 10000 annual entries in the 2011-2016 period).

with the vestibular system, a component of the inner ear labyrinth sensitive to rotational movements and linear acceleration. These mechanosensitive systems are coupled to effector pathways –eye movement, muscle contraction/relaxation- to ensure a proper function. Importantly, these mechanisms are mainly unconscious, i.e. we are not continually integrating this perceptive information into thoughts, but our body is constantly probing its surroundings and using this information to locomotive and postural control through feedback circuits. The importance of all these systems is evidenced in diseased states, e.g. vertigo, the sensation of loss of balance and spinning, can be triggered by malfunction of vestibulo-ocular connections.

At a cellular scale, the mechanical properties of the environment (rigidity, topology, pore size, sessile ligand density) are key determinants of cell behaviour. Also, cells themselves have state-dependent physical properties necessary for their function and survival (DuFort et al., 2011; Moore et al., 2010). As in the case of the whole organism, aberrant single cell mechanotransduction<sup>5</sup> underlies several diseased states by disturbing normal cell function, e.g. idiopathic pulmonary fibrosis (Liu et al., 2015a; Rahaman et al., 2014) or cancer (Elosegui-Artola et al., 2014; Miroshnikova et al., 2016; Paszek et al., 2005).

During mechanotransduction, proteins with force-sensing ability change their function, triggering a signalling cascade that modifies cell activities. The aforementioned mechanore-

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<sup>5</sup> In this thesis, mechanotransduction is used *latu senso* as the conversion of mechanical stimuli into classic biochemical cellular responses.

ceptors are in essence cells with specialized mechanosensitive machinery (in this case, ion channels) whose function leads to membrane depolarization, triggering action potentials and synaptic transmission. Therefore, local changes in cell activity are ultimately responsible of responses at both cell and organism levels.

The best described way for force to trigger protein responses is by conformational changes, but changes in interprotein distances and phase separation are common as well (Hyman et al., 2014). Among proteins exhibiting force-induced conformational changes impacting protein function, mechanobiology has largely focused on ion channels and integrins.

Since they are the main subjects of this thesis, I will pay more attention to mechanically-activated (MA) ion channels. Nevertheless, I will introduce integrins and other mechanosensitive proteins in more detail when I tackle cell adhesion and migration mechanisms.

## 1.1 Mechanically activated channels

A generic ion channel is a transmembrane protein forming a pore whose open probability depends on specific stimuli, e.g. agonist binding, membrane depolarization or, as in the case of MA channels, increases in membrane tension. Ion channels exhibit short latencies<sup>6</sup> and this makes them essential for early responses to sudden changes that could be deleterious upon short exposition. Once open, the pore allows the transit of specific ions, that flow down an electro-chemical gradient and trigger responses ranging from inter-bacterial communication in biofilms (Prindle et al., 2015) to plant gravity sensing and

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<sup>6</sup> Time interval between stimulus application and response start.

root growth (Yamanaka et al., 2010) or animal sensorial perception (Corey and Hudspeth, 1979; Ikeda et al., 2014; Ranade et al., 2014).

Virtually every living species genome contains at least one gene encoding a MA channel. This is in part the result of billions of years of evolutionary pressure on living organisms to deal with mechanical stimuli (Brunet and Arendt, 2016). Bacterial Msc proteins are the best studied MA channels and act as osmolyte escape valves activated in response to turgor pressure. Purified Msc proteins inserted in artificial lipid bilayers respond to bilayer deformations. Since those experimental preparations only contain lipids and the purified protein, the authors concluded that Msc are inherently mechanosensitive (Sukharev et al., 1993). In addition, Msc activity is modulated by amphipaths such as chlorpromazine or trinitrophenol. These molecules contain hydrophobic and hydrophilic parts. Their biased insertion into membranes changes local curvatures, altering membrane tension and in consequence Msc channel activity (Martinac et al., 1990). These observations led to the *force-from-lipid* paradigm, which suggests that the force opening MA channels comes from the lipid bilayer constituting the cell membrane.

Identifying and studying eukaryotic MA channels has proven more difficult. The majority of cell lines exhibit MA currents, and this reduces signal-to-noise ratios in experiments based on transcript overexpression, as the ones used in the late 1990s by the group of David Julius at UCSF, that lead to the identification of several ion channels involved in thermosensation and pain and boosted the TRP ion channel field (Caterina et al., 1997; McKemy et al., 2002). The development



of reliable and affordable siRNA screening technologies, combined with contemporary bioinformatics tools, solved the problem. Bertrand Coste and collaborators of Ardem Pataoutian's group at The Scripps Research Institute individually knocked down candidate genes in a cell line that exhibited the MA cationic currents of interest. After 72 candidates, *FAM38A*<sup>7</sup> knockdown suppressed MA currents. The existing references only showed increased transcript expression in senile plaque-associated astrocytes (Sato et al., 2006) and integrin activation by its protein product (McHugh et al., 2010). Due to its response to pressure, πίεση (piesi) in Greek, the authors re-named it Piezo1 (Coste et al., 2010).

### 1.1.1 Piezo genomics and expression

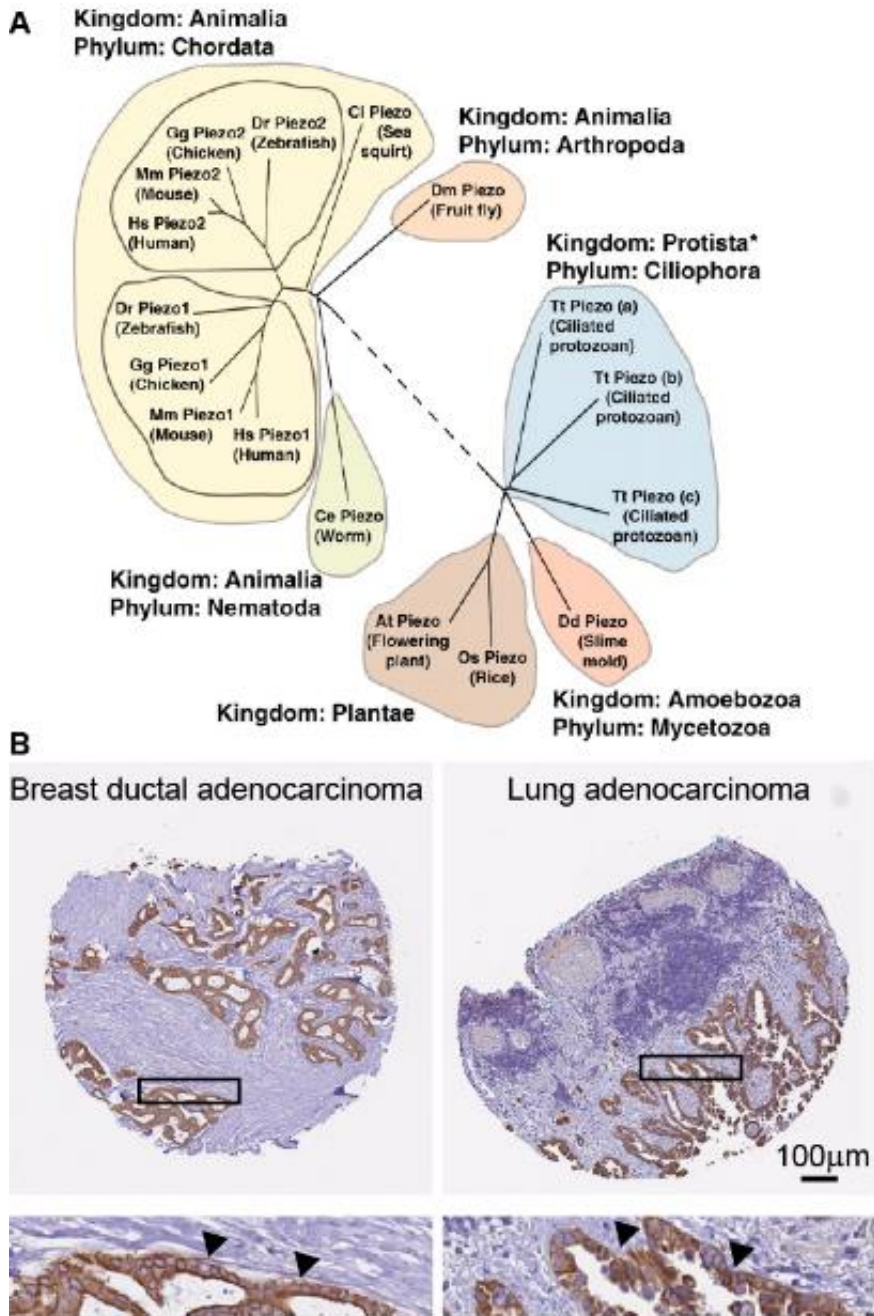
Piezo genes are conserved in eukaryotic genomes (yeast is the exception, where no orthologue is found, Fig.1A). Sequence similarity to other genes, including ion channels, is minimal. This impedes domain function identification based on sequence comparison. Human Piezo genes (*PIEZO1* and *PIEZO2*) encode large proteins (>2500 amino acids) that are expressed in a wide variety of tissues (Coste et al., 2010).

For the interest of this thesis, we must keep in mind that both human Piezo channels are highly expressed in epithelia, the tissue type originating the majority of cancers.

According to the [Human Protein Atlas](#) (Uhlén et al., 2015), these channels are prominently expressed in breast glandular

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<sup>7</sup> Family with sequence similarity 38, also known as KIAA0233 or MIB.



**Figure 1. Piezo expression.**

(A) Unrooted phylogenetic tree of Piezo genes of selected species. (B) Immunohistochemical Piezo2 stainings of sections of breast ductal (left) and lung adenocarcinomas (right). Arrows mark cancer cells with intense staining levels. Adapted from (Coste et al., 2010; Uhlén et al., 2015).

and myoepithelial cells, small cell lung cancer samples, melanocytes (all of them common sources of brain metastasis), fibroblasts and cells of lymphoid origin (Fig.1B). All these cells are subject of intense mechanobiological study because mechanical forces control several of their functions, with important consequences in diseases like cancer (Elosegui-Artola et al., 2014, 2016; Lämmermann et al., 2008; Skau et al., 2016; Thiam et al., 2016).

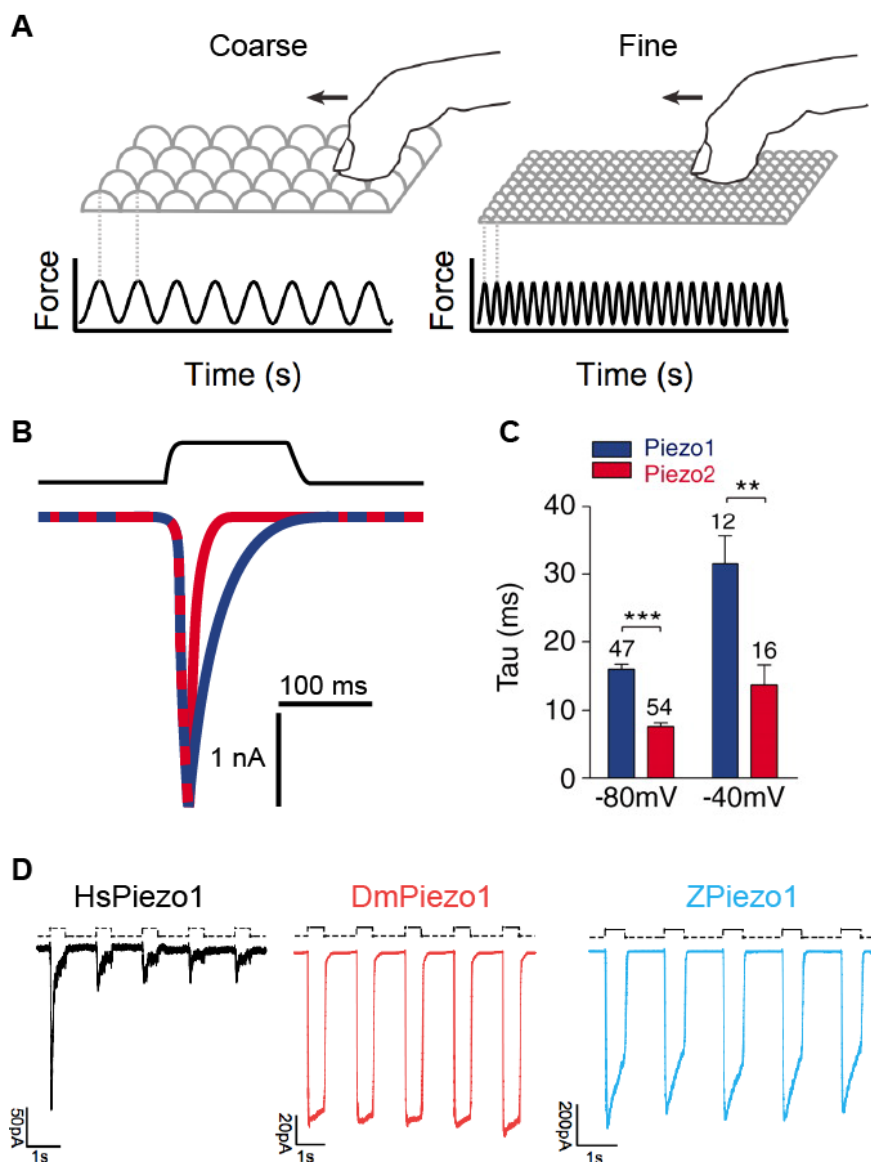
### 1.1.2 Piezo currents

Whole cell MA currents elicited by mammalian Piezo1 and Piezo2 activation are essentially similar. Large, non-selective cationic inward<sup>8</sup> currents appear in response to both positive (pressing) and negative (suction) pressure (Coste et al., 2010) and shear stress (Li et al., 2014). These currents show fast desensitization (reduced response to sustained stimulation) and linear voltage-current relationships. Nonetheless, there is a very important difference: Piezo2 inactivates twice as fast as Piezo1 at physiologically appropriate membrane potentials (Coste et al., 2010).

Inactivation is a current decay shortly after channel activation due to pore obstruction by a specific structure that is different from the main (open/closed) gate.

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<sup>8</sup> By agreement, inward or negative currents are generated by cations entering or anions exiting the cell, or a combination. We can discern between these two by replacing extracellular inorganic salts in the experimental solution by organic N-methyl-D-glucamine (NMDG) salts. NMDG does not fit into the pore of ion channels and thus cannot permeate across them. Consequently, inward current suppression by NMDG while there are still intracellular anions is an indicator of cationic permeability. In turn, cationic preferences are evaluated by individually modifying extracellular inorganic cations and quantifying current reversal potentials.



**Figure 2. Piezo inactivation and adaptation.**

(A) Schematics of force fluctuation during exploration of mechanical environmental features. (B) Superposed Piezo1 and 2 currents elicited by a mechanical pulse (top, black). Dotted lines, current commonalities (resting and inactivation). Solid lines, inactivation phase. (C) Time constants of a mono-exponential equation fitting the inactivating phase of the currents. (D) Human (Hs), Fruit fly (Ds) and Zebrafish (Z) Piezo1 responses to repetitive stimuli. Hs currents inactivate and desensitize rapidly. Dm neither inactivate nor desensitize and Z are slowly inactivating but do not desensitize. Adapted from (Coste et al., 2010; Lewis et al., 2017; Moroni et al., 2017).

It is essential for proper nerve impulse conduction or muscle contraction, by acting as a frequency filter for periodic stimulation (Fig.2A-D) (Lewis et al., 2017). Positive membrane potentials and repetitive stimulation slow down and ultimately abolish the inactivation of mammalian Piezo1 and Piezo1/Piezo2 chimeras (Coste et al., 2010; Gottlieb et al., 2012; Moroni et al., 2017). Fruit fly (*Drosophila melanogaster*) and zebrafish (*Danio rerio*) Piezo channels do not show desensitization and are much more sensitive to membrane voltage (Fig.2C). Considering that these functional states correlate to specific conformations, and the outward direction of currents at positive potentials, it is plausible that voltage and cation exit hold the channel in a prolonged open channel configuration (Moroni et al., 2017). This is also pertinent for diseases caused by mutations slowing Piezo channel inactivation kinetics, activation threshold, and trafficking (Andolfo et al., 2013; Bae et al., 2013; Coste et al., 2013; Glogowska et al., 2017; Zarychanski et al., 2012).

### 1.1.3 Piezo structure-function studies

Going back to the generic ion channel proposed at the beginning of this section, these proteins can be separated into two functionally-coupled elements: a sensor and a pore. In the case of Piezo, recent functional and structural studies have shown that pore properties (ion selectivity, single channel conductance, blocker sensitivity, inactivation kinetics) are determined by the last C-terminal ~500 aminoacids, while the force sensor resides in the N terminus previous ~2000 residues (Coste et al., 2015; Moroni et al., 2017; Zhao et al., 2016). Importantly, cancer genomic data retrieved from the cBioPortal cancer genomics database shows accumulated mutations in this same region in many cancer types (Fig.3). Yet,

the impact of these mutations on channel function and their relevance for cancerous traits remains unknown.

