UNIVERSITÀ DEGLI STUDI DI TRIESTE

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Regulation and function of tumor suppressor DAB2IP in the cellular response to mechanical inputs

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ABSTRACT

The crosstalk between tumor cells and their microenvironment is a major determinant of cancer progression. Thus, understanding and eventually interfering with the dynamic interplay between external cues and cellular responses may lead to more efficient chemotherapeutic approaches. The tumor suppressor DAB2IP encodes a RasGAP and cytoplasmic adaptor that controls specificity, amplitude, and duration of intracellular signaling events in response to multiple extracellular inputs such as inflammatory cytokines, growth factors and hormones. Accordingly, loss of DAB2IP function amplifies multiple oncogenic pathways and fosters cancer aggressiveness.

In addition to secreted factors, also mechanical forces from the extracellular environment significantly affect tumorigenesis, metastasis and chemoresistance; here I asked if DAB2IP may contribute to sensing and/or reacting to mechanical inputs, and if loss of function of DAB2IP would alter the response of cancer cells to mechanical cues.

Using tumoral and normal mammary cell lines, I found that DAB2IP protein levels are reduced when cells are grown on a soft surface, suggesting that ECM rigidity positively affects its expression. I also observed that cell attachment sustains DAB2IP expression – possibly via Focal Adhesions (FAs). Similarly, I found that DAB2IP is downregulated in low density cultures, while upregulated at confluency, indicating that cell-cell contact controls DAB2IP levels - possibly via Adherens Junctions (AJs). Finally, I verified that integrity and contractility of the actin cytoskeleton also impact on DAB2IP protein levels.

Inspired by this evidence, I asked whether DAB2IP may in turn modulate the cell's mechanical properties and/or the cell's response to mechanical inputs. Preliminary experiments suggest that DAB2IP depleted cells are more resistant to density-induced shrinkage and have larger nuclei when grown to confluency. Under the same conditions, I found that DAB2IP knockdown cells exhibit enhanced activation of YAP, a key transcriptional effector of mechanical inputs and powerful oncogene. Together, these observations suggest that DAB2IP might contribute to restrain YAP activity under conditions of contact-inhibition.

The data collected in this Thesis provide the basis for further studies aimed to verify the above hypothesis, and its possible mechanism.

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INTRODUCTION

Mechanical signaling in epithelial cells

Structure and function of epithelia

Tissues are complex structures where aggregates of cells with functional similarities are caged into extracellular matrix (ECM). Epithelial tissues are a continuous sheet of tightly linked cells anchored on the basal membrane (BM); the mechanical support of cells polarization. Upon the stimuli derived from basal membrane and from neighbors, epithelial cells polarize along the apico-basal axis (Hinck & Näthke, 2014). This results in a cell spatial definition: the basal membrane separates epithelial cells from the underlying connective tissue while the apical membrane lines the external surface of organ and lumen of epithelial tubes (Coopman & Djiane, 2016). Consequently, the architecture of epithelium reflects its physiology role. At first epithelial cells acts as a physical barrier between the internal and external environments. In addition, epithelial cells control exchange of macromolecules, nutrients and metabolites trough the apical portion in contact with extracellular fluids (Coopman & Djiane, 2016). Finally, epithelia also constitute most of the tissue in glands, where specialized cells secrete fluids (Hinck & Näthke, 2014).

Cell architecture is dictated by the organization of cytoskeleton that lead cell shape and cell organelles localization (Ong et al., 2020). Compared to microtubules and intermediate filaments, the microfilaments are fundamental in determine cell shape and polarity. Microfilaments, filamentous polymer of actin (F-actin), form a cell cortex that underling the plasma membrane. Cell shape as well as adhesion, motility, exocytosis and endocytosis, derive from a dynamic and tightly regulated polymerization and depolymerization of the globular actin (G-actin) (Rao & Li, 2005). Furthermore, microfilaments are tensile structure able to stretch and relax in response to extracellular stimuli. This tensional property is intrinsic of actomyosin bundle structures. In details, the actin filaments are cross-linked by α-actinin but they also interact and operate with myosin motor proteins. Through the ATP hydrolysis, Myosin II pulls on actin filaments resulting into sliding motion of the bundles. Thus, Myosin II converts metabolic energy into tensile force with consequent effect of actin filaments contraction and cell shape modification (Tojkander et al., 2012).

The actomyosin properties make epithelial tissue a tensile structure. Here, the robustness of actin filament results in rigid structure that acts a physical barrier between different compartments. However, the flexibility of actomyosin governs the cell's tensional homeostasis in response to extracellular mechanical stimuli. Mechanical stimuli from the neighbor cells and ECM are principally sensed through the Adherens Junctions (AJs) and Focal Adhesion (FAs) (Yap et al., 2018) (Kechagia et al., 2019). Located in the cell membrane, these structures link the cell inner cytoskeleton with the cytoskeleton of neighboring cells (AJs) and with components of the ECM (FAs).

In response to external stimuli perceived by AJs and FAs, cells rearrange the structure and the contraction of actin filaments with the aim to better adapt its morphology and functions to the perturbed environment. Thus, every tissue builds an own distinctive architecture, which is the result of equilibrium between cell extrinsic and intrinsic forces. Extrinsic forces are generated by the rigidity and topology of ECM (by cell-ECM contact) and by the geometry and shape of the cells (by cell-cell contact). The internal forces are dictated by tension and organization of the cytoskeleton in response to external stimuli (Humphrey et al., 2014). In response to external forces the actomyosin cytoskeleton also regulates cell fate and behavior through a process referred to as mechanotransduction; in this context, the tensional state of cells translates the external inputs into adaptive gene transcription that mediate biological responses.

External forces are translated by cells via contraction of actomyosin cytoskeleton, however different biological responses result from the signal transduction of specific AJ and FA related actomyosin structure. Indeed, the microfilament cell cortex is composed by several structures distinguishable from their point of origin. At level of cell-cell junctions (AJs), microfilaments organize into a cortical actin ring below the apical plasma membrane. This structure, referred to as circumferential actin belt, is the major determinant in cell shape, polarity, and integrity of epithelial cell layers (Yap et al., 2018). At the level of cell-ECM contact (FAs), actomyosin cytoskeleton takes form into actin filament bundles that allow cells to brace against the underlying basal membrane. These filaments, called stress fibers, arise from the FAs and govern cell adhesion and migration. Currently, stress fibers are the cytoskeleton components better characterized for signal transduction of external mechanical inputs (Kechagia et al., 2019).

The biological responses upon mechanical inputs depend on the cytoskeleton structure that perceive and resolve the insult. For example, forces perceived at cell-ECM contact by FAs promote cell proliferation, while forces perceived at cell-cell adhesion by AJs have a growth inhibitory effect (Hirata et al., 2017) (Furukawa et al., 2017). However, stress fibers and circumferential actin are interconnected, resulting into a unique contractile apparatus; thus, in a more complex way, the biological response to mechanical inputs is the result of equilibrium among different components of cytoskeleton.

Cell adhesion complexes

Focal Adhesion and Adherens Junctions are multifunctional structures that allow cells adhesion with the surrounding environment. In particular, the transmembrane receptors Integrins and cadherins associate the inner cytoskeleton respectively with the fibrous component of the ECM and the cytoskeleton of adjacent cells. In addition to the transmembrane component, cell adhesion complexes are characterized by more than 150 proteins with a spatial distribution between the plasma membrane and the actin cytoskeleton, that can be divided in three different functional modules (Bachir et al., 2017):

The *actin linkage module* allows the physical interaction of inner cytoskeleton with extracellular environment. Molecular components depend on Integrin and E-cadherin receptors and reflect the stage of adhesion to ECM or neighbor cells.