Piezo1 forms homotrimers with a three-blade propeller shape (Fig.4A). The 14-18 transmembrane helices connected by al-

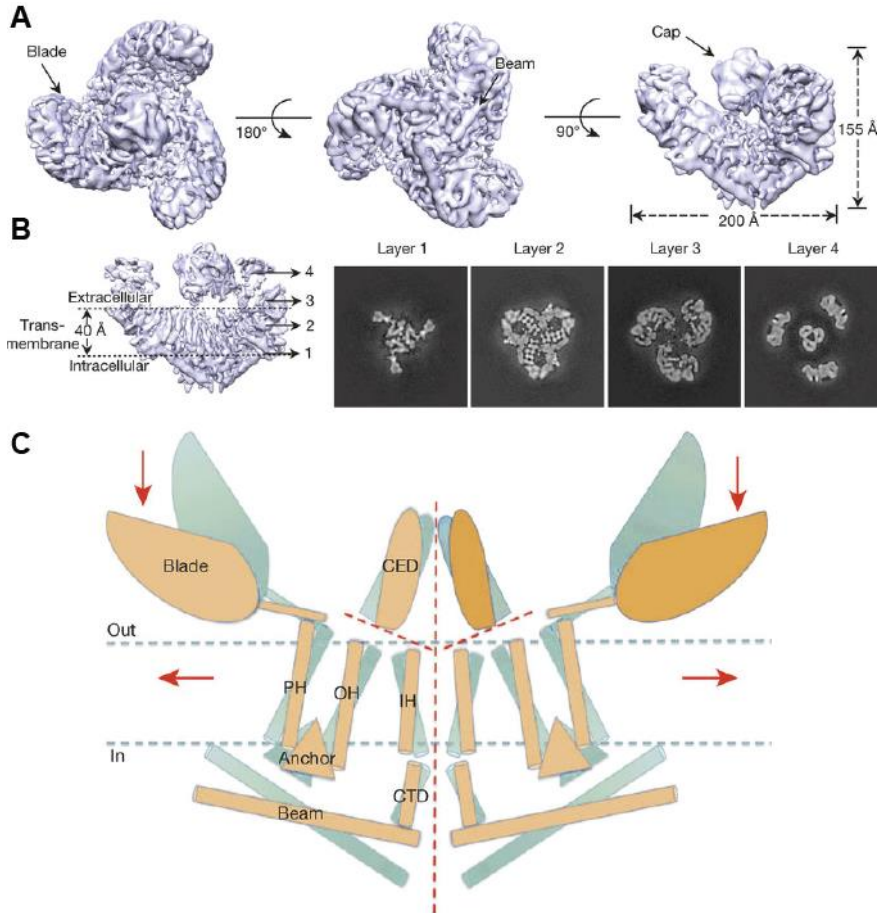


**Figure 3. Piezo mutations in lung and breast cancer samples.** cBioportal data shows a striking accumulation of Piezo2 mutations at 600 C terminal 600 residues. Extracted from [cBioportal](#) on 9/1/2017.

pha-helices that run parallel to the membrane in a single sub-unit have been classified in three sets: peripheral, outer and inner. Outer helices and their following extracellular segments are swapped between monomers, conferring structural stability to the full, trimeric channel. The C-terminal inner helices line the pore, and harbour the aforementioned disease-causing mutations (Fig.4B,C)(Coste et al., 2013; Ge et al., 2015).

Direct force application of  $\sim 10$ pN to this region mimics the inactivation defect and slows activation kinetics. Yet, it does

not affect deactivation. This suggests that activation/deactivation (open/close) and inactivation mechanisms work separately (Zhao et al., 2016).



**Figure 4. Piezo1 structure.**

(A) Top, bottom, and side views of a Piezo1 structural map. (B) Side view with numbered slices shown to the right. (C) Cartoon depicting the different parts of the Piezo1 structure and arrows suggesting its activating mechanism. Adapted from (Ge et al., 2015).

The N-terminal portion corresponding to the propeller blades is extracellular and, due to its flexibility and distance from the pore, it is a clear candidate to be the force-sensing module triggering channel activation. The mechanosensitivity of a

chimera containing the Piezo1 non-pore coding region and the pore of a trimeric mechano-insensitive channel supports this idea (Zhao et al., 2016) but this is not exempt of polemics (Dubin et al., 2017; Zhao et al., 2017). It is possible that the channel works as a lever with allosterically coupled edges at the force sensor and the pore (Ge et al., 2015; Sukharev and Corey, 2004; Wu et al., 2016). This propeller motif is also found in the structure of TRPA1, a channel proposed to mediate slowly-adapting MA currents in somatosensory neurons (Paulsen et al., 2015). Since, as I already mentioned, Piezo protein sequences do not resemble any other protein sequence, it is tempting to think about Piezo1 and TRPA1 propeller-like portions as analogous structures conferring mechanosensitivity. How they achieve this remains to be explored.

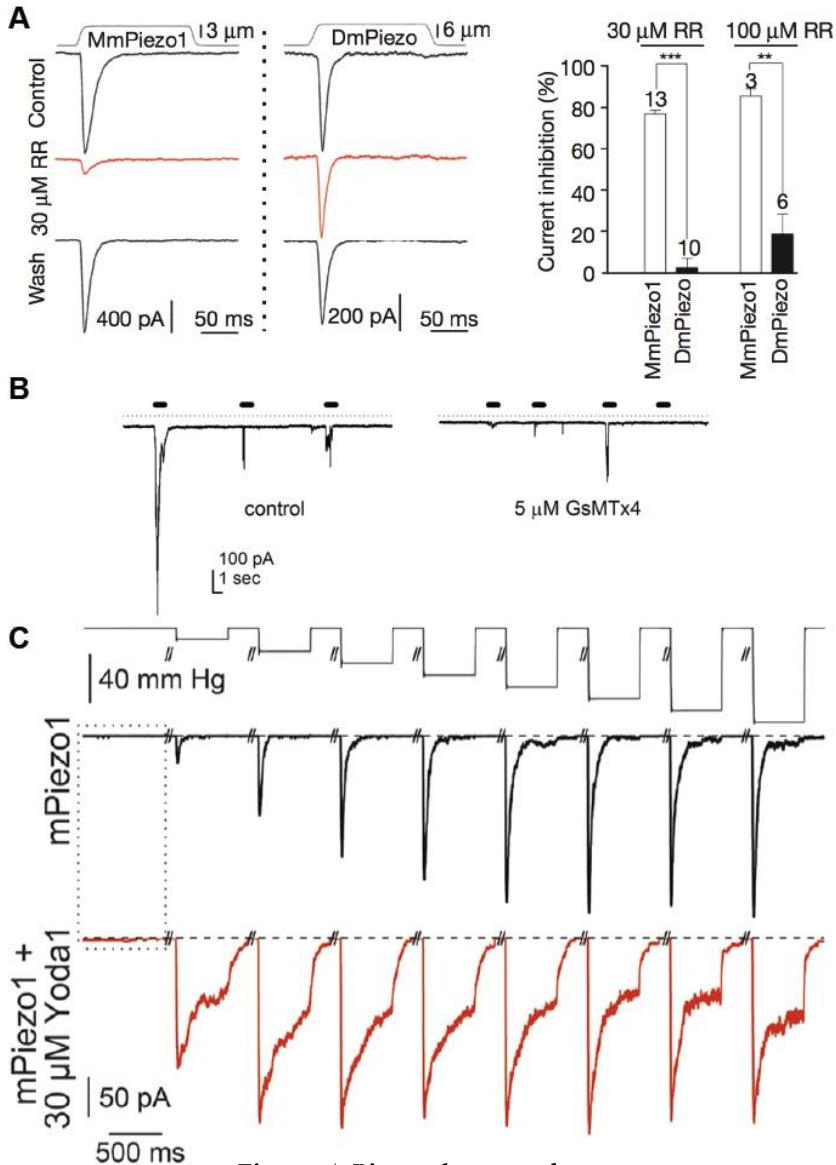
#### 1.1.4 Piezo pharmacology

Mammalian MA current blockage by ruthenium red (RR), gadolinium and streptomycin was already known before Piezo identification as the main mammalian MA ion channels (Bowman et al., 2007). These compounds are not specific and work on a state-dependent manner: RR and gadolinium also block the widely expressed TRP channels and RR only blocks Piezo inward currents (Coste et al., 2012).

The inhibitory effect of GsMTx4, a peptide isolated from the tarantula (*Grammostola spatulata*) venom, was also already known (Suchyna et al., 2000), but this toxin specifically targets Piezo channels in eukaryotes and Msc in bacteria (Alcaino et al., 2017; Bae et al., 2011; Hurst et al., 2009). GsMTx4 binds the outer membrane leaflet near the lipid-water interface. When membrane tension increases and reduces lateral pressure on lipids, the toxin penetrates the membrane and acts as



a retractor opposing lateral lipid collapse (Gnanasambandam et al., 2017). Oddly, the Patapoutian lab observes no GsMTx4 effects on Piezo1 (Syeda et al., 2015).



**Figure 5. Piezo pharmacology.**

(A) Effects of ruthenium red on mouse (Mm) and fruit fly (Dm) Piezo currents in response to mechanical pulses (left) and current inactivation percentage (right). (B) Inhibition of mechanically-induced currents by GsMTx4 in A375-SM melanoma cells. (C) Mouse Piezo1 sensitization (higher peak current, inactivation deceleration) by Yoda1. Adapted from (Coste et al., 2012; Hung et al., 2016; Syeda et al., 2015)

The perspective on Piezo agonists is no better. To date, we only know one compound specifically activating Piezo1: Yoda1<sup>9</sup>. This molecule activates Piezo1 in preparations only containing lipids and purified channels, and has no effect on Piezo2. Yoda1 also slows channel inactivation and reduces its threshold for mechanical activation (Syeda et al., 2015).

### 1.1.5 Piezo activation

Like Msc channels, Piezo1 is inherently mechanosensitive: purified protein insertion into lipid droplets rendered functional channels responding to osmotic shocks, direct injection-induced droplet swelling or when membrane symmetry was broken by addition of lysophosphatidic acid (LPA) (Syeda et al., 2016). Piezo2 lacks these experimental evidences. Also, some reports claim that this channel cannot be activated in excised patches, an experimental configuration where a small patch of the cell membrane is ripped-off for isolated electrical analysis (Moroni et al., 2017). Those same reports show activity when cell integrity is preserved or when chimeras encoding the proposed Piezo1 sensor and Piezo2 pore are expressed and tested in excised patches. Others show that for a stimulus of 500nN with an atomic force microscopy (AFM) tip, Piezo1-only expressing cells respond mildly, Piezo2-only expressing cells do not respond at all but Piezo1-Piezo2-expressing cells respond robustly (Lee et al., 2014). Therefore, seems that Piezo1-Piezo2 can have synergistic effects. This work and our own data show that both channels are abundant in articular

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<sup>9</sup> Yes, Yoda, the dyslexic green Jedi Master. *May the force be with you* and so on. The compound was initially termed Obi1, which was even more appropriate, existing only 1 effective compound among the 3.25 million tested (*you're my only hope*). See editor-author correspondence in (Syeda et al., 2015).

chondrocytes, cells constantly bearing dynamic mechanical loads. These authors also showed that cytochalasin D (a mycotoxin that inhibits Actin polymerization) and verapamil (a voltage-gated calcium channel inhibitor) abolished AFM-induced calcium entry in chondrocytes. These results evidence that although Piezo channels respond to mechanical stimulation by their own, several pathways modulate their function.

How Piezo function is modified by signalling pathways and how this influences cell behaviour will be discussed in the context of the other main themes of this thesis: cell migration and metastasis.

## 1.2 Cell migration

Many cell types migrate, i.e. change their spatial location along time. They do this by combining several modes of locomotion, always influenced by chemical and mechanical environmental cues that drive the intermodal transitions (Bergert et al., 2012, 2015; Diz-Muñoz et al., 2016; Hung et al., 2013; Liu et al., 2015b; Ruprecht et al., 2015; Wolf et al., 2003, 2007). Cell migration is absolutely required during physiological processes as fertilization, body development and growth, immune surveillance or tissue repair (Arboleda-Estudillo et al., 2010; Brugués et al., 2014; Denissenko et al., 2012; Lämmermann et al., 2008; Weber et al., 2013) and is a hallmark of metastasis (Friedl and Alexander, 2012; Gopal et al., 2017; Haeger et al., 2014; Labernadie et al., 2017), the main cause of death by cancer, characterized by cancer cell dispersal throughout the body and later invasion of specific secondary organs (Gavrilovic and Posner, 2005; Maher et al., 2009).

### 1.2.1 Mesenchymal locomotion

Unconfined cells migrating on 2D structures (e.g. a culture dish) exhibit a prototypical mode of migration that coordinates Actin polymerization with adhesion deposition and actomyosin contraction generating traction and promoting adhesion disassembly to move the cell body. This ideal cell is polarized (Fig.6A). The leading edge comprises everything from the advancing edge to nucleus and can be divided in two: the lamellipodium (LP) and the lamella (LA). The rear part of the cell is called trailing edge and must be released to enable forward cell movement (Franco et al., 2004; Mrkonjić et al., 2015).

The LP is a wide, thin, and flat structure generated by Actin polymerization driven by the RhoA/Rac1 GTPase system and the Actin regulating (nucleating, polymerizing) machinery mDia1, Arp2/3, and Ena/VASP (Fig.6B). Initial Rho activity initiates mDia1-dependent linear Actin polymerization that pushes the membrane forward, generating protrusions. LP Rho is rapidly inhibited by Protein Kinase A (PKA)-mediated phosphorylation and this halts protrusion, defining a protrusion-retraction pacemaker (Lee et al., 2015; Machacek et al., 2009; Tkachenko et al., 2011). As the protrusion gets to its maximal speed, Rac1 GTPase activity increases 2  $\mu\text{m}$  behind the cell edge, where adhesions either disassemble or get reinforced and engaged to retrograde Actin flow (Machacek et al., 2009; Oakes et al., 2012; Ponti et al., 2004; Swaminathan et al., 2016). Since the Rho-Rac crosstalk regulates the function of both proteins, proteins modifying one GTPase function will modify the other and thus alter cell migration.

Of the different signalling pathways participating in cell migration it is worth a word on the cAMP-PKA pathway. It is

activated in response to cyclic-adenosine monophosphate (cAMP), that also activates the Exchange Protein Activated by cAMP (EPAC). As mentioned above, PKA inhibits Rho, but it also activates Rac1. Interestingly, Piezo2 activity is sensitized by PKA and EPAC signalling in response to inflammatory mediators (Dubin et al., 2012; Eijkelkamp et al., 2013).

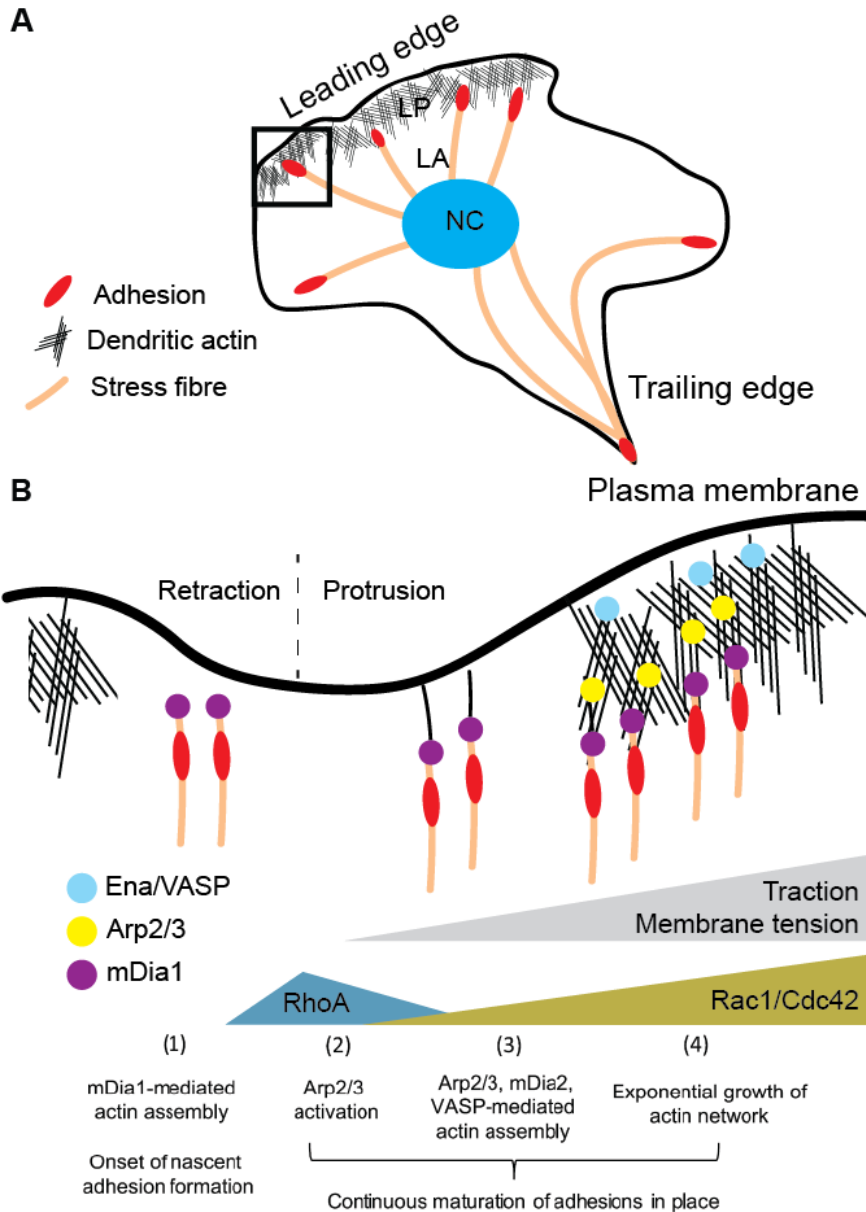
Therefore, specific sensors acting upstream of these GTPases signal into the feedback mechanism in response to diverse stimuli, such as cell adhesion or confinement (Hung et al., 2013; Lawson and Burridge, 2014). Whether these functional links are coupled in migrating cells is not clear, but calcium-PKA interactions impacting cell migration have been known for decades (Howe, 2004, 2011).

Rho/Rac/actin-dependent protrusions contain integrins, transmembrane receptors whose engagement with ECM ligands (e.g. fibronectin, collagen) promotes the formation of cell-matrix adhesions. The intracellular tail of integrins contains docking sites for several signalling and adaptor molecules that enable adhesion-actin engagement and subsequent cell-substrate traction force transmission, rigidity sensing and mechanotransduction. As an example, we have already commented that FAM38A had been identified as an activator of integrin-mediated CHO cell adhesion. Accordingly, its knock-down in HeLa cells reduced  $\beta$ 1-Integrin activation and cell adhesion strength (McHugh et al., 2010). Moreover, Piezo1 suppression reduced the function of the calcium-dependent calpain proteases, that cleave several adhesion proteins and hence regulate adhesion dynamics, both at the leading and trailing edges (Franco et al., 2004; Mrkonjić et al., 2015). The authors hypothesized that Piezo1-dependent Calpain2 increased cleavage of adhesion proteins that were then able to

activate integrins and reinforce cell adhesion (McHugh et al., 2010).

Actin polymerization underlying protrusion increases membrane tension. Meanwhile, traction forces and adhesion deposition increase (Lee et al., 2015). Inhibiting formin-dependent Actin polymerization or modifying membrane tension both reduce protrusion growth, traction force generation and adhesion formation. Tension also controls periodic adhesion placement in rows as the cell edge advances (Pontes et al., 2017). According to all these observations, leading edge dynamics depend on actin-driven membrane tension increases.

In parallel, leading edge protrusion/retraction and adhesion deposition events correlate with calcium signalling (Tsai and Meyer, 2012). Whether Piezo ion channels couple actin-dependent leading edge protrusion and membrane tension increases to calcium entry, calpain function and actin-integrin engagement remains to be demonstrated, but it is a tempting hypothesis due to several observations (Munevar et al., 2004; Wei et al., 2009). First, migrating fibroblasts exhibit local calcium increases (flickers) dependent on extracellular calcium influx coupled to intracellular calcium store depletion. Second, flickers increase in response to shear stress or the integrin ligand RGD, and are abolished when mechanosensitive ion channel function, myosin II activity, or Actin polymerization are reduced, suggesting that a mechanical component is present. Third, flickers are more likely to happen at the frontal part of the LA, where adhesions form. Plus, they appear near integrin spots. Fourth, upon exposure to a chemoattractant gradient, flickers accumulate at the region of the cell proximal



**Figure 6. Cell protrusion during cell migration.**

(A) Actin structures in migrating mesenchymal cells. LP (lamellipodium), LA (lamella), NC (nucleus). (B) Schematic of a retraction-protrusion cycle driven by Actin polymerization under control of GTPases (RhoA, Rac) and Actin nucleating/branching proteins (mDia1, Arp2/3, Ena/VASP) at the lamellipodial leading edge. Adapted from (Lee et al., 2015).

to the source and this results in cell turning. Lastly, Piezo1 inhibition with GsMTx4 reduces MCF-7 breast cancer cells speed (Li et al., 2015).

To sum up, we know that mechano-responding calcium signals, localized near adhesions, guide migration. They could achieve this by promoting biased adhesion deposition at regions where mechanical and chemical signals are prominent.

A role for mechano/osmo-sensitive channels in trailing edge retraction was described almost twenty years ago, even when the molecular identity of these channels was far from being identified. In adherent cells, protrusion-dependent tension increases lead to calcium entry through a gadolinium-inhibited pathway and subsequent retraction of the cell margin (Lee et al., 1999). This is achieved by two complementary pathways. First, calcium transients are followed by increases in traction forces that last until trailing edge retraction, suggesting increases in contractility triggered by calcium entry (Doyle et al., 2004). Second, calcium activates calpain-dependent adhesion disassembly at the trailing edge (Franco et al., 2004). Impairing calcium entry reduces activity of calpains and impedes trailing edge retraction, resulting in elongated cell shapes and stalling migration (Mrkonjić et al., 2015).

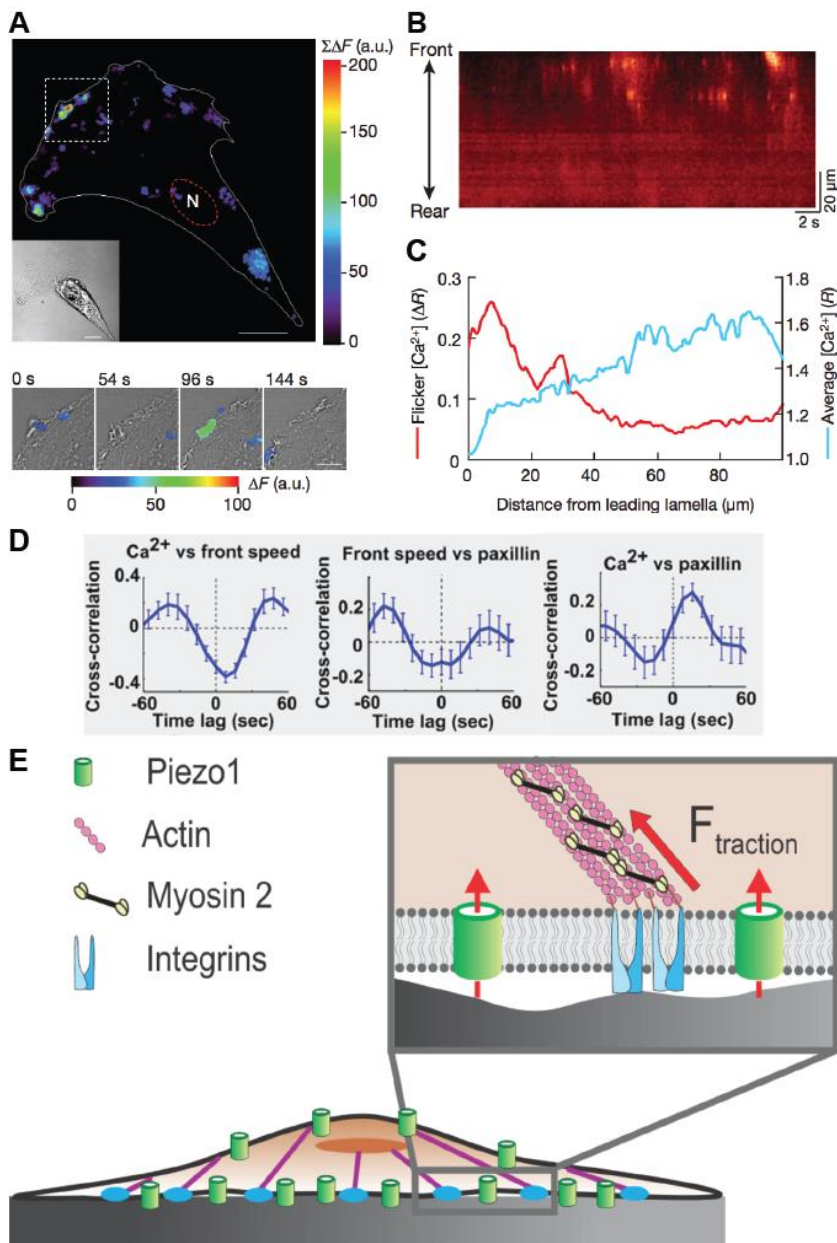
Apart from lamellipodial dendritic Actin structures driving protrusion, cell cycling, adhesion dynamics and migration also require the proper assembly of lamellar cytoskeletal structures termed stress fibres (SFs). These actin-based filaments show diverse structures and compositions, but as common traits we must retain first that their core is composed by short Actin filaments, placed with alternating polarity and bundled by periodically-distributed  $\alpha$ -Actinin and non-muscle Myosin II, resembling the structure of the skeletal muscle contractile



apparatus. Second, that SFs connect adhesions and nuclei or pairs of adhesions. This enables direct matrix-to-nucleus mechanical transmission, but also cell-to-matrix transduction due to the contractile activity. Third, Rho GTPase is a master regulator of SF assembly (Ridley, 2015). Rho is activated by GDP→GTP exchange by Guanine Exchange Factors (GEFs) in response to force and extracellular signalling molecules and activates two target proteins: the formin mDia1, that polymerizes Actin filaments, and Rho-associated protein kinase (ROCK). ROCK reduces Actin filament depolymerization via LIM kinase-dependent ADF/Cofilin inhibition. It also phosphorylates Myosin light chain (MLC) and inhibits MLC phosphatase, leading to increased Myosin II contractile activity. Altogether, Rho activation results in an increase in stress fibres and their contractile activity, and this is required for proper cytoskeletal dynamics and adhesion turnover, as SF disassembly or Myosin inhibition impair these mechanisms (Elosegui-Artola et al., 2016; Hung et al., 2013, 2016; Oakes et al., 2012). Actin fibre-disrupting agents cytochalasin D and latrunculin A also impair whole cell Piezo activity, but do not affect channel activity in excised patches (Eijkelkamp et al., 2013; Gottlieb et al., 2012). The actin-crosslinking protein Filamin A tonically inhibits<sup>10</sup> Piezo1, as its deletion increases calcium entry in response to mechanical stimulation of smooth muscle cells (Retailleau et al., 2015).

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<sup>10</sup> Tonical inhibition defines a constant and long-lasting inhibitory effect in contrast to phasic, interminent ones.



**Figure 6. Calcium transients and cell adhesion.**

(A) Calcium flickers in the lamellipodia of migrating cells. (B) Kymograph showing frontal polarization of calcium flickers. (C) Calcium flickers and global concentrations are oppositely located. (D) Cross-correlation analysis of calcium, Paxillin, and cell front signals. Paxillin peaks after the calcium pulse. (E) Forces at the cell-substrate interface involve integrins, Actin and Myosin and activate Piezo channels. Adapted from (Nourse and Pathak, 2017; Tsai and Meyer, 2012; Wei et al., 2009)

This suggests that the cortical cytoskeleton acts as a mechano-protector, limiting the structural load born by the plasma membrane. This vision is supported by results showing easier Piezo1 activation in blebs, structures devoid of cortical cytoskeletal elements (Cox et al., 2016). Contractility also contributes to Piezo1 activation in adherent cells. In a paper that has definitively influenced the present thesis, Medha M. Pathak and her collaborators showed that non-muscle Myosin II inhibition with blebbistatin reduces Piezo1-dependent calcium signals in human neural stem cells (Pathak et al., 2014). Considering all this, we see that the Actin cytoskeleton plays contradictory roles on Piezo gating: cortical Actin structures shield the channel from external forces, but contracting Actin filaments (a source of internal force) are necessary for Piezo activation in adherent cells. The latter is in agreement with data showing that stretching a stress fibre with optical tweezers activates a gadolinium-blocked calcium entry pathway whose molecular entity remains unclear (Hayakawa et al., 2008).

Importantly, Rho and MLC phosphorylation activation in some cell types require calcium entry through MA channels inhibited by RR and HC067047 (Seminario-Vidal et al., 2011). As previously mentioned, both blockers inhibit Piezo channels. Accordingly, Rho activation in response to certain stimuli could depend on Piezo activation. This would have drastic consequences also for our understanding of another mode of migration widely used during metastasis.

### 1.2.2 Amoeboid migration

The term *amoeboid* refers to the similarity of these cells to amoebas, whose irregular shape changes constantly, resembling a fluid<sup>11</sup>. Cells migrating by this mechanism diverge in shape and protrusion rate but they all share periodic expansion-contraction cycles and faster speeds than mesenchymal cells. Recent research has shown that reducing cell-substrate adhesion and increasing actomyosin contractility trigger a mesenchymal-to-amoeboid (MAT) transition in many cell lines, both *on vitro*<sup>12</sup> and *in vivo* (Bergert et al., 2012, 2015; Liu et al., 2015b; Ruprecht et al., 2015). These conditions promote the cortical accumulation of Actin and Myosin and a rounded, non-spread cell shape. A spatial fluctuation in contractility provokes cell polarization and actomyosin and force gradients. The region with higher actomyosin accumulation and function becomes the cell rear (the cell becomes polarized). For example, LPA addition at one pole of an almost-spherical cell rapidly elongates it in the direction of the gradient, with actomyosin accumulation at the edge next to the source of the chemoattractant. Rearward actomyosin flows reinforce this asymmetry and at the same time transmit forces to the substrate, enabling cell advance. Importantly, vertical confinement of the cells is required for appropriate cell-substrate friction (force transmission) and forward movement, similar to the chimneying climbing technique, in which a climber embedded between two parallel, grip-less walls is able to move by applying force to both walls.

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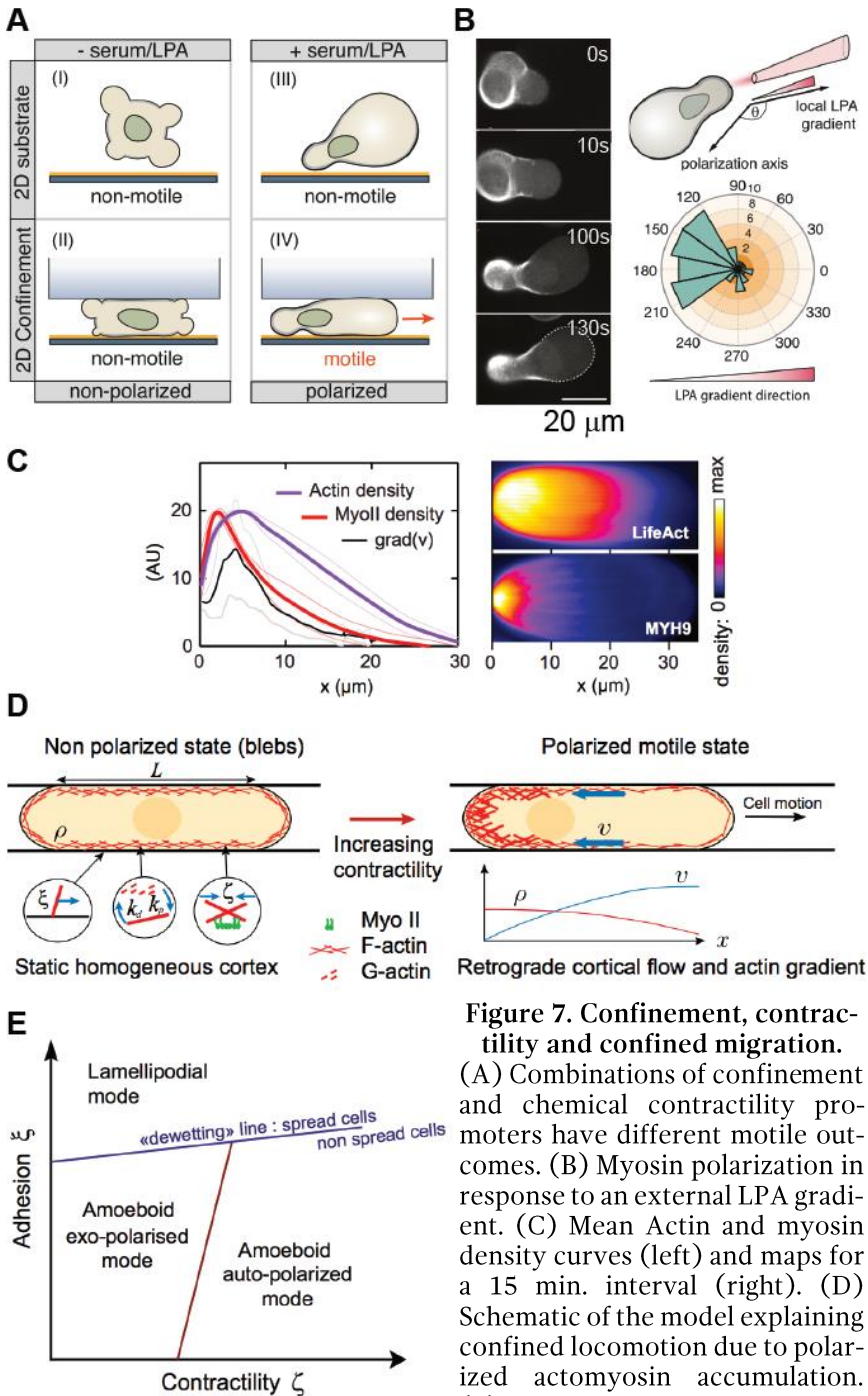
<sup>11</sup> The ancient Greek word for amoeba, ἀμοιβή, means change.

<sup>12</sup> Term coined by Dr. Joachim Goedhart, from University of Amsterdam, referring to experiments performed on glass coverslips.

### 1.2.3 Mechanical inputs in cell locomotion

Diverse stimuli of mechanical nature affect mesenchymal locomotion. First, confinement increases contractility (Hung et al., 2013; Liu et al., 2015b). Ergo, besides enabling force transmission during amoeboid migration, confinement triggers transition into this mode of migration by mesenchymal cells. Second, substrate ligand concentration modulates adhesion strength, and this has biphasic effects on cell migration speed due to an adhesion-actomyosin feedback (Gupton and Waterman, 2006). According to the authors, the amount of ECM ligand determines the amount of nascent adhesions formed. For scarce ligand situations, few adhesions would form, and those forming would be short-lived, impairing force transmission and cell advance. Conversely, abundant ECM ligands would form too many adhesions for the contractile system to manage. And so, the force per adhesion would be low, slowing adhesion turnover. This would impair cell detachment and would stall cell movement. As a proof, authors recover cell migration in highly adhesive surfaces by artificially increasing actomyosin contractility. These effects result in a biased displacement towards ligand-rich regions, termed haptotaxis, with consequences in cancer invasion (Gopal et al., 2017; Oudin et al., 2016). Importantly, integrin-ligand binding during cell adhesion and forward advance triggers calcium entry (Matthews et al., 2010; Sjaastad et al., 1996; Thodeti et al., 2009; Wu et al., 1998), and we have already mentioned calcium regulates actomyosin contractility. An adhesion-contraction coordinating role for MA channel-mediated calcium entry is interesting but remains to be studied.