- In Cell-ECM adhesion, talin and/or α-actinin leads to integrin association with actin. These bind to vinculin that is directly associated with actin cytoskeleton.
- In Cell-cell adhesion, the junction between E-cadherin and actin is mediated by α-catenin. Cooperation with vinculin and α-actinin reinforces the binding to cytoskeleton

Vinculin, an acting binding protein recruited both at AJs an FAs, is responsive to junctional stability and transmission of external force through both cell-cell and cell-ECM contact. In details, upon tensional stimuli at cell junctions, vinculin allows several actin linkage proteins to associate with the actin cytoskeleton.

The *actin regulatory module* governs the organization and dynamicity of actin to establish intact adhesion. The most relevant component is the complex, Arp2/3 complex (Actin Related Protein 2/3), a vinculin interacting protein that induces the assembly of branched actin arrays both in cell-cell and cell-ECM adhesion. Other protein such as Formin, Dia1 and ENA/Mena/VASP promote formation, elongation, and stabilization of linear actin bundles at FAs and AJs.

The *signaling module* triggers intracellular pathways related to actomyosin contractility and function of cell adhesion complexes. Component of this module are kinases, phosphatases, and small GTPases that impinging on the actin regulatory protein elicit polymerization and contractility of cell adhesions.

- At cell-ECM adhesion, the FAK-paxillin signaling regulate nucleation, polymerization and actin organization co-operated by Src, Abl, PI3K and Rho family GTPases.
- At cell-cell adhesion, components of signaling module are less defined. However, SRC, PI3K and Rho GTPases (Rac1, RhoA, Cdc42) are known to modulate the myosin II-dependent actin contractility.

Focal Adhesions

Epithelial cells adhere to the basal lamina via the Focal Adhesion. Integrin, the transmembrane receptor of FAs, links the components of basal lamina outside the cell to the inner actomyosin cytoskeleton. The basal lamina is a thin, tough and flexible layer of extracellular matrix that provide a mechanic support to the overhead epithelium. The contact of cells with the basal lamina is essential to epithelial cell survival; indeed, if lamina-anchoring is lost, cells undergo apoptosis. In addition to this, polarity, morphology, differentiation and migration of cells are also affected by fluctuation in the rigidity and composition of basal lamina (Hinck & Näthke, 2014).

The extracellular matrix (ECM) is a non-cellular structure principally composed by polysaccharides, such as GAG (glycosaminoglycans) which retains water. In addition to these, ECM is composed also by several kinds of fibrous proteins such as collagen, fibronectin, elastin and laminin. These proteins confer physical properties of tensile strength, resilience and elasticity to the ECM. However, the cell-secreted ECM is

characterized by a dynamic molecular network where the ratio among its two main constituents, proteoglycans and fibrous proteins, can vary, thus remodeling the tissue's physical property (Frantz et al., 2010). Notably, the dynamic structure of ECM has a fundamental role in the regulation of cell's anchored to it. Indeed, changes in the physical properties of ECM (strength and elasticity) govern cell fate by altering cell's biochemical and biomechanical process(Frantz et al., 2010).

Among the integrin adhesion complexes (IAC), the best characterized are the Focal Adhesions. Focal Adhesions have elongated and oval shape with size of 2-5um and are principally located at the periphery of cells (Geiger et al., 2001). In addition to stress fibers that link two opposite FAs, other integrin-related filaments anchor the nucleus through the opposite end of strand, allowing a direct mechanical regulation of the nucleus by external forces (Kirby & Lammerding, 2018).

Integrins are heterodimeric receptors composed by two noncovalently associated glycoprotein, called α and β subunit; both characterized by a single pass transmembrane helix. Since there are many isoforms for the α and β subunit, a plethora of Integrin receptor (24 integrin receptors) are possible (Hynes, 2004). The extracellular domain of integrins binds different ligands in the ECM, depending on the heterodimeric structure. For example, the most relevant α5β1 integrin receptor specifically binds the RGD (arginine, glicine and aspartate) motif of fibronectin and vitronectin (Takagi et al., 2003). In the cytoplasm, the short C-terminal tail of integrin interacts with complexes of many different proteins, resulting in the formation and regulation of actin filament binding (Geiger et al., 2001). In particular, the intracellular adaptor protein Talin mediates the linkage of β subunit of integrin to the actomyosin filaments; moreover Paxillin recruits and anchors host proteins such as RAC1 Arp2/3, and FAK that rearrange the actin structure (Bachir et al., 2017) (Fig. A).

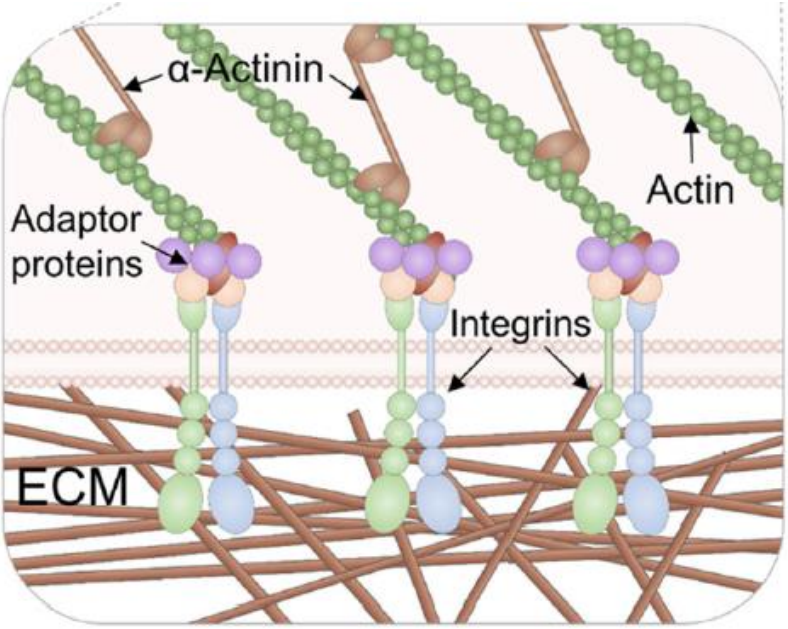


Figure A. Schematic representation of interaction among ECM, Integrins, adaptor proteins and actin cytoskeleton in Focal Adhesions (Saffioti et al., 2020)

In response to changes in the status of ECM binding, integrins activate many downstream signaling such as FAK, SRC AKT and ERK, and sustain the regulation of small GTPases of the RHO family (Hamidi & Ivaska, 2018). These molecular pathways regulate the formation and activation of stress fibers. Although actin filaments are softer than microtubules, high concentrations of parallel stress fibers generate organized rigid structures (Fletcher & Mullins, 2010) that increase and are proportional to cell stiffness (Rajagopal et al., 2018). Thus, maturation of FAs results in formation and contraction of stress fibers, while the stretching of stress fibers stabilizes FAs. What is the starting point of this positive feed-back loop remains to be defined (Kechagia et al., 2019). Regardless the origin of FAs signaling, ECM supports adhesion of cells and elicits their survival. In addition, cytoskeleton rearrangement by FAs also impinge on cell polarity, tissue integrity, intracellular transport and, obviously, cell migration (Horton et al., 2015).

The role of FAs and cytoskeleton remodeling upon alteration of ECM rigidity, despite being mainly studied in tissue culture models, is also relevant in vivo, affecting cell adhesion, migration, tissue remodeling and cell differentiation. Technical progress in tissue culture demonstrated that culturing cells on a matrix adhesion in 2D, can recapitulate the more physiological 3D condition of tissues. Since the formation of focal adhesions requires ECM rigidity, when cells are grown in 2D or 3D on a soft gel matrix, FAs are unstable and the phenotypes triggered by tension of adhesion are dampened,

recapitulating what occurs in vivo. For instance, FAs mediated cell migration is rare in 3D cultures, where more frequently cells undergo ameboid migration. Ameboid cells adopt a propulsive mode based on the protrusion of cortical actin in a manner that is independent of adhesion to the substratum. In contrast, cells cultured on a stiff ECM acquire a typical mesenchymal migration resulting on integrin–based cell adhesion and cytoskeleton contractility (Harunaga & Yamada, 2011).