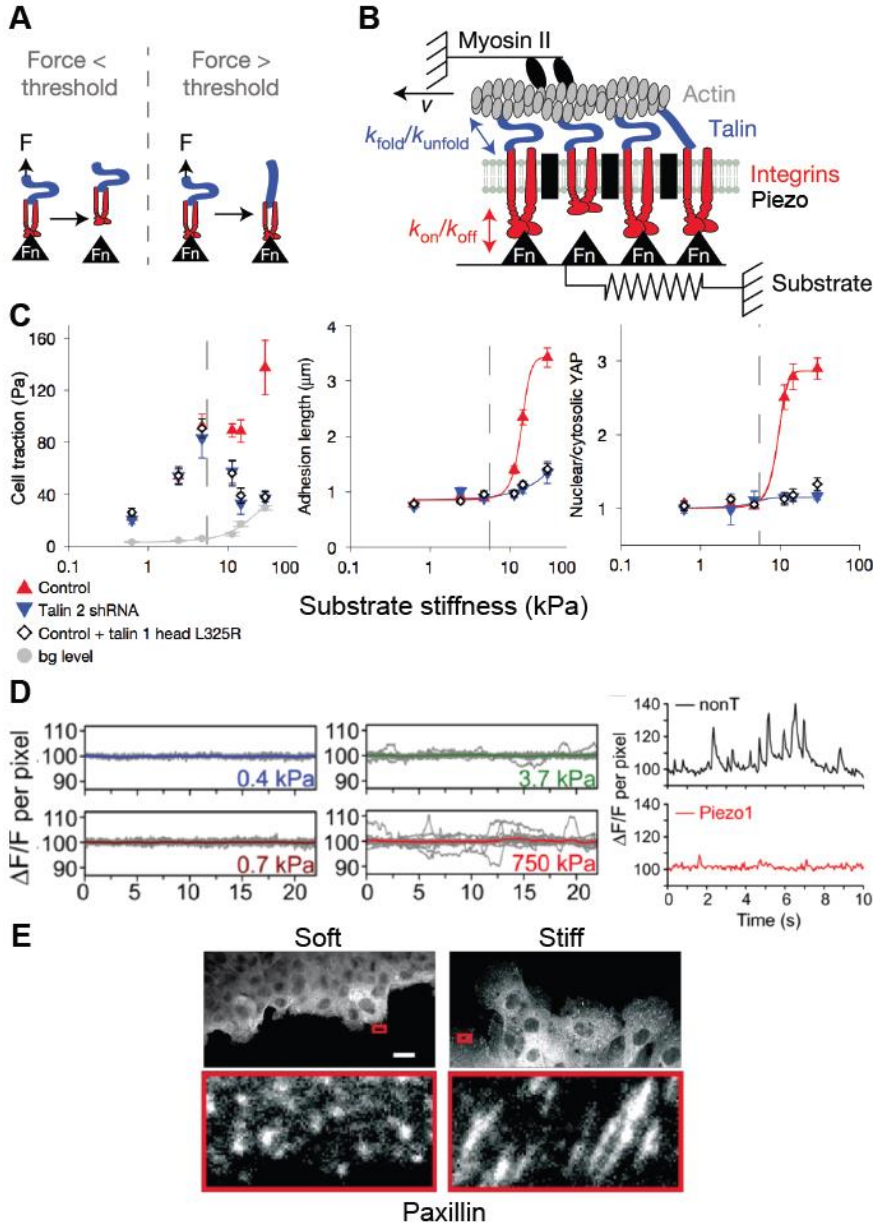
A third parameter affecting migration is matrix rigidity.



**Figure 7. Confinement, contractility and confined migration.** (A) Combinations of confinement and chemical contractility promoters have different motile outcomes. (B) Myosin polarization in response to an external LPA gradient. (C) Mean Actin and myosin density curves (left) and maps for a 15 min. interval (right). (D) Schematic of the model explaining confined locomotion due to polarized actomyosin accumulation. (E) Phase diagram explaining motile modes as a function of adhesion and contractility. Adapted from (Liu et al., 2015b; Ruprecht et al., 2015)

The first evidence showed fibroblasts moving from softer to stiffer regions, but cells also showed directional responses towards the source of external stretching of the substrate, that first caused movement cessation and later protrusion from that region. This shows that external mechanical cell manipulation promotes re-polarization. In their model, the authors already proposed a role for mechano-dependent calcium entry in these processes (Lo et al., 2000). A steep-enough rigidity gradient should be able to polarize the locomotive machinery of the cell and thus promote durotaxis, as was later shown for mesenchymal stem cells (Vincent et al., 2013).

Having said that, the situation is more complicated when analysing the migratory behaviour of multicellular clusters. In this ideal monolayer of cells, contractile Actin filaments form a physical continuum through cell-cell junctions. Cluster edges are placed at soft and stiff regions, i.e. face mechanically different environments. Soft matrices deform further than rigid matrices for a given traction force, and in these monolayers, traction forces at both monolayer edges are similar in magnitude. Therefore, seems that reduced substrate deformation at stiff edges improves Actin polymerization and engagement with adhesions, enabling cell advance. Nevertheless, this model does not explain why adhesions are larger on stiffer regions. Importantly, Piezo1 activity increases with substrate rigidity (Pathak et al., 2014) and this is essential for axonal pathfinding and growth (Koser et al., 2016). Also, confinement promotes collective migration of neural crest cell (Szabó et al., 2016). Increased MOS activity at the rigid edge of monolayers or under confinement could contribute to the observed phenomena.



**Figure 8. Substrate rigidity tunes cell-substrate interaction and Piezo function.**

(A) Talin unfolding stabilizes fibronectin-integrin interactions. (B) Model of force transmission by engagement of the rigidity-dependent clutch upon talin unfolding. (C) Rigidity-dependent force transmission, adhesion maturation and mechanotransduction of single cells requires talin unfolding. (D) Piezo channels mediate rigidity-dependent calcium signals. (E) Stiffness gradients locally regulate adhesions of cell monolayers. Adapted from (Elosegui-Artola et al., 2016; Pathak et al., 2014; Sunyer et al., 2016).



Actin-adhesion engagement is a deeply studied force-dependent phenomenon. The clutch analogy was initially proposed to explain why, while the Myosin-dependent F-actin retrograde flow was constant, forward movement of the cell was intermittent. The authors proposed that the forces derived from Actin polymerization could be transmitted to the substrate when Actin was coupled to matrix-bound adhesions, and that this coupling slipped (Mitchison and Kirschner, 1988). Later refinement has incorporated several parameters to this model (Chan and Odde, 2008; Elosegui-Artola et al., 2014, 2016). First, each type of integrin interacts with its matrix ligand with specific binding and unbinding rates. Second, inside the cell, Myosin contracts stress fibres, i.e. pulls on the filaments. If these filaments were not bound to adhesions, they would flow as fast as Myosin pulls on them. This is the case for cells on soft substrates. When the Actin-adhesion clutch gets engaged, the movement of the filament is transmitted to the substrate through adhesions, slowing retrograde Actin flow. Substrate rigidity determines the rate at which Actin pulling on engaged clutches deforms the substrate and thus how force loads at integrin-matrix bonds. At low rigidities, force loading takes longer than spontaneous integrin-matrix unbinding, and there is little cell-substrate force transmission. Upon reaching a given threshold, force loading unfolds an adhesion adaptor protein named Talin before integrin de-adhesion. Talin unfolding recruits Vinculin and enables engagement with the contractile Actin cytoskeleton. It also recruits more integrin units, increasing integrin-matrix binding probabilities. This process, known as reinforcement, promotes adhesion growth, increases force transmission, and activates mechanotransducing pathways involved in cell proliferation and cancer, e.g. YAP (Aragona et al., 2013; Dupont et al., 2011; Elosegui-Artola

et al., 2016). We will mention them when discussing metastasis.

We have already learnt about the role of Piezo-dependent Calpain activation on integrin activation and adhesion reinforcement. In addition, Calpain-mediated Talin cleavage increases its ability to bind the cytoplasmic tails of integrins (Yan et al., 2001). Importantly, also shear stress-dependent cell alignment, as seen in endothelial cells orienting parallel to blood flow, depends on Piezo1 expression and function, coupled to Calpain activation (Li et al., 2014). Moreover, integrin-mediated adhesions sensitize Piezo to pulling forces of 30nN<sup>13</sup> (Gaub and Müller, 2017), suggesting a role for integrin-mediated adhesions in Piezo modulation and strengthening the potential role of this ion channel in adhesion or Actin dynamics regulation. All these results show that local mechanical activation or modulation of Piezo function, coupled to Calpain activation, is able to orchestrate whole-cell adhesion and cytoskeletal dynamics.

Summarizing, we know that proper cell migration requires MA ion channel function, and we know some of the underlying mechanisms, such as RhoA activation, cell contractility or protease activation controlling adhesion dynamics. We have also seen that chemical and mechanical inputs influence the way cells organize their adhesive and contractile machinery, determining the mode of cell migration. In light of these observations, MA channels emerge as potential unifying sensors

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<sup>13</sup> Accordingly, in our hands, cells seeded on plates coated with the non-specific ligand poly-D-lysine are less sensitive to mechanical stimulation than cells seeded on fibronectin- or collagen-coated plates.

where confinement, ligand density, matrix rigidity, shear stress or even chemical signals (e.g. LPA) converge to trigger unified responses, i.e. polarization and migration along a gradient (haptotaxis, durotaxis, chemotaxis), by impacting adhesion and cytoskeletal dynamics. This would have very important repercussions for the understanding and approaching a disease where cell environmental rigidity and migration are fundamental: cancer.

### 1.3 Cancer

Cancer is a group of complex diseases with non-physiological proliferative and spreading capabilities as common traits of cancerous cells. The mainstream theory conceives that these deleterious features are acquired due to DNA or epigenetic changes. Indeed, many cancers share common mutations inactivating DNA damage-induced cell cycle arrest pathways (tumour suppressor genes: Retinoblastoma, p53) or activating pro-survival genes and pathways (oncogenes: Ras) (Hanahan and Weinberg, 2000). The development of high throughput genome sequencing strategies during the first decade of the 21<sup>st</sup> century revealed that cancer genes are scattered throughout almost every process in cell biology. Many genes identified by these means are components of signalling pathways that are nowadays under study as potential therapeutic targets for specific cancer types. Nevertheless, the complexity of cancer cannot be explained only by means of mutations affecting the protein-coding genome of specific cells, which accounts, in turn, for less than the two percent of the total genome. Adding this

to intratumoural genetic heterogeneity, pleiotropy<sup>14</sup> and polygenes<sup>15</sup> hinders establishing causal relationships between gene alterations and tumour phenotypes and helps to explain the failure of many targeted therapies.

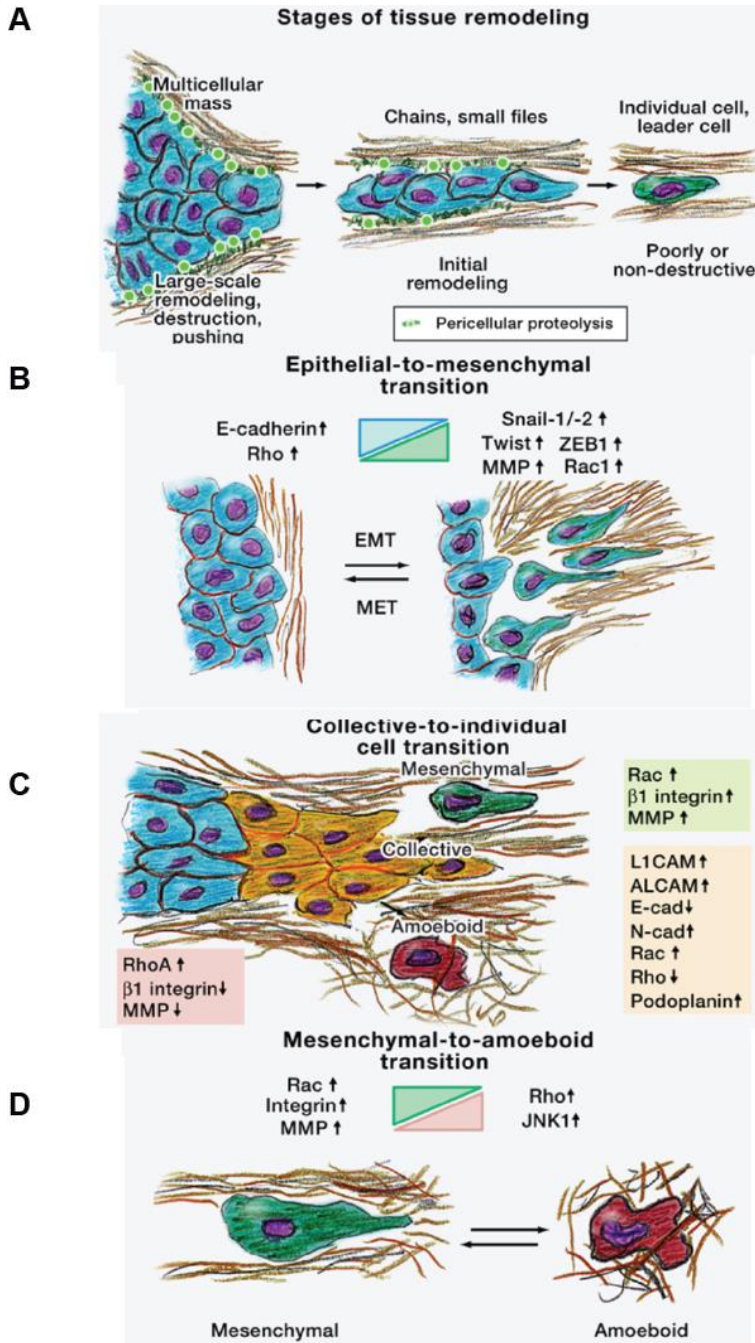
The Genome Theory of Cancer Evolution uses evolutionary concepts to envision a more accurate framework to understand and tackle cancer (Greaves and Maley, 2012; Kareva, 2011; Maley et al., 2017). It is important to remind that natural selection is driven by local environmental conditions acting on phenotypes, which in turn can be the convergent outcome of several genotypes. During cancer development, hypoxia, reactive oxygen species (ROS) or tissue mechanics select for specific phenotypes but, at the same time, promote genomic instability (all three can provoke DNA lesions), potentially increasing phenotypical variability and eventually favouring malignant traits (Irianto et al., 2016, 2017a, 2017b; Plodinec et al., 2012).

Since the main cause of death by solid cancers is metastasis, i.e. the spreading of cancer cells (CC) throughout the body (Gavrilovic and Posner, 2005; Nguyen and Massagué, 2007), I will focus here on the influences of the environment on pro-metastatic features such as phenotypic heterogeneity, migration, matrix remodelling capability or defence-evading mechanisms.

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<sup>14</sup> Situation in which a single gene affects several phenotypic traits.

<sup>15</sup> Group of genes that interact to influence a phenotype



**Figure 9. Transitions in metastasis.**

Pericellular proteolysis and cell-cell interactions dynamically modulate tissue invasion during metastasis. Adapted from (Friedl and Alexander, 2012).

### 1.3.1 Metastasis

Any living tissue, healthy or diseased, can be thought as a container (the extracellular matrix, ECM) and a continent (several cell types) with interwoven cell-cell and cell-matrix interactions.

Healthy, epithelial cells sit on top of a collagen-rich fibrous sheet called Basement Membrane (BM). Cell-cell and cell-matrix adhesions ensure timely cell renewal and matrix deposition and that makes them essential for proper epithelial function (selective permeability barrier, secretion). During tumorigenesis, adhesions become labile and transformed cells perforate the BM, gaining access to the underlying structure, termed mesenchyma, a connective tissue containing fibroblasts, immune cells and blood and lymphatic vessels. This process is called Epithelial-Mesenchymal Transition (EMT) and involves drastic changes in gene expression conferring new morphological and functional features to transformed cells<sup>16</sup>. Once in the mesenchyma, invading cells remodel the matrix while migrating, and ultimately intravasate. The necessity of cells undergoing EMT to metastasize is not clear, because key drivers of EMT can be suppressed without affecting the metastatic potential of cells (Ye et al., 2017). In these cases, metastatic cells remain bounded by epithelial cell-cell junctions and require mesenchymal cells (Cancer Associated Fibroblasts, CAFs) to remodel the matrix and collectively invade their environment. CC-CAF cooperation is mediated by cell-cell mechanically-active links: upon cell to cell contact,

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<sup>16</sup> EMT is essential during physiological processes as gastrulation or neural tube formation.

CAFs repolarize and pull CC masses, enabling collective invasion (Labernadie et al., 2017). Hence, independently of their origin (cancer cells *per se* or accessory cell types), locomotive and matrix-remodelling activities are indispensable for metastasis. As in many other situations in biology, these properties are functionally linked.

- Matrix degradation in metastasis

In response to ECM-integrin engagement, metastatic breast cancer cells in culture activate an integrin→Src→Tsk4/5→N-WASP axis promoting specific patterns of Actin polymerization from the ventral face of the cell as bundles perpendicular to the substrate. These protrusions, termed invadopodia, concentrate enzymes (e.g. metalloproteases) that locally degrade the underlying matrix. *In vivo* experiments in mice show that interfering with invadopodia biology impairs metastatic extravasation and colonization (Leong et al., 2014). Once again, mechanical stimuli as matrix rigidity linearly correlates with ECM degradation as a function of RhoA-mediated Actin polymerization and Myosin contractility (van den Dries et al., 2013; Jerrell and Parekh, 2014; Lizárraga et al., 2009).

Mesenchymal ECM is rich in cross-linked collagen networks whose small pore size impedes free cell movement. Work at S.J. Weiss' lab identified the membrane-tethered collagenases MT1-, MT2- and MT3-MMPs as the main players of pericellular matrix degradation enabling basement membrane transmigration and metastatic cell invasion and proliferation (Hotary et al., 2006, 2003; Sabeh et al., 2004). Pioneering work at Peter Friedl's lab inhibiting these enzymes showed that invading cells transit into an amoeboid mode of migration characterized by a loss of adhesive structures and a cortical distribution

of the contractile actomyosin machinery that ensures cell migration (Wolf et al., 2003). I have previously introduced the potential role of MA channels on amoeboid migration. Joint work by both groups later showed that MMP-independent migration speed decreased linearly with pore size and that it required nuclear deformation (Wolf et al., 2013). Each cell type showed a matrix degradation VS nuclear deformability balance that resulted in characteristic minimal pore sizes inhibiting migration, e.g. cancer cells lose pore transmigration efficiency for sections under  $10\mu\text{m}$ , while polymorphonuclear immune cells keep migrating until  $4\mu\text{m}$  (Fig.10A). This is likely due to different nuclear-deforming capability, because the nucleus diameter acts as the limiting factor during pore transitions (Petrie et al., 2016; Wolf et al., 2013).

Integrating these results with the observations on the pro-MAT effect of inhibiting the pericellular matrix degradation suggests that the mechanical stimulation of cells by confinement (which is increased when proteases are inhibited because cells can no longer degrade the matrix and widen the existing pores) is what triggers MAT. On that account, the failure of MMPs inhibitors as antimetastatic drugs could be explained, at least in part, by the fact that cells are still able to invade even in the presence of these compounds because cancer cells are experiencing MAT in response to the confinement imposed by the stroma (Sabeh et al., 2009). Also, pores can be negotiated by transient decreases in cell volume followed by volume recovery. Dealing with osmotic stress is absolutely required for cell viability. Cells have evolved under this selective pressure favouring efficient volume-regulating mechanisms. Cells passively swell when water enters them, e.g. when placed in a hypotonic solution. This activates a fast,



conserved response termed Regulatory Volume Decrease (RVD), consistent on solute (frequently, ions) exit from the cell and subsequent, osmotically-driven water efflux, leading to basal volume recovery (Hoffmann et al., 2009). Solute loss is achievable by many ways, but they all ensure neutral electrical result, i.e. equal anion and cation exit. In mammals, the ubiquitous activation of Swell1, the mammalian volume-sensitive chloride channel, ensures anion exit (Qiu et al., 2014; Voss et al., 2014). One of the mammalian cation exit mechanisms starts with Piezo1 activation in response to membrane stretch (Cahalan et al., 2015). Calcium entry through these channels activates calcium-dependent potassium channels, that allow potassium ions to exit down their electrochemical gradient. The global loss of KCl is what ultimately drives water exit and volume recovery. In the case of Red Blood Cells (RBCs), aforementioned gain-of-function mutations in Piezo1 lead to cell dehydration by excessive water loss and lysis haemolytic anaemia seen in xerocytosis (Andolfo et al., 2013; Bae et al., 2013; Zarychanski et al., 2012), clearly showing the volume-reducing effects of Piezo activation.

Altogether highlights again the relevance of mechanical inputs for cell migration and points to Piezo as a potential unified sensor triggering adapting responses (locomotive changes, matrix degradation, volume regulation) that enable cell invasion.

After remodelling the mesenchyma, cells gain access to blood vessels and intravasate. Once in the bloodstream, circulating tumour cells (CTCs) are transported throughout the circulatory system, virtually visiting all organs. These events are frequent in cancer, even before tumour diagnosis, and millions of

cancer cells can be released into the bloodstream. Nevertheless, many cancer patients do not suffer from metastasis, even when CTCs are detected in a blood analysis (Kim et al., 2009; Massagué and Obenauf, 2016a). These observations suggest that metastasis is a very inefficient process, which can be explained by the obstacles faced by invading cells. As in the case of an infection, the initially identified obstacles were cellular and chemical. Recently, the mechanical nature of the invaded tissue has emerged as an additional barrier. Allow me to describe a metastatic cascade using a specific target organ.

### 1.3.2 Brain metastasis

Given that local and systemic approaches against other metastasis are improving their efficacy, brain metastasis is an increasing problem (Gavrilovic and Posner, 2005; Maher et al., 2009). As an example, it is the main cause of death by lung and breast cancer, two top frequent cancers in the Western World, and it is the subject of study of the second chapter of this thesis.

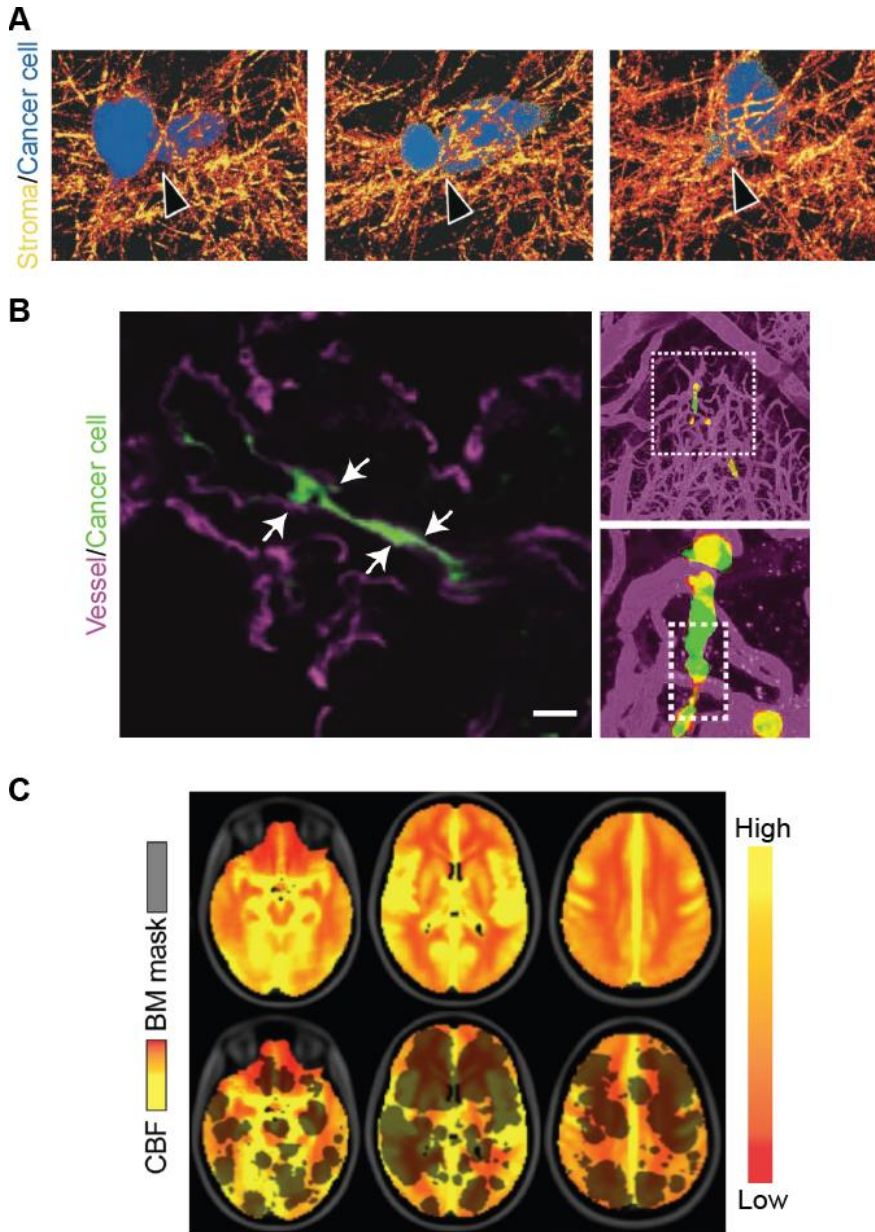
CTCs get lodged in brain microvessels (Fig.10B, *left*), where traumatic deformation kills most of them (Furlow et al., 2015; Kienast et al., 2010; Zeidman, 1961). Some of the survivor cells start to transmigrate the vessel wall and encounter a second barrier. The brain vasculature presents a specialized structure called Blood Brain Barrier (BBB), characterized by tight junctions between endothelial cells sitting on top of a specific basement membrane that is in turn surrounded by brain defensive cells (pericytes and astrocytes). The BBB restricts trans and paracellular transport between the bloodstream and the brain milieu, ensuring that only convenient

compounds access the brain<sup>17</sup>. Experiments in living mice to which metastatic cells had been injected into the systemic circulation showed that, during transmigration, cells protrude into the brain parenchyma while retaining the opposite edge inside the vessel, thus creating a narrowing along the vessel wall (Fig.10B, *right*) (Kienast et al., 2010). Successful brain metastatic (BrM) cells take 1-3 days to cross the BBB, but transmigration can be observed up to 14 days after cell injection. Why the latter group of cells is not able to proliferate once in the brain is unknown, but one option is that so long transition times impose too much mechanically-induced DNA damage (Irianto et al., 2017b), compromising cell survival.

Once inside the brain, BrM cells adhere to, spread along and wrap around the abluminal surface of microvessels in a process called vascular co-option, resulting in metastatic outgrowth along brain vascular structures (Carbonell et al., 2009; Kienast et al., 2010; Valiente et al., 2014). Importantly, in experiments seeding BrM cells directly on brain slices, cells preferentially adhere and spread along vessels within 2h, whereas cells not adhering to vessels do not spread (Carbonell et al., 2009). The basement membrane around these vessels is rich in collagen and laminin, cell adhesion molecules that trigger cell-substrate force transmission, cell spreading and activation of proliferative pathways. Interfering with specific cell-matrix adhesion molecules expressed by BrM cells ( $\beta$ 1integrin, L1CAM) impaired cell spreading and metastatic outgrowth (Carbonell et al., 2009; Valiente et al., 2014).

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<sup>17</sup> Ironically, these properties impede the delivery of several chemotherapeutic agents to the brain.



**Figure 10. Volume changes during metastasis: invasion, blood-borne dissemination and extravasation.**

(A) Migration through a narrow pore (black arrowhead) involves volume regulation. (B) *Left*: Intravascular lodging deforms circulating cancer cells. *Right*: Cancer cell extravasating from a brain microvessel. (C) Brain metastatic outgrowth preferentially takes place at places of low cerebral blood flow (CBF). Adapted from (Follain et al., 2017; Furlow et al., 2015; Wolf et al., 2003)

In recent years, YAP/TAZ is under intense study as a mechanotransducing pathway correlated to cancer cell malignancy (Aragona et al., 2013; Cordenonsi et al., 2011; Dupont et al., 2011; Elosegui-Artola et al., 2016; Wada et al., 2011; Zanconato et al., 2015). These transcriptional co-activators accumulate inside the nucleus of cells grown on rigid substrata and promote a transcriptional program that enables oncogenic and metastatic growth. Recent data suggest that the main mechanism of nuclear YAP accumulation is nuclear flattening-induced nuclear pore opening by forces exerted by contractile Actin fibres connecting the nucleus to adhesions (Elosegui-Artola et al., 2017). The connection between mechanical inputs and actin-adhesion engagement has been introduced previously. The conservation of this mechanism in BrM cells remains unknown, but nuclear YAP accumulation is high in human brain metastatic samples and is associated with reduced prognosis (Kim et al., 2015). Additionally, the main up-regulated genes when comparing BrM to parental counterparts are YAP/TAZ signature target genes *CTGF* and several *SERPINS* (Dupont et al., 2011; Valiente et al., 2014). Lastly, Piezo1 activity and Myosin-dependent contractility in response to matrix rigidity promote nuclear YAP accumulation in neural stem cells and consequent differentiation towards the neuronal lineage (Pathak et al., 2014).

Besides the structural features (mechanical deformation by microvessels, BBB tightness and the necessity to co-opt vessels to proliferate), BrM cells must overcome brain defence mechanisms. Reactive astrocytes, that outnumber neurons – and certainly BrM cells- release plasminogen activator (PA), which converts the zymogen plasminogen into Plasmin, a protease with fatal consequences for invading cells. First, Plasmin

cleaves FasL at the plasma membrane of astrocytes into soluble FasL (sFasL), a death signal that kills BrM cells. Second, Plasmin sheds L1CAM from BrM cells, impairing cell spreading and, consequently, vascular co-option and metastatic proliferation (Valiente et al., 2014) (Fig.11).

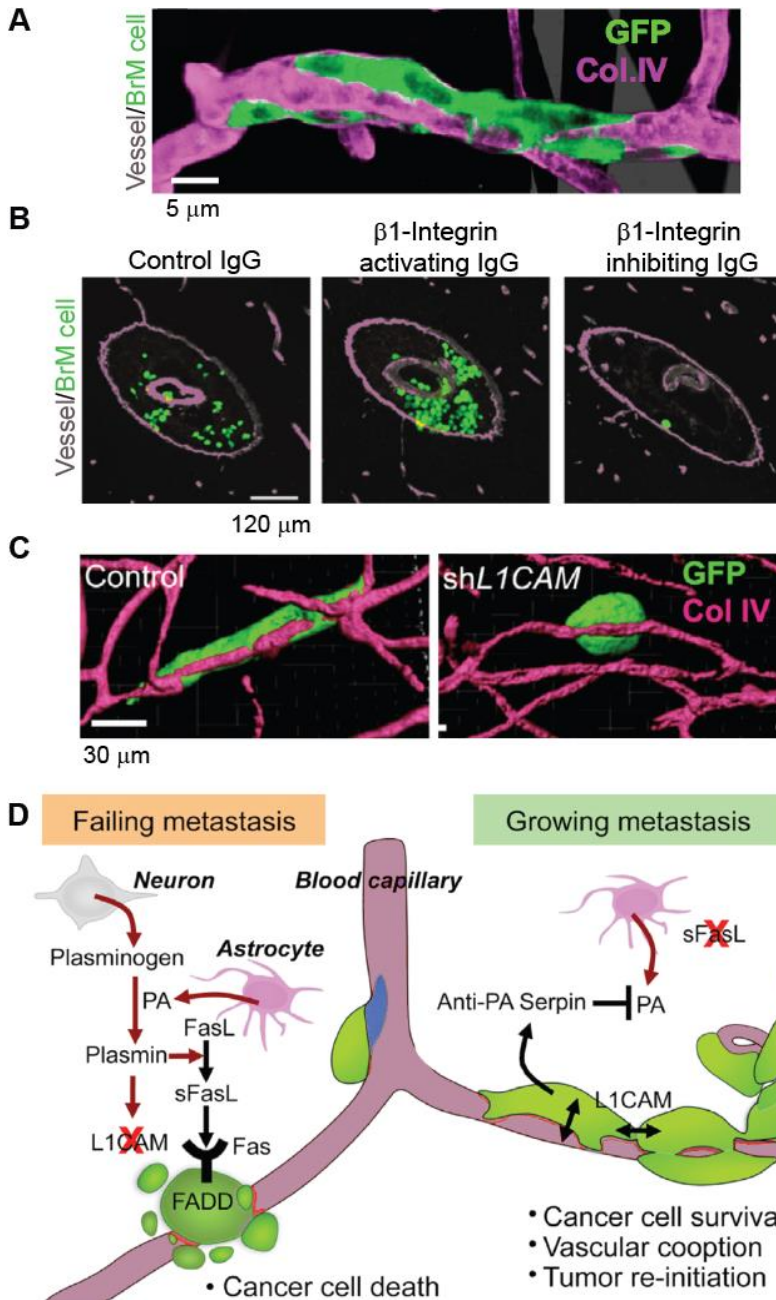
Despite the biological mechanisms protecting the brain from metastatic invasion, the colonization of the brain parenchyma by cancer cells is a final complication of up to 40% of metastatic cancers and it is associated with poor quality life due to neurological distress and reduced life expectancy (median < 1 year). Successful BrM cells bypass all defensive mechanisms and we are just starting to know how.

- Surviving inside the vessels

Inside blood vessels, cells are immersed in blood and exposed to contact with immune cells, oxidative stress or shear forces. Metabolic changes promote survival under oxidative conditions and mechanotransduction of shear forces can ultimately activate proliferative pathways via auto/paracrine ATP signaling. Interestingly, mechanically-induced, pannexin-mediated ATP release, which results essential for metastatic cancer cell survival (Furlow et al., 2015) is dependent on Rho activation after calcium entry through TRPV4 (Seminario-Vidal et al., 2011) or Piezo1 (Cinar et al., 2015; Wang et al., 2016). Moreover, as in the case of pore transmigration, volume regulation permits cell transit through narrow vessels. Therefore, MOS are likely essential for cancer cell intravascular survival by promoting cell survival in response to mechanical deformation and contributing to narrow passage negotiation.

- BBB transmigratory mechanisms

Comparative genome-wide expression analysis between BrM



**Figure 11. Cell-ECM adhesion, vascular co-option in brain metastasis.** (A) BrM cancer cell (green) spreading along a brain microvessel. This interaction is mediated by  $\beta$ 1-integrin (B) and L1CAM (C) and is necessary for tumour outgrowth. (D) SERPINS shield metastatic cells from brain parenchymal defence mechanisms. Adapted from (Carbonell et al., 2009; Valiente et al., 2014).

lung and breast cancer cells and their parental (non-metastatic) or metastatic, non-brain targeting counterparts followed by filtering by association to brain relapse identified COX2, ST6GALNAC5, and the EGFR ligand HBEGF as key mediators of BBB transmigration (Bos et al., 2009). According to the authors, HBEGF promoted cell motility, while COX2 increased BBB permeability and ST6GALNAC5 increased cell surface sialylation which in turn augmented cancer cell adhesion. Additional work identified collagen-targeting metalloproteases as key mediators of BBB disruption during metastatic transmigration, working in invadopodia-like structures (Leong et al., 2014; Wu et al., 2015).

These evidences suggest that BrM cell ability to penetrate the BBB depends on adhesive and junction-remodelling machineries, that are in turn modulated by ion channels like Piezo.

- Vascular co-option and SERPINS

BrM cells overcome plasmin lethal action by up-regulating and secreting PA-inhibiting Serine Protease Inhibitors (SERPINS) B2, E1, E2, and I1. These proteins prevent sFasL production and L1CAM shedding, shielding BrM cells and allowing their spreading along blood vessels, which enables metastatic proliferation (Valiente et al., 2014). What controls SERPIN up-regulation and secretion is currently unknown. SERPINI1 is normally expressed in neurons and is up-regulated, with beneficial effects, during processes of synaptic plasticity or damage (learning, stroke or seizures). SERPINI1 is directed to the regulated secretory pathway by a N-terminal sequence absent from SERPINE1 or SERPINB2. This suggests that different secretory mechanisms are active during BrM invasion. In general terms, secretion requires loaded vesicles to fuse with the plasma membrane to expel their content to the extracellular



space. Intermembrane fusion demands the transient dissolution of the actomyosin cortex lining the inner face of the plasma membrane and mechanical forces to fuse both membranes. These processes usually depend on protein conformational changes induced by calcium entry following membrane depolarization.

Recent data showing that mechanically-induced serotonin secretion by gastrointestinal enterochromaffin cells depends on Piezo2 activation (Wang et al., 2017) and MOS-dependent SERPINE1 secretion by lung cells (Henry et al., 2016) prompted us to investigate whether Piezo2 regulates SERPIN secretion enabling brain invasion.



## 2.Methods



### Cell line generation, culture, transfection, and treatments

CHO- $\alpha$ 4WT and CHO- $\alpha$ 4S988A cell lines were generated by stably transfecting CHO cells with pQN4G and pQN4S988AG plasmids, respectively, in which wild-type or mutant  $\alpha$ 4 integrin cDNA was tagged with GFP by inserting into a PGBI25-fN1 GFP vector. MDA-MB-231 BrM2 cells were kindly provided by Joan Massagué (Memorial Sloan Kettering Cancer Center, New York City). We transfected HEK293-T cells (ATCC) with pGIPZ lentiviral shRNA control and Piezo2-targeting plasmids (clone V3LHS-305314, Dharmacon) to obtain lentiviral particles. After collection, centrifugation, and titration, culture supernatants were used to infect BrM2 cells.