Adherens Junctions

Cell-cell junctions link neighboring cells together and result into a structural and functional continuum among cells in tissues (Garcia et al., 2017). Cell junctions preserve tissue homeostasis by regulating the integrity of tissues, the diffusion of ions and other solutes across the tissue, and controlling cell proliferation and migration (Garcia et al., 2017). These functions are strictly mediated by cadherins that reinforce cell-cell contacts via anchoring junctions (e.g. adherens junctions, desmosomes). Differently to connective, muscular, and neuronal tissues, epithelia have peculiar and well-recognizable cell junctional structures. However, cell-cell junctions are not exclusive of epithelial cells; indeed, also fibroblasts and neuronal cells are linked together via cadherin-adhesion systems.

Adherens junction (AJs) are one of the most important and ubiquitous type of cell-cell adhesion complexes (Gumbiner, 1996). AJs are anchoring sites for actin filaments, connecting the actin cytoskeleton of adjacent cells. The core components of AJs are cadherins, that mediate Ca2+- dependent cell adhesion by their intercellular homophilic binding. These molecules are transmembrane proteins: the extracellular region is composed of five repetitions of the extracellular cadherin domain and is involved in the homophilic binding; the cytoplasmic tail is responsible for actin filament anchoring. To make the adhesion effective, AJs are formed by cadherin clusters (Harris & Tepass, 2010).

Among cadherins, E-cadherin is expressed in most epithelial tissues, where it helps cells to form a tight and polarized layer. The interaction between E-cadherin and actin filaments is mediated by various catenins associated with its cytoplasmic domain. In particular, α-catenin is the key mediator that physically links E-cadherin to the actin cytoskeleton, since it can bind actin filaments directly or via interaction with actin-

binding proteins such as vinculin. α-catenin interaction with E-cadherin occurs at the distal part of the cytoplasmic tail through β-catenin binding. In contrast, the more proximal region of E-cadherin cytoplasmic domain interacts with p120 catenin, that regulates cadherin stability and function (Gumbiner, 2005) (Fig. B)

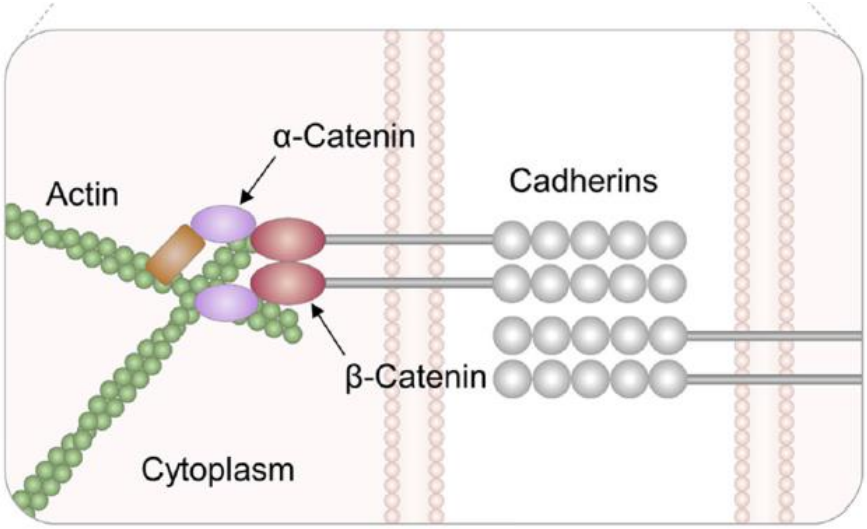


Figure B. Schematic representation of interaction among E-cadherin, catenins and actin cytoskeleton in Adherens Junctions (Saffioti et al., 2020).

Importantly, catenins associated to the cytoplasmic tail of E-cadherin entail regulation of the growth inhibitory Hippo pathway but also mediate downstream effects on signaling pathways such as Wnt/β-catenin, TGF-β, NF-κB (Mendonsa et al., 2018). Relevant, E-cadherins can transmit anti-growth signals by sequestering β-catenin, therefore avoiding its entrance in the nucleus and the transcription of several genes regulating cell cycle progression (Padua & Massagué, 2009).

In addition to modulation of cell signaling, AJs organize the actin cytoskeleton, which in turn supports AJ formation and stability (Harris & Tepass, 2010). In epithelia, actin filaments connect all AJs, forming an actin ring. This structure, called circumferential actin belt, is a contractile ring beneath the apical face of cell (Juankun Zhang et al., 2005). It controls intercellular surface tension and cell shape within epithelial monolayers, acting as a direct linker between neighboring cells (Furukawa et al., 2017). Consistently, when cells are tightly packed in a monolayer, with reduction of their basal area, they have lower stiffness at their center and higher stiffness at the cell border, as a result of reduction of stress fibers and formation of circumferential actin belt (Nehls et al., 2019). Mechanical inputs from neighbor cells are sensed by AJs that via the contraction of the actin belt

actomyosin lead to a tensional response and activation of intracellular signaling cascades (Yap et al., 2018). Adhesive forces of cell-cell junctions must be strong, in order to preserve tissue structure under external stresses, and at the same time dynamic, to allow formation of new cell contacts during development, tissue renewal, and wound repair (Harris & Tepass, 2010). Despite much knowledge on the protein components involved in AJs, regulation of signals deriving from cell-cell contact and their biological consequences on cell behavior remain poorly understood. Above all, the phenomenon of growth inhibition by forces generated by cell-cell adhesion is controversial. Indeed, there are contradictory evidences in the scientific literature. For instance, some evidences indicate that contraction of the circumferential actin belt sustains contact inhibition of cell proliferation (Furukawa et al., 2017), while other evidences indicate that growth inhibition by cell-cell contact is initiated by force-independent signaling that indirectly reduce the available space to establish cell-ECM junctions, resulting in reduced adhesion forces (Goodwin et al., 2017).

Plasticity of cell junctions

FAs and AJs show a remarkable plasticity since they are continually formed and disassembled. This ability is crucial for preservation of epithelial integrity under the pressure of constant changes in cell packing and ECM stiffness that occur during the physiological processes of cell division and cell death, essential for development and tissue homeostasis (Baum & Georgiou, 2011).

The dynamic regulation of FAs and AJs is ensured by the continuous turnover of Integrins and E-cadherins, that undergoes cycles of endocytosis, sorting, and recycling back to the plasma membrane (Brüser & Bogdan, 2017) (Kechagia et al., 2019). These receptors can be internalized through different endocytic pathways, depending on the cellular context. The most studied is clathrin-mediated endocytosis, that allows spatially controlled internalization (Baum & Georgiou, 2011). Clathrin does not bind directly E-cadherin/integrin, so endocytic adaptors, such as AP2 and DAB2, are needed to mediate internalization (Brüser & Bogdan, 2017) (De Franceschi et al., 2016).

Notably, the internalization of clathrin-coated vesicles is driven by cytoskeleton activity; in fact, the polymerization of actin filament at the rim of endocytic pits sustains the invagination and scission of the underlying membrane (Brüser & Bogdan, 2017) (Ezratty

et al., 2009). Thus, a crosstalk between AJs/FAs and actin cytoskeleton governs tissue homeostasis: by organizing the actin cytoskeleton, AJs and FAs regulate the structure of epithelia; on the other hand, by sustaining E-cadherin and Integrin turnover, the actin cytoskeleton impacts on tissue plasticity. Alterations of this finely regulated crosstalk can destroy the physiological processes of proliferation and cell survival in tissues, with implications for cancer development and progression.