For transient transfection, we used:

Plasmid	Cell	Provider	Transfecting agent
PDE1 siRNA	CHO	Santa Cruz	Lipofectamine 2000 (Thermo)
	A375-SM	OriGene	
Piezo1 siRNA	CHO	Santa Cruz	
	A375-SM	Life Technology	
pRK5-Myc-RhoA-Q63L	MDA-MB-231-BrM2	Addgene #12964 (Gary Bokoch)	
pRK5-Myc-RhoA-T19N		Addgene #12963 (Gary Bokoch)	
RhoA-FRET sensor		M. Matsua (Osaka University)	
GFP-CA-mDia1		Addgene #45583 (Klaus Hahn & Ronen Zaidel-Bar)	

Table 1. Cell transfection reagents and plasmids.

Cells were maintained in an incubator at 37°C, 5% CO<sub>2</sub> using the following media:

Cell line	Medium (provider)	Supplements (Gibco)
CHO- $\alpha$ 4WT	Ham's F12 (Cellgro)	10% FBS 1 $\mu$ g/ml Pen/Strep
CHO	DMEM High Glucose (Life Technologies)	
CHO-B2	MEM (Invitrogen)	
MDA-MB-231-BrM2	DMEM High Glucose and Calcium-Free DMEM High Glucose (Life Technologies)	10% FBS 1 $\mu$ g/ml Pen/Strep 1x GlutaMAX 2 $\mu$ g/ml Puromycin

Table 2. Cell culture media and supplements.

When needed, we used the following compounds:

Objective	Compound	Concentration	Provider
PKA activation	Forskolin	50 $\mu$ M	Santa Cruz
PKA inhibition	Rp-cAMPs	50 $\mu$ M	
Piezo inhibition	GsMTx4	10 $\mu$ M	Abcam, Alomone
PDE1 inhibition	IBMX	100 $\mu$ M	Santa Cruz
	8MM-IBMX	100 $\mu$ M	Axxora
PDE4 inhibition	Rolipram	10 $\mu$ M	Cayman Chemical
PDE3 inhibition	Milrinone	1 $\mu$ M	Enzo Life Sciences
Actin depolymerization	Latrunculin	1 $\mu$ M	Santa Cruz
Actin polymerization	Jasplakinolide	500 nM	Tocris
Glass coating	Rat tail Collagen type I	1, 10, 50, 100 $\mu$ g/mL	Corning
Glass and hydrogel coating	Human Fibronectin	1, 10, 20, 50, 100 $\mu$ g/mL	Sigma

Table 3. Compounds used during pathway manipulation.

## Electrophysiology

Cells were seeded on collagen type I (10ug/mL for 1h at 37°C) coated plastic Petri dishes previously coated with rat tail. Before each experiment, culture medium was replaced with an isotonic solution containing 140 mM NaCl, 5 mM KCl, 1.2 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>, 5 mM glucose, and 10 mM Hepes, pH 7.4, at 300 mosmol/liter. The intracellular (pipette) solution contained 140 CsCl, 1 mM EGTA, 10 mM Hepes, 4 mM ATP, and 0.1 mM GTP (300 mosmoles/liter, pH 7.3). Whole-cell currents at -80mV were acquired at 10 kHz and low-pass-filtered at 1 kHz using an Axopatch200B amplifier. As a mechanical stimulator, we used a heat-polished glass pipette mounted on a piezo amplifier & position controller (E-665, Physik instrumente) synchronized with the recording program (pClamp10, Molecular Devices) to move the pipette in 0.5 μm increments. Inactivation percentage of mechanically activated whole-cell currents was measured at the end of the stimulus. All experiments were performed at ~23°C between 24-72 after seeding.

## Gene expression profiling of A375 melanoma cells

We employed the GEO2R platform to explore the expression levels of the channels of our interest in the GSE1845 dataset-accession number GSM29663, corresponding to control A375 cells.

## RT-qPCR

We used the NucleoSpin RNA kit (Macherey-Nagel) for RNA isolation, Superscript II (Thermo) for retrotranscription and SYBR Green PCR Master Mix (Thermo) for quantitative PCR. *β-actin* was used as an internal control for the quantification of gene expression. Real-time PCR was performed with SYBR

green PCR master mix (Applied Biosystems) in 7900 HT Sequence Detection System (Applied Biosystems). Relative mRNA abundance was calculated using  $\Delta\Delta\text{CT}$  method (in triplicates). Primers are available on request.

### **Immunostaining**

After medium washing with TBS, we fixed cells with 4% paraformaldehyde diluted in Cytoskeleton Buffer (CB; 10mM MES pH 6.1, 138mM KCl, 3mM  $\text{MgCl}_2$ , 2mM EGTA, 320mM sucrose) followed by permeabilization with 0.5% Triton X-100 in TBS for 10min. Next, we sequentially incubated primary and secondary antibodies (see Table 5) diluted in blocking solution (2% BSA in 0.1% Triton X-100 in TBS) in a humid chamber for 1h and DAPI for 10 minutes, all at room temperature. To visualize Actin filaments, we included Dylight 554-labelled phalloidin during secondary antibody incubation. Samples were mounted using Fluoromount-G (Thermo). We used 0.1% Triton X-100 in TBS as washing solution between steps.

### **Staining confocal imaging and analysis**

We imaged immunostaining samples using SP5 or SP8 Leica laser scanning confocal microscopes with 63x 1.40 immersion oil objectives. Z-stacks containing all the nuclear height were defined using the DAPI signal. Conditions remained constant during acquisition of data later to be compared.

Nuclear and cytoplasmic YAP quantification was performed on maximum intensity projections of DAPI and YAP image Z-stacks with CellProfiler (Carpenter et al., 2006). We used the DAPI image for nuclear region of interest (ROI) identification and the rest of the cell was considered cytoplasm. Mean intensity values of each ROI were used to calculate nuclear/cytoplasmic ratios.



Compound	Provider	Dilution or volume	Vehicle
PFA	VWR	$\frac{1}{4}$ from 16% stock	CB
Triton X-100	X100, Sigma	0.5%, 0.1%	TBS
Mouse anti-paxillin	610051, Millipore	1/200	Blocking
Mouse anti-YAP clone 63.7	sc101199, SCBT		
Dylight554-phalloidin	21834, Thermo		
Donkey anti mouse 647 IgG	A31571, Thermo	1/500	
Goat anti rabbit 647 IgG	A21244, Thermo		
DAPI	D1306, Thermo	1/1000	
Fluoromount-G	00-4958-02, Thermo	7 $\mu$ L	

Table 5. Tools used for immunostaining

For adhesion and F-actin analysis, we used FIJI (Schindelin et al., 2012). Images of both stainings were first bandpass filtered and then manually thresholded for binarization and automatic particle detection and description with *Analyze particles*. For adhesion orientation, first we subtracted  $\text{angle}_{\text{adhesion}} - \text{angle}_{\text{cell}}$  to obtain the deviation of the adhesion relative to the cell major axis. For comparing purposes, we normalized these angles from  $0^\circ$  to  $90^\circ$  using their cosine. For F-actin quantification, we added the areas of all detected particles and divided it by the cell area.

### Calcium, PKA and RhoA activity imaging

Cells transfected with biosensors, AKAR4-Kras, AKAR3-TA-Kras or Yellow Cameleon were washed 2X with Hanks' balanced salt solution buffer and maintained in the dark at RT.

Transfected cells were plated in microchannel devices. Unconfined cells (located outside the 3 $\mu$ m channels) and confined cells (inside 3 $\mu$ m channels) were imaged on a Zeiss Axiovert 200M microscope with a cooled charge-coupled device camera (MicroMAX BFT512, Roper Scientific, Trenton, NJ) controlled by METAFLUOR 6.2 software (Universal Imaging, Downingtown, PA). Dual cyan/yellow emission ratio imaging used a 420DF20 excitation filter, a 450DRLP dichroic mirror, and two emission filters [475DF40 for CFP and 535DF25 for YFP]. These filters were alternated by a filter-changer Lambda 10–2 (Sutter Instruments, Novato, CA). Exposure time was 50–500 ms, and images were taken every 10–30 s. Fluorescence images were background-corrected by subtracting the fluorescence intensity of background with no cells from the emission intensities of cells expressing fluorescent reporters. The ratios of yellow/cyan emissions were then calculated at different time points. Values were normalized by dividing with the average basal value before drug addition.

Cells transfected with a RhoA-FRET sensor were resuspended in phenol red-free medium and seeded on 10 $\mu$ g/mL collagen I-coated glass-bottomed 35mm dishes (Mattek) and allowed to adhere and spread for 1h in the incubator. Then, we used a SP5 laser scanning confocal microscope with a 40x immersion oil objective to collect CFP and FRET (YFP) emission images of the basal plane of cells while illuminating cells with the 458nm (CFP) laser. A recording chamber allowed environmental control at 37°C and 5%CO<sub>2</sub>. For image analysis, we followed a previously described strategy (Reffay et al., 2014): first, we generated a mask of the cell (cell-containing pixels =

1; background pixels = 0) and multiplied CFP and FRET images by it. Then, we divided the resulting acceptor (FRET) and donor (CFP) images.

### **Cortical flow analysis**

Cells seeded on fibronectin-coated hydrogels were imaged every 5 seconds for 2-5 minutes. We traced straight lines perpendicular to the advancing edge and generated kymographs using the Kymograph tool of FIJI. From kymographs, we fitted a line to observed cortical features and calculated speeds from the angle  $\alpha$  relative to the horizontal, according to  $\tan(\alpha)=\text{slope}=\text{dt}/\text{dx}$ .

### **Atomic force microscopy and stiffness measurement**

Force spectroscopy experiments were conducted using a Molecular Force Probe (MFP-1D; Asylum Research, Santa Barbara, CA). Using thermal oscillation method, a triangular cantilever (nominal spring constants of 10 pN/nm) was calibrated, with its deflection (degree of bending) measured by laser reflection onto a split photodetector. Cells were seeded on a glass slide patterned with 8 $\mu\text{m}$  fibronectin lines or a uniform fibronectin 2D surface. Cells were cultured in the appropriate serum-free medium solution in the presence of a chemical agent or its corresponding vehicle control. The cantilever height was adjusted such that each approach cycle generated a slight force ( $\sim 1\text{--}2$  nN) onto the cell surface before reproach. Reproach velocity was 25 mm/s, and the dwell time was set to 20 ms. For quantifying stiffness, the point of contact between the AFM tip and the cell surface was identified by a custom MATLAB program. The approaching curve (deflection as a function of indented position) was then fitted by Sned-

don/Hertz model for corresponding tip geometry. The Sneddon/Hertz model of indentation force was used to calculate the elastic modulus (i.e. the stiffness) of the cell.

### **Traction force microscopy**

Hydrogel manufacturing, cell seeding and image acquisition and analysis were performed as previously (Elosegui-Artola et al., 2014), but we functionalized hydrogels with Sulfo-SANPAH (Sigma) for later incubation with 100 $\mu$ g/mL Human Fibronectin (Sigma).

### The experiment

The basic set-up implies first, fabricating matrix-coated gels with embedded fluorescent microscopic beads and seeding cells on them. Polyacrylamide offers optimal properties for building these gels: it is unexpensive, transparent (excellent for imaging purposes), elastic (a requisite for the kind of analysis performed), safe and easy to manipulate in a standard biological laboratory, stable at the desired timescales and its stiffness is easily modulated by adjusting the acrylamide/bis-acrylamide ratio. Second, cells and beads are imaged for the desired amount of time (*preT* images), ideally in a timelapse microscopy set-up with automated stage and controlled atmosphere. Third, cells are de-attached (e.g. using trypsin) and forces dissipate causing beads return to their resting location. This offers a reference image (*postT*) for comparison.

The approach used in this thesis derives from Butler's Fourier Transform Traction Cytometry (FTTC) (Butler et al., 2002) and consists on building a displacement field from *preT* and *postT* images to then calculate a traction field in the Fourier Space, easing computational analysis. Images are first automatically corrected for translational shifts produced by stage

drift or sample manipulation. In our case, this is achieved using cross-correlational analysis of the high bead density *preT-postT* image pairs, that are translated one with respect to the other in order to match pixels with correlation maxima. Then, each image is divided in partially overlapping  $n \times n$  square windows called Interrogation Windows (IW). *preT* and *postT* IW pairs are compared also by cross-correlational analysis. The coordinates yielding the maximum of the cross-correlation function between images are set as the centre of the IW and the translation necessary for this determines the displacement vector. Repeating this procedure for all IW pairs renders a uniformly discretized displacement field, which enables the use of simple Fourier Transform algorithms that work well in uniform lattices like protein crystals. This method also offers the option to discard those IW with correlations lower than a threshold. Butler's approach offers two types of tractions. Unconstrained tractions are calculated for every pixel in the field of view. Constrained tractions are calculated after building a second traction field that sets cells outside a ROI to 0. The second mode is the one used in this thesis.

### **Fabrication of 1D Protein Micropatterns**

Standard lithography was used to create a silicon wafer with an array of features of prescribed dimensions (50  $\mu\text{m}$  wide, 5  $\mu\text{m}$  tall, and 20 mm long), separated from each other by 8  $\mu\text{m}$ . Replica molding was used to create a PDMS stamp bearing an array of 8  $\mu\text{m}$ -wide lines. Stamps were functionalized with 100  $\mu\text{g}/\text{mL}$  fibronectin (Sigma-Aldrich) in Dulbecco's Phosphate Buffered Saline (PBS) (Life Technologies). After 1 h at room temperature (RT), this solution was removed and the stamps were again dried under an air stream. Stamps were then inverted onto tissue culture dishes (Falcon) under steady

pressure. After 30 min, the stamps were removed and the dish was backfilled with 2.5% Bovine Serum Albumin (BSA; Sigma-Aldrich) in PBS to prevent cell adhesion outside of the fibronectin-patterned areas. After 1 h the BSA solution was removed and the stamps were rinsed 3X with PBS. 2D areas were created using a flat PDMS stamp.

### **Cell migration**

For confined migration experiments, the cell migration chamber was fabricated by standard multilayer photolithography as previously described (Balzer et al., 2012; Hung et al., 2013; Tong et al., 2012)(Hung et al., 2016; Tong et al., 2012). For the features of the first layer containing 3  $\mu\text{m}$  channels of 10  $\mu\text{m}$  height we spun-coated SU-8 3010 while for the secondary features containing 50  $\mu\text{m}$  height parallel channels for cell seeding we used SU-8 2025. Poly(dimethyl siloxane) (PDMS, Sylgard 184 kit, Dow Corning, Midland, MI, USA) devices were obtained by casting a mixture of PDMS prepolymer and curing agents (10:1) over the photoresist wafer mould which were then degassed and cured at 85°C for 1 h. The PDMS was cut, oxygen plasma treated, sealed to a coverslip and coated with 20  $\mu\text{g}/\text{mL}$  collagen type I (BD Biosciences, San Jose, CA, USA) for 1h at 37°C, as previously described (Stroka et al., 2014). BrM2 *shControl* and *shPiezo2* cells were harvested from tissue culture dishes, resuspended to a concentration of  $10^7$  cells/mL and added to the cell inlet well of the device ( $10^5$  cells/device). After cells were allowed to adhere, either one of the top right wells was filled with full medium, while the rest of the top right and lower right ones with plain DMEM in order to create a FBS gradient through the microchannels, or all the top right wells were filled with full medium containing 100nM bradykinin and the lower right with DMEM in order to create

a bradykinin gradient through the channels. The devices were moved to a stage-top live cell incubator with a controlled cell culture environment (5% CO<sub>2</sub>, 37°C, and relative humidity), mounted on a motorized stage of an inverted Eclipse Ti microscope (Nikon). Migration experiments were visualized with a DS-Fi1 camera head and a 10x objective. NIS-Elements was set to capture phase contrast images every 10 min for the duration of each live cell experiment.

We manually tracked every cell using the MtrackJ plug-in in ImageJ and calculated the cell speed and persistence based on a custom-made MATLAB code. Percentage of cell entry and entering time were manually calculated from the videos obtained. Cell entry time was defined from the time point the first cell protrusion entered the channel until the entire cell was migrating fully within the channel.

For planar, unconfined migration, glass-bottomed 12-well plates (P12G-0-14-F, MatTek) were treated with collagen I for 1h at 37°C. After three washings with PBS, we seeded 15,000 cells in 2mL of medium per well and allowed them to adhere for 2h in the incubator. Then, we placed the plate on an inverted microscope (Zeiss Cell Observer) equipped with an automated plate holder and controlled environment (humidity, 37°C, 5% CO<sub>2</sub>). We randomly selected 10 XY positions per cell line and matrix concentration and imaged them in the phase contrast and GFP channels every 20 minutes for up to 24h.

Cell trajectories were analyzed in a blind manner using the Trackmate platform (Tinevez et al., 2017) in Fiji (Schindelin et al., 2012). From these readouts, we calculated the mean average speed per cell line and matrix concentration.

### **Tracking cells embedded in 3D collagen I matrix**

A375-SM cells were embedded in 1mg/ml type-I collagen gels,. Briefly, 10000 cells suspended in 1:1 (v/v) ratio of cell culture medium and reconstitution buffer (0.2M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Sigma-Aldrich, St. Louis, MO), 0.26M sodium bicarbonate (NaHCO<sub>3</sub>) (Sigma-Aldrich), and water as solvent) were mixed with appropriate volume of soluble rat-tail collagen I (BD Biosciences, Franklin Lakes, NJ) to obtain the desired target collagen concentration. NaOH (1M) was added quickly and the final solution was mixed well to bring the pH to ~7. The cell suspension was added to a 24-well cell-culture dish and immediately transferred to an incubator maintained at 37°C to allow polymerization. This cell density was chosen so as to minimize cell collisions. Fresh medium was added after the collagen gel had solidified (approximately after 20 min of incubation). After 5 h of incubation, a Nikon TE2000 microscope with a phase contrast 10-X objective (Nikon, Melville, NY) was used to image the motility of living cells through a CCD camera (Hamamatsu, Hamamatsu, Japan). Images were collected every 5 min for > 8 h. For cells embedded in 3D collagen matrices, the focus plane was at least 200 μm away from the bottom of plates to diminish edge effects. Custom software made in MATLAB was used to track displacement of individual cells.