The transcription factors YAP and TAZ

YAP/TAZ in Physiology and cancer

YAP (Yes-associated protein) and TAZ (transcriptional co-activator with PDZ-binding motif) are two mammalian transcriptional co-regulators that emerged in cell biology in the early 2000s (Sudol, 1994) (Yagi et al., 1999)(Kanai et al., 2000). At first, the transcription factor Yorkie, the homolog of YAP and TAZ, has been identified in *Drosophila melanogaster* during a genetic screening aimed to identify regulators of organ size, eventually demonstrating that Yorkie is the final effector of the Hippo pathway, a kinase cascade that controls organ size and tissue homeostasis (J. Huang et al., 2005).

Successively Dong discovered that the core architecture and functions of the Hippo pathway are conserved and active in mammalian cells. Indeed, YAP/TAZ overexpression leads to giant liver size in conditional transgenic mice (Dong et al., 2007). In addition, YAP hyperactivated mice also develop hepatocellular carcinomas and metastatic dissemination of tumors cells, revealing a role for this pathway in cancer development and progression (Dong et al., 2007) (Camargo et al., 2007) (Lamar et al., 2012). For this reason, YAP/TAZ are mentioned as "master regulator of organ growth".

Pleiotropic and tissue specific is the YAP/TAZ regulation of organ growth and cell plasticity during animal embryonic development and regeneration. YAP/TAZ mediated organ growth is dictated by the increasing of cell number and not cell size (hypertrophy). Accordingly, YAP/TAZ -induced tissue hyperplasia is due to increased cell proliferation and survival. Indeed YAP/TAZ transcribe for genes involved in cycle progression, DNA duplication and DNA repair, mitosis and anti-apoptotic genes. In addition, YAP/TAZ signaling sustains cell activity potentiating energetic demands via a metabolic reprogramming.

Physiologically, in addition to organ growth and development YAP/TAZ signaling occurs in tissue repair and organ regeneration in non-regenerating tissue in response to cell damage from trauma, disease and aging. Notably, tissue regeneration and wound repair not only depend on tissue residents stem cells but also terminally differentiated cells participate in this process. Indeed, YAP/TAZ sustains cellular de-differentiation of somatic cells into resident stem cells through transcription of pluripotency genes (SOX2, NANOG; OCT4, MYC and component of Polycomb group proteins (Mannaerts et al., 2015). The concomitant expansion of progenitor and stem cell compartments lead to

wound healing repair and organ regeneration. Thus, YAP /TAZ principally are not required for cell maintenance but are activate under injury to resolve tissue damage.

YAP/TAZ hyperactivity leads to tumor formation and progression. Notably, cancer has been defined as a "wound that do not heal" because cancer cells activate same regenerative pathway used to normal cells to repair tissue damage. If normal cells activate YAP/TAZ signaling to resolve tissue damage, neoplastic cells under chronic tissue irritation and inflammation take advantage from cell-fate reprogramming of YAP/TAZ signaling to escape from tissue tumor suppressive stimuli. Indeed, rather than from corruption of resident stem cells, cancer could originate from YAP/TAZ mediated de-differentiation of normal cells, that turn into cancer stem cells under pressure of pro-tumoral stimuli from the microenvironment (Zanconato et al., 2019).

In addition to cancer cells proliferation and plasticity, YAP/TAZ activation results also in drug resistance and metastasis. Indeed, resistance to chemio-therapeutic drugs, radiation, and molecularly targeted drugs is mediated by YAP/TAZ activation of survival pathways. Regarding metastasis, YAP/TAZ activation results in cytoskeleton remodeling, F-actin dynamic, limitation of focal adhesion maturation, resistance to anoikis and also cell metabolic adaptation (Zanconato et al., 2019). All these phenotypes lead to high cancer cells aggressiveness; indeed, patients with YAP/TAZ hyperactivated tumors have a poor outcome (Patel et al., 2017).

Finally, there is an emerging role of YAP/TAZ in regulating the cross talk between tumor and stromal cells. Cancer cells with hyperactivated YAP/TAZ signaling convert the healthy microenvironment into malignant, by stiffening of ECM and recruiting pro-tumorigenic fibroblasts and immune cells. Reciprocally, stromal cells can sustain cancer malignancy favoring activation of YAP/TAZ signaling (Zanconato et al., 2019). For example, cancer associated fibroblasts (CAFs) make a rigid ECM that leads to enhanced cancer cells proliferation (Calvo et al., 2013). Moreover, upon YAP/TAZ signaling stimulation, the immune cells Treg secrete TGF-β factor that suppress anti-tumor immunity mediated by CD8+T cells (Ni et al., 2018).

Thus, YAP and TAZ represent a central hub for crosstalk between cancer cells and tumor microenvironment, highlighting the new concept that cancer does not occur from alteration of single cells, but it is the effect of wide and heterogenic tissue corruption.

Structure, activity, and biological effect

The paralogue genes YAP and TAZ are mapped on chromosome 11q22 and 3q23-q24 respectively (Y. A. Chen et al., 2019) (Kanai et al., 2000). They are ubiquitously co-expressed in mammalian adult tissue and during development but with some exceptions. For example, TAZ is absent in the thymus and peripheral blood leukocytes while the hippocampus and parathyroid gland lack YAP expression (Heng et al., 2021).

YAP and TAZ share 46% amino acid identity and for this reason they are considered as equivalent proteins (YAP/TAZ) with structural similarity and redundant genetic function. Indeed, in several tissue the concomitant depletion of YAP and TAZ results in a stronger phenotype than single knockout, or early embryo death, suggesting an overlapping and fundamental biological function. However, emerging evidences indicate that YAP and TAZ possess also distinct attributes, as mutual depletion of YAP or TAZ gives different phenotypes.

For instance, in embryonic development YAP knock-out (KO) mice are not viable due to lethal defect of yolk sac vasculogenesis and embryonic axis abnormalities. On the contrary, TAZ KO mice are viable, but develop kidney disease and lung emphysema (Heng et al., 2021). In addition, also in non small cell lung cancer (NSCLC) it has been reported that YAP and TAZ coordinate non-overlapping transcriptional programs and consequent phenotypes: YAP sustains cell proliferation while TAZ cell migration (Shreberk-shaked et al., 2020).

If YAP and TAZ divergent functions are dictated by differences in protein structure is not demonstrated. Differently to YAP, TAZ has only one WW domain and lacks the proline rich and SH3 binding domain. This leads to a variability in the pattern of TAZ protein interactors compared to YAP. In addition, differently to YAP that has a unique degradation sequence in the TAD domain, TAZ presents an additional phosphor-degron motif in the TEAD sequence. Thus, TAZ protein is more unstable compared to YAP and different regulatory mechanism impinge on YAP and TAZ half-life. However even if regulated by different mechanisms, YAP and TAZ often respond in a coherent manner to the same regulatory inputs, so the biological relevance of their functional divergences remains unclear.

For instance, in non small-cell lung cancer cell lines YAP activity leads to cell division and cell cycle progression, whereas TAZ regulates ECM organization and adhesion to

foster cell migration (Shreberk-shaked et al., 2020). Moreover, in HEK293 cells only YAP, through strong expression of Cyr61, has been associated to increased cell proliferation, volume and granularity but also cells spreading and glucose uptake (Reggiani et al., 2021).

Both YAP and TAZ lack a DNA binding domain but orchestrate their transcriptional activity thanks to a TEAD-binding domain, WW domains, and a transcription activation domain (TAD). In detail, the TEAD-binding domain, located at the N terminal of proteins, is responsible for the recognition of the transcription factors of TEAD family. There are four isoforms of TEAD (TEAD 1-4) that are expressed in cell specific manner and lead to the transcription of different subsets of effector genes. The TAD domain, located at the C terminal of proteins, promotes YAP/TAZ activity after the binding to TEAD and other transcription factors (Varelas, 2014)(Yagi et al., 1999)(Levy et al., 2008).

Importantly, in the core of the protein the proline-rich sequence in WW domain (two and one WW domains respectively for YAP and TAZ) mediates protein-protein interactions with other transcription factors. Indeed, during physiological development and/or cancer progression, YAP/TAZ interact and sustain transcription program of p53-related proteins p63 and p73, RUNX 1/2, SMADs, PAX3, TBX5 and AP-1 (Yagi et al., 1999)(Alarcón et al., 2009) (Strano et al., 2005). Notably, the transcription factor AP-1 cooperates with YAP/TAZ to sustain TEAD mediated gene transcription. Mechanistically, YAP or TAZ/TEAD/AP1 complex binds on super enhancer element that are distant from the target gene promoter (Zanconato et al., 2018).

Subsequent YAP/TAZ mediated recruitment of RNA polymerase II results in gene transcription (Galli et al., 2015). Finally, YAP/TAZ co transcriptional activity can be affected by nuclear inhibitors: vGLL4, SWI/SNF subunit ariD1a, tiaM1, and the long non-coding RNA MaLat1. All of them are nuclear factors that compete with YAP/TAZ to TEAD binding (Zanconato et al., 2019).

YAP/TAZ drive the expression of multiple gene subsets that fit together into the complex biological process of tissue growth (Pan, 2010). YAP/TAZ dependent genes have been classified in six categories: (i) cell proliferation, cell cycle and tumorigenesis, (ii) cell migration, (iii) stemness/dedifferentiation, (iv) cell lineage fate determination and differentiation, (iv) cytoskeleton and cell morphology, and (vi) anti-apoptosis and cell survival (Moya & Halder, 2019).

Among these, there are a number of canonical YAP/TAZ target genes; these include CTGF (Cystein-rich protein connective Tissue Growth Factor) and CYR61 (Cystein-rich angiogenic inducer 61), secreted proteins involved in extracellular matrix deposition and remodeling, myofibroblast activation, angiogenesis and migration (Zhao et al., 2008)(Y. A. Chen et al., 2019); BIRC5, also called survivin, involved in anti-apoptotic signaling (Dong et al., 2007); AREG (Amphiregulin), a member of the EGF family (Pan, 2010) but also fibroblast growth factor (FGF1) and the transcription factor ankyrin repeat domain 1 (ANKRD1), which plays an important role in endothelial cell activation; cyclin D1 and cyclin E, whose expression promotes cell cycle progression(Mizuno et al., 2012) (Stein et al., 2015) (Lei et al., 2008) (Zhao et al., 2008) (Totaro et al., 2018).

Hippo dependent and Hippo independent YAP regulation

Structural composition of YAP/TAZ proteins unveils their physiological function of transcriptional regulator. In addition to specific domain for co-transcriptional activity, YAP/TAZ presents also functional domains to interact cytoplasmic protein. In details, YAP/TAZ present a 14-3-3 binding domain, a coil-coiled region and PDZ-binding domain. Differently to TAZ, YAP also present a proline-rich sequence at N-terminal and a SH3-binding motif that amplify the set of possible interactors. The co-transcriptional function of YAP/TAZ is dictated by these protein-protein interaction domains that allow YAP/TAZ retention and degradation into the cytoplasm. In resting cells, cytoplasmic YAP/TAZ anchor to protein complexes that limit their nuclear translocation. Instead, under activating stimuli YAP/TAZ move from cytoplasm to the nucleus where the binding with TEAD transcription factors sustains their transcriptional program activation. Thus, YAP and TAZ shuttle between the nucleus and cytoplasm and YAP/TAZ function is strictly correlated to nuclear localization (Piccolo et al., 2014). YAP/TAZ respond to a wide spectrum of extracellular stimuli, determining YAP/TAZ as key mediators of cellular interaction with the microenvironment. In detail, YAP/TAZ nuclear translocation is fostered by (i) biomechanical cues, (ii) extracellular ligands, such as growth factors and lipids, (iii) energy, osmotic and hypoxic stress, and (iv) inflammation and tissue injury. All these stimuli can be integrated by other pathways, and for this reason a complex signaling network controls YAP/TAZ activity (Heng et al., 2021).

The Hippo pathway is one fundamental YAP/TAZ regulator. This signaling occurs principally at the apical domain of polarized epithelial cells (Elbediwy et al., 2016) and it is highly conserved in different species ranging from fruitflies to mammals (Heng et al., 2021) (Piccolo et al., 2014). There is no canonical upstream receptor in this pathway, but many inputs roll in the core kinase cascade. Indeed, Hippo signaling and YAP/TAZ function depend on the balance in the activity/quantity of several upstream regulatory kinases, which can function in a complementary manner and which may substitute each other (Y. A. Chen et al., 2019).

The canonical Hippo pathway is a kinase cascade where LATS1/2 proteins are the direct regulators of YAP/TAZ, impacting on their localization and function (Zhao et al., 2007)(Lei et al., 2008). When Hippo pathway is off and LATS1/2 are inactivated, YAP/TAZ are unphosphorylated and translocate to the nucleus (Zhao et al., 2008). Upon activation of the Hippo pathway, LATS1/2 phosphorylate YAP/TAZ proteins, which are inactivated through their nuclear exclusion, sequestration in the cytoplasm, and proteasomal degradation (Zhao et al., 2007)(Lei et al., 2008)(C. Y. Liu et al., 2010) (Zhao, Li, Tumaneng, et al., 2010) (Fig. C)

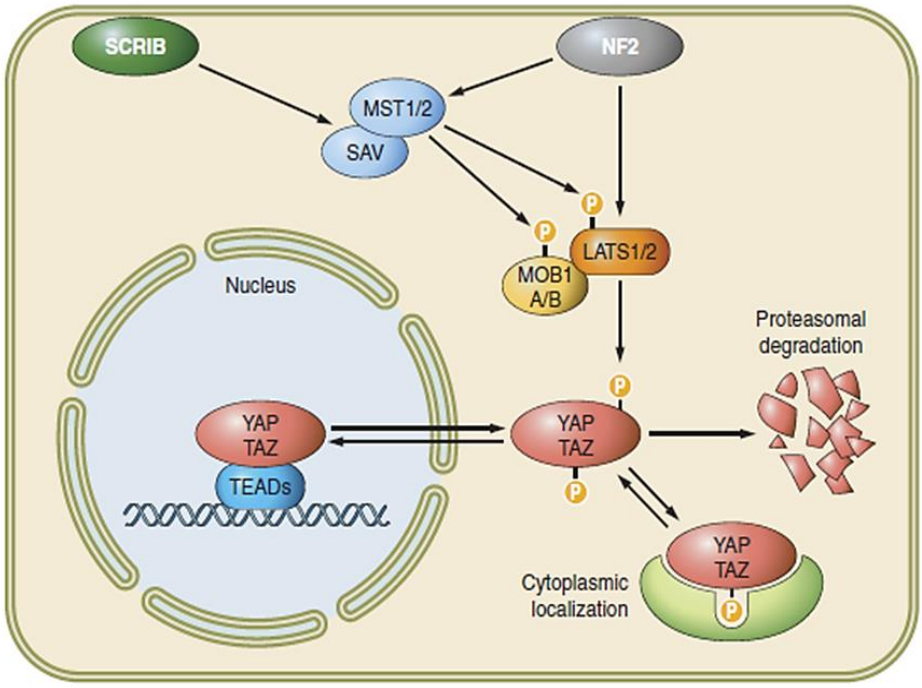


Figure C. Overview of Hippo pathway cascade. See text for details (Piccolo et al., 2014).