### **Matrix degradation assay and quantification**

Glass coverslips were prepared by sequential coating (1 mg/ml gelatin-Cy3, RT, 10min.), gelatin crosslinking (4% paraformaldehyde/0.5% glutaraldehyde, 4°C, 30 min.), washing (30 mg/ml NaBH<sub>4</sub> in PBS), sterilization (70% ethanol), and washing (PBS). Cells were grown on those glasses overnight



and then fixed and imaged with an Axiovert 200 M microscope using a MicroMax 5-MHz and LD plan 10× (NA 0.25) objective. For quantification, we counted the number of matrix-degradating spots and spot and cell areas using FIJI.

### **Proliferation**

We seeded 5000 per well in 24-well plates. 3, 5 and 7 days later, cells were counted with a Neubauer chamber after trypsinization and 1/2000 dilution.

### **SERPINB2 secretion**

We seeded 200000 cells per well in a 6-well plate and cultured them for 24h. 100  $\mu$ L of supernatant were placed on a nitrocellulose membrane with a 0.45  $\mu$ m pore size (Amersham Protran) and allowed to adsorb for 1h at room temperature. After aspiration of the remaining liquid, we blocked the membrane for 1h with 5% dry milk in 0.1% Tween-TBS (TTBS) followed by overnight incubation with an anti-SERPINB2 antibody (Ab47742, Abcam, 1/500 in blocking solution) at 4°C. After washing thrice with TTBS, we incubated the membrane with an anti-rabbit Horseradish Peroxidase-coupled IgG (GE Healthcare, 1/2000 in blocking solution) for 1h, and repeated the washing steps. Finally, a SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Scientific) was used for signal detection. For Western Blot, we lysed cells in 50mM Tris, pH 7.4, 150mM NaCl, 0.5 % NP-40 and protease inhibitors. We loaded 50  $\mu$ g of protein per well in a 8% polyacrylamide gel and transferred the results of the electrophoresis to a nitrocellulose membrane using an iBlot dry blotting system (Thermo). Blocking and antibody incubation steps were per-

formed as in the aforementioned case, but including a monoclonal anti-Tubulin antibody (T6074, Sigma) as loading control, and a 1/10000 secondary antibody for its detection.

### **Statistics**

All experiments (i.e. cell seeding, manipulation and data acquisition) were performed at least three times.

In all cases we performed a D'Agostino-Pearson omnibus normality test prior to any hypothesis contrast test. We used unpaired Student's t-test to compare 2 cases. Otherwise, we chose 1-way analysis of variance (ANOVA) followed by Tukey or Dunn post-hoc tests, where appropriate. All tests and graphs were done in Graphpad Prism 5 and later exported to Adobe Illustrator for figure design. Bars and error bars represent mean values  $\pm$  SEM and are reported along with p-values in each figure.

## 3.Results



## Chapter I: Confinement-sensing and Signal Optimization via Piezo1/PKA and Myosin II pathways.

Wei-Chien Hung, Jessica R. Yang, Christopher Yankaskas, Bin Sheng Wong, Pei-Hsun Wu, **Carlos Pardo-Pastor**, Meng-Jung Chiang, Zhizhan Gu, Denis Wirtz, Miguel A. Valverde, Joy T. Yang, Jin Zhang and Konstantinos Konstantopoulos

*Cell Reports*. **15**, 1430–1441 (2016).

DOI: [10.1016/j.celrep.2016.04.035](https://doi.org/10.1016/j.celrep.2016.04.035)

This work was highlighted as the issue cover with the following commentary:

*On the cover: Hung et al. describe two cooperating signalling modules, Piezo1/PKA and Myosin II, by which cells sense and traverse confined spaces. Signalling output is optimized through complex feedback loops ultimately leading to efficient cell motility. Artist Jun Cen (cenjun.com) depicts a small diver exploring confined migration, which is symbolized by the large size and tangled arms of the octopus trying to squeeze into the cave.*

Hung W-C, Yang JR, Yankaskas CL, Wong BS, Wu P-H, Pardo-Pastor C, et al. [Confinement Sensing and Signal Optimization via Piezo1/PKA and Myosin II Pathways](#). Cell Rep. 2016 May 17;15(7):1430–41. DOI: 10.1016/j.celrep.2016.04.035

## **Chapter II: Piezo2 regulates RhoA and Actin cytoskeleton to promote mechanobiological responses of cancer cells**

**Carlos Pardo-Pastor**, Fanny Rubio-Moscardo, Marina Vogel, Selma A. Serra, Sanela Mrkonjic, Olivier Destaing, Alexandros Afthinos, José M. Fernández-Fernández, Xavier Trepapat, Konstantinos Konstantopoulos, Corinne Albiges-Rizo, Miguel A. Valverde.

Submitted

Pardo-Pastor C, Rubio-Moscardo F, Vogel-González M, Serra SA, Afthinos A, Mrkonjic S, et al. [Piezo2 channel regulates RhoA and actin cytoskeleton to promote cell mechanobiological responses](#). Proc Natl Acad Sci. 2018 Feb 20;115(8):1925–30. DOI: 10.1073/pnas.1718177115

# 4. Discussion





#### 4.1 Piezo1, Myosin II, and confinement sensing

Many times, biology studies cells cultured on plates, but *in vivo*, cells frequently live confined in tissues. Although this fact has enabled the identification of important differences between cell behaviour (e.g. migration) in 2D and 3D, the underlying mechanisms detecting confinement remain elusive.

Combining microfabrication techniques and time-lapse microscopy on cells expressing a FRET-based calcium sensor, we have shown that confinement induces an elevation of intracellular calcium. After MA channel expression profiling for candidate selection, we provided two evidences indicating that this response requires Piezo1: calcium entry inhibition by the Piezo-targeting toxin GsMTx4 and by specific downregulation of this channel using siRNAs. Those same experiments revealed reduced migration velocities in confined Piezo1 knock-down cells but not in cells migrating unconfined. These cells also showed increased entry times to the confining channels. All this suggested a role for Piezo1-mediated calcium elevation in detecting physical confinement and adapting the cell migration machinery to this situation. Calcium increases upon confinement correlated with PKA activity decreases, recorded with an additional FRET-based sensor. The calcium chelator BAPTA and the PDE1 inhibitor IBMX reduced confinement-induced inhibition of PKA, but none of them abolished it. Therefore, confinement reduces PKA activity via this newly described Piezo-calcium-PDE1 pathway, paralleled by an additional, independent mechanism. Treating *siPiezo1* cells with blebbistatin abrogated PKA inhibition by confinement, demonstrating that this alternative pathway depends on Myosin II, which emerges as a direct mechanosensor.

Confining cells on 1D fibronectin-printed lines reproduced calcium elevations and PKA activity reductions and enabled direct cell manipulation. AFM on cells in this configuration showed increased cell stiffness in response to confinement. This response was reduced but not suppressed by individual inhibition of Myosin II, Piezo1 or PDE1 or by direct activation of PKA using forskolin. Conversely, inhibiting this kinase with Rp-cAMP exacerbated stiffness increases in response to 1D confinement. Importantly, these pharmacological manipulations altered stiffness even in unconfined cells, suggesting mechanisms working independently of microenvironmental dimensionality cues. As in the case of PKA activity, dual blocking of Piezo1 and Myosin II fully suppressed stiffness increases due to confinement. Importantly, latrunculin dropped cell stiffness in all conditions, demonstrating that this parameter depends on Actin polymerization.

Narrow channels and 1D adhesive lines both promote calcium increases and PKA inhibition, but the exact nature of the stimulus triggering these changes is not clearly identified in our work. We can hypothesize that both manipulations elongate the cells and this increases membrane tension, leading in parallel to Piezo1 activation and cortical actomyosin re-arrangement causing Myosin II activation, increasing contractility and cell stiffness. Using inhibitors, we have seen that Myosin II effects do not require Piezo1 activation. This indicates either direct mechanosensing by Myosin II or that it is activated by another yet-to-be-identified mechanosensor, but does not discard a Piezo1 contribution in Myosin II activation. I have already introduced that, although Piezo1 is a mechanosensor *per se*, integrin-mediated adhesions promote its activation (Gaub and Müller, 2017). Similarly, although it is expendable, calcium

entry through Piezo1 could contribute to Myosin II activation by confinement, either by Rho→ROCK→MLCK-dependent MLC phosphorylation, enhanced myofilament polymerization or still undescribed mechanisms.

We should also consider the dynamics of the calcium signal observed in these cells. Piezo channels show fast, transient (in the range of tenths to hundreds of milliseconds) responses to mechanical stimulation. Yet, the calcium increases we observed last much longer. Therefore, Piezo1 seems to be the confinement sensor triggering calcium increases that need to be maintained by additional mechanisms. Transient calcium entry through membrane channels can promote calcium exit from the intracellular stores in the Endoplasmic Reticulum (ER) in a process termed Calcium-Induced Calcium Release (CICR), involving ryanodine and IP<sub>3</sub> receptors (RyRs and IP<sub>3</sub>Rs, respectively). CICR in response to adhesion dynamics is involved in cell migration (Tsai et al., 2014; Wei et al., 2009) and thus it is a possible mechanism to explain the prolonged calcium increase found in confined cells.

To sum up, here we present a circuit where Piezo1 and Myosin II emerge as mechanosensors enabling cell responses to confinement, e.g. cell stiffening and adaption of locomotive strategies. Its nature as a MA ion channel and our data support a role for Piezo1 as a direct sensor of confinement and position this channel as an important regulator of several functions during relevant processes in biomedicine, such as cancer metastasis.

## 4.2 Piezo2, Rho, and the Actin cytoskeleton

In the second chapter of the thesis, we use quantitative microscopy on living and fixed transgenic cell lines to discover that

Piezo2 is essential for Rho-dependent Actin cytoskeleton organization and mechanical signalling of brain metastatic (BrM) MDA-MB-231 breast cancer cells.

Traction force microscopy revealed reduced *shPiezo2* cell-substrate force transmission. These forces are generated by Myosin II contraction of Actin filaments and transmitted to the substrate through adhesions. Western blots showing equal pMLC/MLC ratios, that take into account the portion of active (pMLC) among total (MLC) Myosin light chain protein, prompted us to discard reduced Myosin II activity as the cause of reduced traction forces in *shPiezo2* cells. This result is in accordance with our previous data showing that Piezo1 and Myosin II signal into common downstream pathways in parallel, and not in series.

Using phase contrast microscopy, we observed faster cortical flows in *shPiezo2* cells. These cortical flows depend on Actin polymerization and actomyosin contraction, and faster speeds in adherent cells correlate with lower traction forces, which can be explained by reduced engagement between flowing Actin and sessile adhesions (Elosegui-Artola et al., 2014, 2016; Maiuri et al., 2015). Therefore, the next step was to evaluate Actin and adhesive structures. To our surprise, stress fibres (SF) were almost absent in *shPiezo2* cells and in control cells grown in calcium-free, magnesium-supplemented medium<sup>18</sup>. These observations suggest that extracellular calcium entry through Piezo2 may be required for SF formation and down-

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<sup>18</sup> Extracellular divalent cation (e.g. calcium) depletion reduces integrin affinity, reducing cell-substrate adhesion. Magnesium supplementation avoids this confounding factor.

stream mechanotransduction in these cells. Since SF polymerization is under control of the RhoA→mDia1 formin axis, we focused our efforts on this pathway. FRET probes for RhoA activity showed reduced activation of this GTPase in *shPiezo2* cells, whose SF were rescued by promoting Actin polymerization by treatment with the drug jasplakinolide or by overexpressing constitutively active variants of the RhoA GTPase (RhoA-Q63L) or the mDia1 formin (CA-mDia1). These results suggest that Piezo2 is a mechanosensitive upstream regulator of the well-known RhoA→mDia1 pathway in charge of Actin polymerization into stress fibres.

RhoA activity increases in response to mechanical stimulation are broadly studied due to their role in several aspects of development, or cancer, mainly by regulating cytoskeletal dynamics. Nevertheless, how this small GTPase is activated is not well understood. The finding that Rho GTPases get activated in response to MA channel function was already proposed and is remarkable due to its consequences (He et al., 2017; Seminario-Vidal et al., 2011). First, cell extrusion in confluent epithelia is essential for maintaining epithelial functions and prevent tumour formation. Extrusion is a Rho-dependent process and is impaired after Piezo1 suppression (Eisenhoffer et al., 2012; Marinari et al., 2012). Second, MA-channel-dependent Rho signalling regulates ATP release, which is essential for lung epithelia signalling regulating mucus hydration and ciliary beating, both involved in defence mechanisms (Andrade et al., 2005; Arniges et al., 2004; Lorenzo et al., 2008; Seminario-Vidal et al., 2011). Third, in cancer cells, this auto and paracrine signalling mechanism promotes intravascular metastatic cell survival, and, in mesenchymal cells, it promotes chromatin condensation in response to stretching (Furlow et al., 2015;

Heo et al., 2015, 2016). This latter work identified calcium signalling and Piezo channels as required elements of this cascade with consequences during cell differentiation, but did not highlight it as one of their main discoveries. In my opinion, an ion channel promoting DNA compaction in response to mechanical perturbations is, at least, eyebrow lifting. Our results show that cells need Piezo $\rightarrow$ RhoA $\rightarrow$ mDia1 signalling to physically connect adhesions and the nucleus through SF. This link has been previously shown to regulate the physical properties of the nucleus and DNA compaction (Buxboim et al., 2014; Heo et al., 2016; Jain et al., 2013; Ramdas and Shivashankar, 2015; Ricci et al., 2015). Since DNA damage by physical inputs is a source of genomic instability (Irianto et al., 2017b), Piezo/Rho-dependent DNA compaction emerges as a hypothetical pathway that needs further study in cancer biology.

Considering the results in Chapter I regarding Piezo1 and PKA, we can anticipate how calcium-dependent PKA $\rightarrow$ Rac1 attenuation would relieve Rac1-dependent RhoA inhibition, constituting indeed a RhoA-activating pathway. A more direct effect exerted by calcium could be explained by its activating effect on PDZ-RhoGEF/ARHGEF11, which results in Rho-GDP exchange by GTP and activation of this GTPase. The same RhoGEF is also involved in RhoA activation in response to LPA, a stimulus known to promote intracellular calcium increases, contractility, and amoeboid motility (Ruprecht et al., 2015). Under those circumstances, LPA is known to activate its specific receptors, coupled to G proteins that specify its signalling outcomes (Ishii et al., 2004). Nevertheless, LPA activates also Piezo channels in artificial bilayers (Syeda et al., 2016), raising the potential existence of a LPA $\rightarrow$ Piezo $\rightarrow$ Calcium $\rightarrow$ PDZ-RhoGEF/ARHGEF11 $\rightarrow$ RhoA axis. PDZ-RhoGEF/ARHGEF11

gets phosphorylated and activated by Proline-rich tyrosine Kinase 2 (*PYK2/PTK2B*) in response to intracellular calcium increases. Knockdown of either protein impairs RhoA activation, indicating the need of both kinase and GEF to link calcium increases to RhoA activation (Ying et al., 2009). *PYK2* is rapidly activated by intracellular calcium increases in several cell lines, and it promotes tissue invasion. This kinase is related to FAK, and their functions are cross-regulated (Du et al., 2001; Kohno et al., 2008; Owen et al., 2007; Soni et al., 2017).

To summarize, we have identified Piezo2 as an upstream activator of RhoA→mDia1 signalling leading to SF formation. Calcium is known to modulate this pathway through specific proteins. Further work to unveil the potential connection between membrane proteins and the contractile module should include the study of LPA effects in cells depleted of LPA receptors, as well as co-localization studies of adhesion proteins -including FAK and *PYK2*- and Piezo channels.

Our results, together with many other previous studies, propose a picture where signalling molecules and adhesions could be integrating chemical and physical stimuli at the level of a master regulator of cell biology, RhoA; and from our biased view, a more interesting observation, that this master regulator is under the control of an ion channel that detect mechanical environmental cues. As an example, this GTPase and its target mDia1 also regulate mitosis and cytokinesis. Cell division is a key process during a cell's lifecycle and it has clear mechanical components. Adherent cells undergo a drastic change in shape, known as Mitotic Cell Rounding, to ensure proper room for DNA condensation and division. RhoA→mDia1 signalling ensures actomyosin-dependent force generation against external deformations. Curiously, mitotic cells swell, and this turgor



also contributes to robust rounding (Sorce et al., 2015; Stewart et al., 2011; Théry and Bornens, 2008; Zlotek-Zlotkiewicz et al., 2015). Rounded cells remain attached to the substrate through Actin-rich retraction fibres, which lead spindle orientation in an external force-dependent manner (Fink et al., 2011; Théry et al., 2005). The study of how forces regulate mitosis is growing, but so far, no attention has been paid to the role of ion channels. Piezo mechanosensitivity and its influence on the RhoA→mDia1 axis seems an appealing subject for this field.

### 4.3 Piezo, mechanotransduction, and cancer

Conditions that impair SF formation (e.g., Piezo2 knockdown or extracellular calcium removal) also reduced nuclear YAP accumulation. Conversely, jasplakinolide, RhoA-Q63L or CA-mDia1 rescued both SF and YAP accumulation. These results match previous observations and highlight the importance of Actin polymerization in mechanotransduction (Dupont et al., 2011). Also, our results place a MA channel as the trigger of a cascade regulating several steps of metastatic invasion.

#### - Cell and tissue mechanics regulating YAP/TAZ

Early discoveries claimed that SF effects on YAP activity relied on the inhibition of the Hippo/LATS phosphorylation pathway regulating specific residues in YAP (Sansores-Garcia et al., 2011; Wada et al., 2011). Regarding YAP/TAZ activation in tumorigenesis, Hippo inhibiting inputs seem negligible. This pathway is rarely altered in tumours, while YAP/TAZ is frequently activated (Harvey et al., 2013; Zanconato et al., 2016). In addition, inactivating components of the Hippo pathway does not guarantee transformation (Zanconato et al., 2016 and references therein). A common feature of YAP/TAZ-induced

tumour growth and malignancy is matrix stiffening, signalling through Rho/ROCK and filamentous Actin. This mode of activation is highly Hippo/Lats-insensitive and is also employed by external soluble cues such as LPA or sphingosine acting through GPCRs (Aragona et al., 2013; Dupont et al., 2011; Elosgui-Artola et al., 2016, 2017; Nowell et al., 2016; Yu et al., 2012). Matrix remodelling and stiffening is common in cancer and promotes malignancy, in part through YAP. Conversely, placing invasive cells on soft 3D matrices reduces their malignant traits and YAP signalling (Aragona et al., 2013; Kumar and Weaver, 2009; Levental et al., 2009; Miroshnikova et al., 2016; Paszek et al., 2005; Schedin and Keely, 2011).