LATS1/2 phosphorylate YAP and TAZ at five and four S/T residues, respectively; among these residues, the most important for inactivation are Ser127 and Ser381 for YAP, and Ser89 and Ser311 for TAZ (C. Y. Liu et al., 2010) (Zhao et al., 2010). The phosphorylation of YAP at Ser127 creates a binding site for 14-3-3 proteins, which contribute to YAP cytoplasmic sequestration (Piccolo et al., 2014). The phosphorylation of YAP-Ser381 and TAZ-Ser311 also influence their stability, functioning as primers for additional phosphorylation by CK1 kinase. As a result, β-TrCP recognizes poly-phosphorylated YAP/TAZ and functions as an adaptor for SCF E3 ubiquitin ligases, leading to proteasomal degradation (C. Y. Liu et al., 2010) (Zhao et al., 2010). In most cells TAZ is a rather unstable protein, with a half-life lower than two hours. In contrast, YAP is a relatively stable protein and is mainly regulated by nucleo-cytoplasmic shuttling (Piccolo et al., 2014).

The Hippo pathway starts from MST1/2 kinases. MST1/2 bind the regulatory protein SAV1, forming an active enzyme that phosphorylates LATS1/2 (Chan et al., 2005). However, MST1/2 are not the only upstream regulators of LATS1/2. In fact, PP2A, TAOK1/2/3, MAPK, AMPK, and PTPN14 are cytosolic signaling molecules that modulate Hippo signaling pathway. Among these, MAP4K family kinases act in parallel to MST1/2 in the regulation of LATS1/2 and YAP/TAZ (Meng et al., 2015). In contrast, Protein phosphatase 2A (PP2A) activates YAP/TAZ through dephosphorylation of MST1/2.

Many are the upstream regulator of LATS1/2 kinases. For instance, the tumor suppressors Merlin/NF2 and angiomotin (AMOT) promote LATS1/2 dependent YAP inhibition (Sabra et al., 2017) (Mana-Capelli & McCollum, 2018). Also, cell polarity complexes regulate in different ways Hippo signaling. In details the aPKC and PAR3 systems sustain YAP activity through inhibition of Hippo pathway. aPKC binds and limits MST1/2 activity while Par3 inhibits phosphorylation of LATS1/2. On the contrary, Crumbs and Scribble that operate at level of Adherens Junction interact with and sustain LATS1/2 activity, with consequent YAP retention in the cytoplasm. Hippo signaling regulation also occurs by surface transmembrane receptors of soluble ligands. For instance, G protein-coupled receptors (GPCRs) can sustain Rho GTPases and actin cytoskeleton organization that lead to LATS1/2 phosphorylation. On the contrary, activation of epidermal growth factor receptor (EGFR), a tyrosine kinase receptor (RTK), results in LATS1/2 inhibition through PI3K-PDK1 signaling. Finally, the status of actin cytoskeleton in response to

ECM stiffness, cell shape, and cell-cell contact has a major role in the Hippo pathway and YAP/TAZ regulation (Gumbiner & Kim, 2014).

The Hippo pathway is not the only regulator of YAP and TAZ nucleo-cytoplasmic shuttling. In fact, YAP and TAZ are frequently overexpressed in cancer cells and accumulate in the nucleus, but this hyperactivity does not always correlate with mutations in Hippo pathway genes. Indeed, human malignancies are rarely due to defects in the Hippo pathway. In addition, YAP phosphorylation by LATS is not sufficient to suppress YAP nuclear accumulation in several conditions; in fact, inhibition of nuclear export resulted in nuclear accumulation of phosphorylated YAP, demonstrating that it can nonetheless translocate to the nucleus (Totaro et al., 2018).

Notably, YAP/TAZ are also regulated by the molecular signaling of Notch, TGF-β, and Wnt-β-catenin. In particular Notch regulates YAP/TAZ activity through Recombination signal Binding Protein for immunoglobulin kappa J region (RBPJ), a transcription factor mediating canonical Notch signaling. Indeed, RBPJ directly controls transcription of the YAP1 protein by binding to its promoter sequence. Instead, TGF-β signaling cooperate with YAP/TAZ through the binding and nuclear translocation of the transcription factors SMAD2/3 protein (Slemmons et al., 2017). WNT signaling regulates YAP/TAZ stability through the β-catenin destruction complex. This complex is composed by a central scaffold protein, Axin, and other proteins such as APC, PP2A, GSK3β and CK1. CK1 and GSK3β phosphorylate β-catenin ensuring its degradation by β-TrCP ubiquitin ligase, thus maintaining Wnt pathway inactive. In the presence of Wnt ligand, activated Fz-LRP6 receptors disassemble the destruction complex through the recruitment of Axin. β-catenin is consequently released and shuttles to the nucleus where it regulates the transcription of Wnt target genes (Clevers, 2006). In resting cells, YAP/TAZ are inactivated by the sequestration in the β-catenin destruction complex, where they play an essential role allowing β-catenin degradation. YAP/TAZ associate with Axin in the same domain used for LRP6 binding and recruit β-TrCP. Depletion of YAP/TAZ impedes β-TrCP association to the complex: β-catenin can be phosphorylated by the complex but cannot be degraded, consequently it accumulates and can shuttle to the nucleus. After Wnt stimulation, activated LRP6 displaces YAP/TAZ from Axin1, leading to both YAP/TAZ and β-catenin nuclear translocation. The overexpression of its protein levels allows YAP to resist LRP6 displacing from Axin1, inhibiting Wnt signaling in a concentration-dependent manner. Therefore, β-catenin destruction complex is a negative regulator of

both YAP/TAZ and β-catenin activity, preventing their nuclear localization (Azzolin et al., 2014).

Therefore, even if the Hippo pathway is a key regulator of YAP and TAZ function, many other LATS-independent mechanisms control their activity (Dobrokhotov et al., 2018). These include inflammation, metabolism, Rho GTPases and others; but perhaps the major stimuli that affect YAP/TAZ activity are mechanical signals coming from the extracellular matrix and adjacent cells (Totaro et al., 2018).

YAP/TAZ regulation by mechanical inputs

YAP/TAZ signaling is activated in response to external forces that lead to shear, tensile, and compression of the actomyosin cytoskeleton. Principally these stimuli are generated from stiffness of substrate, compaction of adjacent cells, and flow of fluids at interfaces between the apical and lumen of the cells. When mechanical stimuli lead to formation and contraction of filamentous actin (F-actin), YAP/TAZ translocate in the nucleus and activate a transcriptional program (Dasgupta & McCollum, 2019). Although the mechanical forces perceived by cell junctions are the major event that strongly affect the cytoskeleton rearrangement, also other microenvironment stress factors can impinge on the ratio of globular (G-actin) vs structured actin (F-actin) with consequent modulation of YAP/TAZ signaling. For instance, in response to activated G-protein–coupled receptors, Rho GTPases increase F-actin levels and induce Hippo inhibition. In this context AMOT binding to F-actin results in the disruption of Hippo protein complex, and consequently YAP translocates into the nucleus. On the contrary, under high levels of G-actin (and low F-actin), AMOT is mostly free in the cytosol, where it forms a Hippo protein complex that retains YAP in the cytoplasm. In addition to AMOT, although the molecular mechanisms are unknow, also the upstream kinases MAP4K, TAO and PAK limit YAP/TAZ activity through LATS1/2 activation under low cytoskeleton tension (Dasgupta & McCollum, 2019).

Thus, despite cells can generate tension autonomously through cytoskeleton contraction in response to microenvironment signals, the better characterized mechanism of YAP/TAZ regulation by actomyosin tension is principally fostered by stress fibers formation upon focal adhesion remodeling.

YAP regulation at Focal Adhesions

YAP/TAZ activity is strongly affected by mechanical stimuli that come from the extracellular matrix. Compared to cells grown at high density or cultured on a soft extracellular matrix, where YAP/TAZ are retained in the cytoplasm, cells grown at low density, that are spread on the substrate, and cells grown on a stiff extracellular matrix show nuclear YAP/TAZ localization and high transcription of their target genes. Both cell spreading and stiff ECM conditions result in increased actin stress fibers, indicating actin cytoskeleton tension. Thus, in response to ECM stiffness, cytoskeleton tension leads to YAP/TAZ activation and consequently cell proliferation and survival (Dupont et al., 2011)(Wada et al., 2011)(Aragona et al., 2013). YAP/TAZ activation in response to ECM stiffness is mediated by focal adhesion assembly and can be Hippo dependent or Hippo independent. One major role is played by FA-associated kinases SRC and FAK. Despite both kinases can directly promote YAP nuclear translocation through activating phosphorylation, FAK and SRC act principally via LATS1/2 inhibition. In fact, they can directly apply an inhibitory phosphorylation on LATS1/2 and its scaffold protein MOB1. In addition, SRC and FAK can inhibit LATS1/2 less directly through integrin-related signaling pathways. For instance, a stiff ECM causes the clustering and activation of β1-integrins, that lead to LATS1/2 inhibition through the FAK-Src-PI3K-PDK1 axis (Kim & Gumbiner, 2015). Also, FAK-dependent activation of a RAC-PAK signaling cascade sustains an inhibitory phosphorylation of Merlin/NF2, that loses its scaffold function for YAP and LATS1/2, with consequent YAP activation (Sabra et al., 2017). Finally, the Hippo pathway is regulated by integrin signaling through modulation of phosphatidyl-inositol-phosphates (PIP2). Mechanistically, stiff substrates reduce PIP2 levels via FAK/PLC axis inhibition. Reduction of PIP2 shuts down a signaling cascade that leads to the activation of RAP2. RAP2 is an upstream regulator of Rho and MAP4K, that sustain the Hippo pathway. Thus, increasing substrate stiffness impedes RAP2 activation, thereby promoting nuclear translocation and activation of YAP. In other words, a soft ECM in turn activates LATS1/2, followed by phosphorylation and inhibition of YAP/TAZ (Meng et al., 2018).

Mechanical forces from the ECM can regulate YAP/TAZ also in a Hippo-independent manner (Dupont et al., 2011). One example is provided by the nucleus acting as mechanotransducer in YAP regulation (Elosegui-Artola et al., 2017). Structurally, the actin cytoskeleton connects FAs to the LINC (Linker of Nucleoskeleton and

Cytoskeleton) complex on the nuclear envelope, allowing the propagation of mechanical forces from the ECM to the nucleus. This connection is present only in cells adhering to stiff substrates, not to soft ones.

Cells grown on soft substrates are round, and also the nucleus has a round shape because it is not coupled to the substrate. In this context, YAP is retained in the cytoplasm. An increase in ECM stiffness results in actomyosin cytoskeleton contraction and consequent changes in cell and nuclear shape. Indeed, contractile forces promote changes in nuclear shape due to increased connections between focal adhesions and LINC complexes in the nuclear membrane via actin stress fibers. This results in nuclear flattening and mechanical opening of nuclear pores, that stimulates YAP/TAZ nuclear import, without affecting their export. Consequently, YAP/TAZ accumulate into the nucleus activating gene transcription (Elosegui-Artola et al., 2017) (Fig. D).

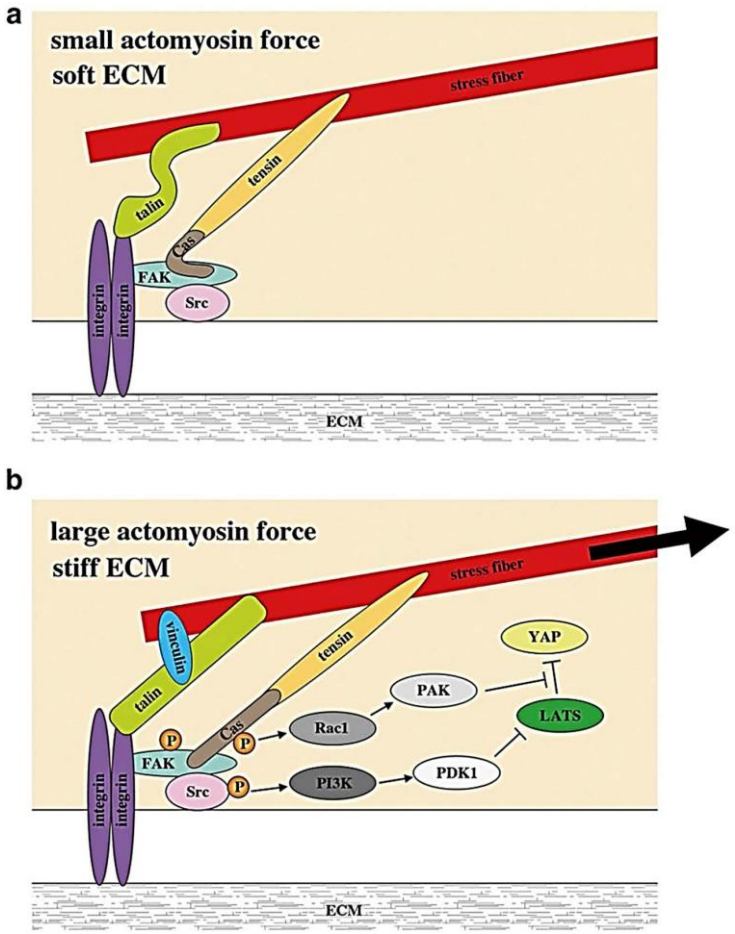


Figure D. Model of FAs-dependent regulation of YAP/TAZ. (a) Low tension on FAs has no effect on YAP/TAZ. (b) High tension on FAs induces the inhibition of LATS, with consequent nuclear accumulation of YAP and TAZ. (Dobrokhotov et al., 2018).

YAP regulation at Adherens Junctions

It is well known that cell-cell contact induces growth arrest, allowing the maintenance of tissue integrity. This phenomenon, termed contact inhibition of proliferation (CIP), involves YAP/TAZ signaling.

In high cell density, when cytoskeleton is organized in a circumferential actin belt, YAP is phosphorylated and sequestered in the cytoplasm, on the contrary, at low cell density, when there is no circumferential actin belt but adherent cells form many stress fibers, YAP translocates in the nucleus to promote cell proliferation (Dupont et al., 2011) (Furukawa et al., 2017)(Zhao et al., 2007).

A molecular mechanism of YAP/TAZ regulation by cell-cell contact has been defined. In particular, AJs sense cell density and recruit Hippo pathway components at cell-cell junctions, eliciting a LATS-dependent YAP/TAZ inhibition.

However, if YAP/TAZ inhibition at AJs incorporates also a further step of mechanical regulation is not fully understood, and contradictory evidences highlight an opposite role for actomyosin tension at AJs in regulating YAP/TAZ activity (Dobrokhotov et al., 2018).

Some evidences suggest that mechanical tension al cell-cell junctions is dispensable for YAP/TAZ inhibition; notably, the stretching of a dense monolayer with uncorrupted AJs, efficiently induces nuclear YAP. The same occurs in response to shear stress in confluent endothelial cells. In these systems the tension across cell-cell junction is enhanced but the reinforcement of cell-ECM adhesion prevails to activate YAP/TAZ (Aragona et al., 2013)(Benham-Pyle et al., 2015). In addition, when cells are cultured at high density on a soft substratum, there is a stronger YAP/TAZ inhibition compared to dense cells grown on a stiff ECM. This indicates that the main myosin pool relevant for YAP/TAZ regulation is the one associated to cell-ECM sensing, and not to cell-cell junctions (Stanton et al., 2019).

Nonetheless, other authors suggest a role for actomyosin tension at cell-cell junctions in negative regulation of YAP/TAZ signaling (Hirata et al., 2017) (Furukawa et al., 2017).