Cancer Associated Fibroblasts (CAFs) perform matrix remodelling during invasion of some cancer types and their generation requires YAP signalling in response to matrix stiffening (Calvo et al., 2013; Labernadie et al., 2017). Importantly, other conditions exhibiting matrix remodelling and stiffening, e.g. pulmonary or hepatic fibrosis, show nuclear YAP activation in response to initial matrix stiffening and remodelling by fibroblasts. YAP activation in these cells promotes their differentiation into myofibroblast, increasing fibrosis (Liu et al., 2015a; Rahaman et al., 2014). Therefore, YAP signalling in non-transformed cells is involved in fibrosis during cancer and non-cancer disease, expanding the therapeutic applications of targeting YAP.

Existing evidences propose that matrix stiffening leads to increased Rho-dependent Actin polymerization and contractility. Since stress fibres form a continuum between the nucleus, adhesions, and the substrate, contractility-derived forces flatten the nucleus, and this deformation is what permits increased nuclear YAP accumulation (Das et al., 2016; Driscoll et

al., 2015; Dupont et al., 2011; Elosegui-Artola et al., 2016, 2017). Our results argue in their favour and expand the observation that Piezo1 inhibition or knockdown reduced nuclear YAP accumulation in human neural stem cells (Pathak et al., 2014). In addition, our work functionally links Piezo activity to Actin polymerization influencing YAP signalling in cancer cells, although we have not studied the Hippo-dependent phosphorylation status of YAP and thus we cannot discard a role for this modifications in the nucleo-cytoplasmic shuttling of this protein under these circumstances.

- YAP and tumour malignancy

YAP and TAZ are usually overlapping transcriptional co-regulators whose study has exploded in recent years<sup>19</sup>, most likely due to their remarkable role in development and, specially, tumorigenesis. For the sake of simplicity, I will only mention YAP, but its functions overlap with TAZ's. Frequent sources of brain metastasis, such as lung and breast adenocarcinomas or melanomas show YAP activation (nuclear accumulation in microscopy samples, target transcript up-regulation in transcriptomic analysis), whose extent directly correlates with poor prognosis. Experimental models of these tumours show that transgenic YAP activation worsens otherwise benign tumours. Conversely, YAP inactivation abrogates oncogene-induced transformation and reduces malignant traits of aggressive cells (Zanconato et al., 2016 and references therein). In addition, nuclear YAP accumulation in human brain metastatic breast cancer is associated with shorter patient survival (Kim et al., 2015). Altogether suggests that brain metastatic cancer

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<sup>19</sup> Annual entries (Pubmed): ~60 in 2006, ~300 in 2014, ~600 in 2016.

cells rely, at least in part, on YAP pathway activation, but which are the underlying mechanisms? In other words, which are the advantages conferred to BrM cells by YAP activation? There are no specific studies on which YAP targets fuel brain metastasis, but the enormous amount of similar evidences in other cells permits to build some hypotheses.

YAP immunoprecipitation followed by sequencing of bound DNA (ChIP-seq) in MDA-MB-231 cells revealed that these proteins associate with TEAD and AP-1 to interact with active enhancers at DNA and promote the transcription of genes involved in S-phase entry and mitosis, fuelling tumour growth by promoting proliferation, one of the main hallmarks of cancer (Zanconato et al., 2015).

AP-1 is a dimer formed by oncoproteins Fos and Jun in response to inflammatory cytokines or UV irradiation (Eferl and Wagner, 2003). Combining matrix stiffening and inflammation, YAP/TEAD/AP-1 seems a principal oncogenic pathway and could contribute to the observed connections between or UV exposition to tumorigenesis.

YAP also targets focal adhesion-related genes and its suppression reduces  $\alpha V$ ,  $\beta 1$  and  $\beta 3$  integrin, Vinculin or Zyxin protein levels and this impairs 2D and 3D migration, processes required during invasion (Liu et al., 2016; Nardone et al., 2017). In addition, YAP promotes another feature of cancer cells: evasion from several death signals, including detached cell anoikis<sup>20</sup> and several apoptotic pathways, e.g. Tumour Necrosis

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<sup>20</sup> Apoptosis in response to loss of attachment to the ECM. It is frequent in healthy cells and almost absent upon transformation.

Factor and Fas in liver tumours (Dong et al., 2007; Zhao et al., 2012).

Focusing on BrM cells, it is important to remember that Fas activation is the ultimate cause of their death in response to astrocyte activation and Plasmin production during brain invasion. Furthermore, some of the BrM signature genes, including those that are essential for BrM sheltering from Plasmin (*SERPINS*), are transcriptional targets of YAP (Dupont et al., 2011; Valiente et al., 2014). Therefore, YAP could be contributing to BrM biology by increasing proliferation, migration and death evasion rates. Heterologous BrM expression of YAP variants constitutively active or unable to bind DNA followed by gene expression profiling, SERPIN secretion or *in vivo* invasion studies would be useful to identify the specific roles of these proteins in brain metastasis. Performing those experiments also in Piezo1/2 knockdown backgrounds would permit us to discern which of the effects described here depend on the concomitant reduction in nuclear YAP accumulation.

We have found that *Piezo2* diminishes SERPINB2 secretion, but it did not reduce *SERPINB2* mRNA levels. Therefore, it is possible that, although we have seen reduced nuclear YAP accumulation in these cells, the remaining levels of transactivation are enough to maintain the transcription of this gene. Alternatively, under these conditions, *SERPINB2* transcription could be under control of other regulators.

So far, we have seen which YAP effects can contribute to tumorigenesis in general and I have proposed some hypothesis about specific effects on BrM cells. Now, it is also understandable to ask what underlies YAP activation in cancer cells and, again, which specific aspects of brain metastasis are relevant for it. Mechanical inputs are ubiquitous during the life of a cell,

even more in the case of a metastatic cell: these cells experience crowding and confinement during exit from the tumour, they get deformed during blood-borne dissemination, and, finally, they get lodged in microvessels and adhere to their walls. In the case of BrM cells,  $\beta$ 1-Integrins mediate cell-endothelium adhesion and blood flow forces promote BBB remodelling and BrM cell extravasation into the brain parenchyma, a densely packed tissue. Successful extravasated cells spread along the vessels during co-option and the resulting tumours grow constantly compressed by the surrounding, reactive tissue. Strikingly, adhering to and spreading along the abluminal face of vessels is absolutely essential to form metastatic lesions in the brain (Carbonell et al., 2009; Chen et al., 2016; Follain et al., 2017; Furlow et al., 2015; Kienast et al., 2010; Valiente et al., 2014; Zeidman, 1961). All these evidences show a determinant role for mechanical forces in the metastatic cascade resulting in brain metastasis. Our results expand this knowledge by showing that Piezo channels are required for detecting several of the aforementioned stimuli (confinement, matrix ligand density and stiffness), and that suppressing these channels impairs almost all these pro-metastatic functions (locomotive adaption to confinement, haptotaxis, matrix degradation, SERPIN secretion and Rho-driven Actin polymerization promoting YAP signalling and proliferation). Future experiments with *shPIEZO2* cells *in vivo* could lead us to identify which of these processes acts as a mechanical bottleneck during metastasis.

Brain metastases tissue mechanics is an unknown field, but studies of primary brain tumours show matrix stiffening by Tenascin C deposition and enhanced mechanosignalling positively correlating with malignancy (Miroshnikova et al., 2016). Metastatic breast cancer cells also secrete Tenascin C during

lung infiltration (Oskarsson et al., 2011). Therefore, Tenascin C seems to promote tumour aggressiveness by increasing tumour stromal stiffness and thus enhancing mechanosignalling. Answering whether this pathway is conserved in BrM cells, how Tenascin C is secreted, or how perivascular Tenascin C contribution regulates mechanotransduction or YAP signalling could identify novel regulatory mechanisms underlying BrM survival and outgrowth. The results could be applied to other cancer processes whose malignancy increased with Tenascin and also to non-tumoural processes in which this matrix-deposited glycoprotein exerts essential roles, e.g. neurogenesis or tissue repair (Midwood et al., 2016).

#### 4.4 Piezo, Actin, and rigidity sensing

Sensing tissue rigidity is necessary for cell function: cells differentiate depending on the stiffness of the underlying substrate, tumour malignancy positively correlates with the stiffness of its matrix, single and clustered cells migrate along rigidity gradients, etc. (Engler et al., 2006; Lo et al., 2000; Paszek et al., 2005; Sunyer et al., 2016). Integrin-matrix binding and Talin unfolding rates are key determinants of this mechanotransductive pathway (Elosegui-Artola et al., 2014, 2016; Sunyer et al., 2016), but Piezo channels are also involved: Piezo1-mediated calcium entry increases with matrix rigidity and this is necessary for stiffness-dependent nuclear YAP accumulation (Pathak et al., 2014). This work found that inhibiting Myosin reduced Piezo1 activity and YAP translocation, but did not study Actin. We propose that in MDA-MB-231 BrM2 cells, Piezo2 activity increases with substrate rigidity and this promotes RhoA $\rightarrow$ mDia1 mediated SF formation. The nucleus-Actin-adhesion-substrate continuum upon SF engagement to adhesions and the nucleus permits force transmission, leading

to nuclear flattening and YAP accumulation. At the same time, SFs properly engaged to adhesions transmit traction forces, enabling cell locomotion. When substrate rigidity is too low, neither Piezo2-mediated calcium entry nor Talin unfolding are favoured. Therefore, SF formation is inhibited, the nucleus is not flattened and YAP accumulation is reduced. Also, traction forces would be lower and this slows cell migration, what would explain durotaxis. Confirmation of this hypothesis will require quantifying rigidity-driven migrational biases of *shPiezo* cells grown on the presence of physiological and pathological stiffness gradients, as in (Sunyer et al., 2016; Vincent et al., 2013). With that experimental set-up we could also evaluate the interplay between stiffness-dependent calcium signalling, Actin polymerization and adhesion deposition.

#### 4.5 Piezo channels in metastatic invasion

Chemical and physical cues bias cell migration and this drives several patterns of tissue remodelling and invasion during development, healing or disease. Cancer cell migration guided by chemical signals, chemotaxis, has received much attention for many years. Recently, mechanical aspects of the cell surroundings have emerged as additional regulators of cell locomotion, likely because they end up altering the same cytoskeletal machinery (Artemenko et al., 2016).

As we have explained in the previous section, when combined with existing data, our work suggests a role for Piezo channels in responses to rigidity and thus durotaxis. More importantly, it also shows that Piezo channels regulate migration in response to ligand density, dimensionality or pore size.



- Haptotaxis

Piezo2 suppression abrogated haptotaxis, i.e. the biphasic relationship between cell migration speed and ECM protein concentration. Haptotaxis is common in development and cancer. Recent work has shown that increasing substrate rigidity and ECM ligand spacing promotes adhesion collapse due to excessive loading of integrin-ECM bonds (Oria et al., 2017). This scenario would be similar to our experiments with low ECM protein concentrations coating glass coverslips. Ligand spacing is reduced as ECM coating concentrations rise, and this optimizes traction force transmission and cell migration. Nevertheless, *shPiezo2* are not able to polymerize Actin connected to adhesions, impairing force transmission and forward movement. After this optimal point, the ECM is so dense that Myosin II power is not enough to properly disassemble adhesions, stalling migration. Under these circumstances, Myosin II activation rescues migration speeds (Gupton and Waterman, 2006). In our opinion, this manipulation would be ineffective in *shPiezo2* cells.

Actin polymerization places active, unbound integrins in the cell front during leading edge advance, optimizing adhesion formation during cell migration (Galbraith et al., 2007). This would also contribute to impaired adhesion turnover and cell migration in our cells. Also, it could help to explain the dispersed distribution of adhesions in *shPiezo2* cells: integrins are no longer placed at the cell front, where they bind matrix ligands and initiate cascades that result in cell polarization and directed migration.

Also, recalling the observation that integrin-mediated adhesions sensitize Piezo channels (Gaub and Müller, 2017), it is tempting to hypothesize that channel activation as a function

of ECM ligand density could be an additional mechanism driving haptotaxis.

We evaluated haptotaxis in cells migrating on glass, but this phenomenon is found also in 3D set-ups and *in vivo*, under conserved regulating pathways, and is involved in cancer cell guidance (Gopal et al., 2017; King et al., 2016; Moreno-Arotzena et al., 2015). Detailed studies showed that  $\beta$ 1-Integrin and Rac1-Actin signalling converging on Arp2/3-dependent dendritic Actin polymerization are essential for 2D and 3D haptotaxis. Importantly, both suppression and constant activation of Rac1 impair haptotaxis (King et al., 2016). According to previous knowledge and our own results, the reduction in RhoA signalling observed in *shPiezo2* cells should increase Rac1 signalling. This could be an additional factor impairing haptotaxis in these cells.

- 3D migration: propulsion, matrix degradation, a combination?

We found the Piezo1/PDE1/PKA confinement-sensing pathway conserved in invasive melanoma A375-SM cells. In this case, Piezo1, PDE1, PKA and Myosin II inhibition all suppress confined migration to equal extent. Therefore, seems that in these cells, Myosin II acts downstream of Piezo1, supporting the notion that MA channels influence contractility. This has important consequences for the mesenchymal-to-amoeboid transition, where low adhesion and increases in contractility trigger a drastic change in cell polarization that enables fast migration driven by a cortical flow that offers forward propulsion (Bergert et al., 2015; Liu et al., 2015b; Ruprecht et al., 2015). According to these models, cell confinement is necessary for amoeboid migration because otherwise the cortical flow cannot be coupled to the substrate, no friction appears and there

is no propulsive force. But, importantly, confinement itself can act as the trigger in contractility increases (Liu et al., 2015b). Our work describes Piezo1 as a confinement sensor triggering adaptive responses that include adhesion dismantling and final increases in Myosin II activity, i.e. contractility. Therefore, Piezo1 activation by confinement triggers changes associated to MAT. Considering all these evidences, it is plausible that Piezo1 acts as a first, unified transducer of chemical and mechanical stimuli leading to increased contractility during MAT. Future studies could evaluate whether Piezo1 or Piezo2 knock-down impairs LPA-dependent polarization and stable-bleb formation in Zebrafish progenitor cells as in (Ruprecht et al., 2015). It will be also important to study the effect of knocking down these channels in HeLa cells, which highly express them (Human Protein Atlas and our own data) and show clear MAT in response to confinement (Liu et al., 2015b).

Amoeboid migration is common in 3D environments (Friedl and Alexander, 2012; Wolf et al., 2003, 2013). Migration speed of A375-SM cells in a 3D collagen gel was reduced by PKA activation or by Piezo1 knockdown. These results highlight the importance of this newly-discovered axis for migration in pathophysiologically relevant confining environments. Having said that, we have not identified yet the ultimate mechanism causing reduced migration velocity in these circumstances. We must take into account that we have also found that Piezo2 interference reduces matrix degradation by MDA-MB-231-BrM2 cells. Additionally, reducing matrix degradation with protease inhibitors reduces 3D migration speed because smaller pore sizes oppose nuclear translocation (Wolf et al., 2013). Therefore, it is legit to ask whether reduced migration in 3D collagen

gels by siPiezo1 is due to impaired propulsive mechanisms, reduced matrix degradation or a combination. 3D migration slowing by PKA activation does not respond this question, because PKA-mediated Rho inhibition could affect both propulsion, by impairing amoeboid migration, and degradation, by precluding invadopodia formation. Other PKA-mediated mechanisms such as indirect MLCK inhibition, reducing contractility (Howe, 2004), could also participate. Experiments in 3D set-ups or, even better, *in vivo* with cells knockdown for Piezo channels would offer us the opportunity to evaluate more properly how these channels participate in this process, including the relevance of matrix degradation or nuclear deformation.

There is the concern of using different cell lines to tackle mechanobiological properties of cells, although signalling mechanisms are frequently conserved. More open questions rise from our matrix-degradation experiments, which are based on 2D gelatin substrata. Our results clearly show reduced gelatinase activity, while during our 3D migration assays, A375-SM cells rely on 3D collagenase activities. Under those circumstances, gelatinase activities are dispensable (Sabeh et al., 2004). Yet, those same experiments offer responses. In parallel to reduced gelatin degradation, *shPiezo2* cells showed nearly-abolished invadopodia formation, as quantified by Actin staining. Since these structures are rich in Actin, independently of their targeted matrix protein, we think that the collagen-degrading capacity of *shPiezo2* cells should also be impaired.

Together, we propose a model in which physical barriers opposing cell advance locally activate Piezo channels. This would localize matrix degradation to increase the pore size in order

to overcome the barrier, similar to a boring machine or a mole. Once the pore is large enough, Piezo signalling should decrease because now there is less resistance to cell advance and thus invadopodia could be dismantled. Sustained Piezo signalling by the matrix opposing cell advance could increase contractility to levels triggering the MAT, as observed when pericellular proteolysis is inhibited (Wolf et al., 2003). Dynamic volume regulation would also permit easier transit through pores. Our results show increased entry times of Piezo1 and Piezo2 knockdown cells into confining channels. Taking into account the role described for this channel in volume regulation, a possible explanation is that siPiezo1 cells are less efficient in reducing their volume, and this hinders the transit through confining cross-sections.

# 5. Conclusions



- Cell confinement activates Piezo1-mediated calcium entry.
- Calcium entering through Piezo1 activates PDE1-dependent cAMP degradation and subsequent PKA → Rac1 inhibition.
- Activation of the aforementioned circuit results in Myosin II inhibition release, allowing confined migration.
- Confinement activates Myosin II in parallel by mechanisms yet-to-be described.
- Suppressing Piezo1 function impairs motility of epithelial and cancer cells under several modes of confinement.
- Piezo2 is necessary for RhoA function in brain metastatic breast cancer cells.
- Piezo2 knockdown suppresses stress fibre formation and, consequently, force transmission.
- Reduced force transmission in Piezo2 knockdown abrogates mechanotransduction and reduces migration efficiency.
- Constitutively active mutants of RhoA or mDia1 rescue stress fibre formation and mechanotransduction.
- Piezo2 knockdown suppresses pro-metastatic features such as matrix degradation or SERPINB2 secretion, likely through its effects on Actin dynamics.





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