In any case, the induction of CIP depends on the presence of all components of AJs, since depletion of E-cadherin, α- or β-catenin prevents cytoplasmic retention of YAP in dense culture conditions (Kim et al., 2011) (Silvis et al., 2011). In fact, breast cancer MDA-MB-231 cells, that do no express E-cadherin, show nuclear YAP even in high cell density

(Kim et al., 2011). Notably, expression of an E-cadherin/α-catenin chimeric protein, able to directly bind to the actin cytoskeleton, lead to cytoplasmatic YAP localization in MDA-MB-231 cells cultured in high density (Kim et al., 2011). Therefore, it is not the homophilic interaction of E-cadherins between adjacent cells, but the consequent actomyosin tension at AJs that mediates YAP cytoplasmic sequestration and CIP (Kim et al., 2011) (Hirata et al., 2017).

Regarding mechanism, one important role is mediated by α-catenin, that changes its structure in response to actomyosin tension. In high density, the minimal cytoskeletal tension promotes a close conformation of α-catenin, that does not interfere with the Hippo signaling complex formed by LIMD1, vinculin, TRIP6, and LATS1/2. In this context LATS1/2 sustained by MOB1 are active in the cytoplasm to inhibit YAP. Ser127-phosphorylated YAP is bound by 14-3-3 proteins, and the YAP/14-3-3 complex is recruited to AJs, where α-catenin stabilizes it, protecting against PP2A-mediated dephosphorylation.

At low cell density, the circumferential actin belt is lost and actomyosin tension generated by pulling forces from neighboring cells sustain stress fiber formation. In this context, α-catenin changes its conformation and releases YAP that translocates into the nucleus. When α-catenin binding is lost, PP2A can efficiently dephosphorylate Ser127, causing YAP nuclear translocation (Schlegelmilch et al., 2011). Importantly, α-catenin complexed with YAP is detected also in soluble form. Mechanistically, tension causes opening of α-catenin structure that increases its binding to LIMD1 and vinculin. Moreover, to this complex also participates LATS1/2 and TRIP6. In this context, TRIP6 competes with LATS1/2 activator MOB1 and binds LATS1/2 at adherens junctions, thereby inhibiting it. Together, LIMD1 and TRIP6 inhibition of LATS1/2 allow YAP to translocate to the nucleus (Dutta et al., 2018).

A second mechanism is based on nuclear translocation of Merlin/NF2. Upon contraction of the circumferential actin belt, Merlin/NF2 is released from cell-cell contact regions, where it normally resides interacting with E-cadherin, F-actin and α-catenin, supporting stable AJs. At high cell density, Merlin/NF2 is released from AJs and shuttles to the nucleus; there, it increases YAP nuclear export functioning as an adaptor that connects YAP to exportins. Therefore, Merlin/NF2 nuclear translocation results in cytoplasmic YAP accumulation in a Hippo-independent manner (Furukawa et al., 2017). Once in the

cytoplasm, YAP is phosphorylated by LATS1/2 and retained in a complex with 14-3-3, that protects it from dephosphorylation (Schlegelmilch et al., 2011) (Fig. E).

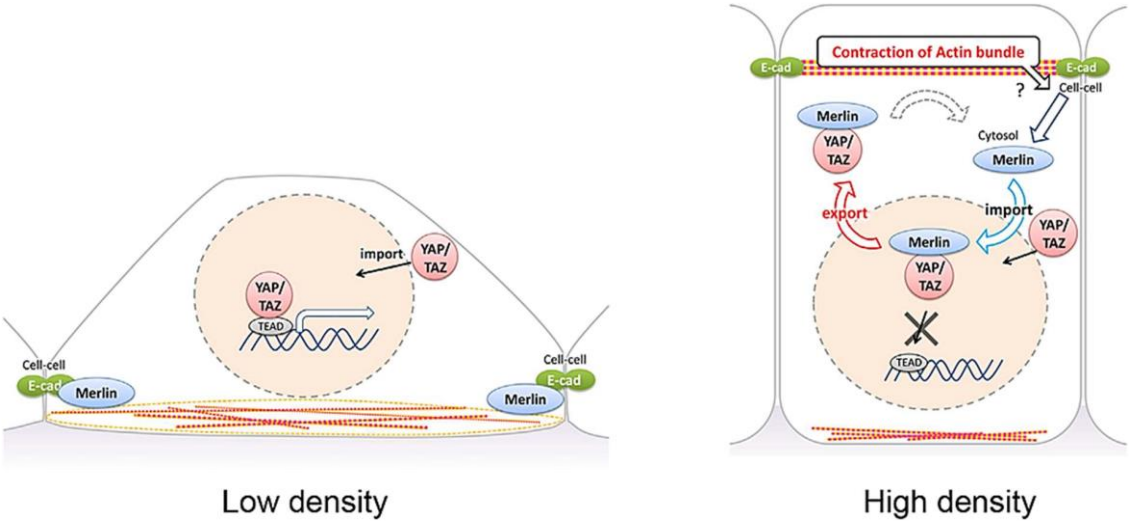


Figure E. Mechanism of YAP/TAZ inhibition by circumferential actin belt. At low cell density conditions,Merlin is located at Adherens Junctions. In contrast, in high cell density conditions Merlin is released and stimulates nuclear export of YAP/TAZ (Furukawa et al., 2017)

In summary, cell-cell contact has an essential role in YAP and TAZ inhibition, although the mechanisms controlling the switch between YAP/TAZ activation and inhibition at AJs remain poorly defined (Dobrokhotov et al., 2018). In conclusion, YAP/TAZ transduce external forces into a cellular response that in healthy conditions aims to restore tissue homeostasis, allowing organ development and repair of injury. Under conditions of aberrant mechanical cues, dysregulation of YAP/TAZ may drive the onset of diseases such us atherosclerosis, osteoarthritis, liver fibrosis, and cancer. Despite a remarkably large number of innovative discoveries have been made in the last decade, our understanding of YAP/TAZ regulation by mechanical stimuli remains incomplete - especially concerning the identity of upstream regulators (Cai et al., 2021).

DAB2IP

The tumour suppressor DAB2IP (Disabled homolog 2 interacting protein) also called AIP1 (ASK1 interacting protein) is a cytoplasmic Ras-GAP and adaptor protein that controls specificity, amplitude and duration of intracellular signalling events in response to many extracellular inputs such as inflammation, growth factors and hormones. In particular, it is a negative modulator that mitigates the oncogenic RAS/MAPK, TNF/NF-kB-and PI3K/AKT pathways, thus attenuating cell proliferation, survival, metastasis, and chemoresistance (L. Liu et al., 2015) (Bellazzo et al., 2016).

The human DAB2IP gene

The hDAB2IP gene is located on the long arm of the chromosome 9; cytogenetically it spans at 9q33.2 locus for approximately 218 kb with a plus strand orientation. (https://www.genecards.org/cgi-bin/carddisp.pl?gene=DAB2IP). DAB2IP is conserved in vertebrates, and has orthologs in invertebrates such as Drosophila melanogaster. At least 258 organisms have recognized DAB2IP orthologs (Cao et al., 2020).

hDAB2IP is a complex gene: it has a long sequence (15 exons and 14 introns) (H. Chen et al., 2002), a high level of polymorphism (1457 polymorphisms)(Yang et al., 2011a), and a high level of mononucleotide repeats in the coding sequence (Son et al., 2018).

Regarding control of gene expression, four different TATA-less transcriptional start sites (TSS) flanked by CpG islands have been predicted at the 5'-end of hDAB2IP gene (H. Chen et al., 2002). Although the transcription factor SP1 has been associated to DAB2IP transcription (Shang Li et al., 2017), it seems that promoter methylation is the key determinant in DAB2IP expression. The differential contribution of CpG islands associated to alternative DAB2IP promoters likely dictate the expression of DAB2IP variants (H. Chen et al., 2002).

In human tissues two are the most abundant DAB2IP transcripts. The hDAB2IP transcript variant 1 (NM_032552.3) and hDAB2IP transcript variant 2 (NM_138709.1) are transcribed from two different promoters. Although these two variants are concomitantly expressed in normal and fetal human tissues, the expression level of the variant 1 (NM_032552.3) is apparently stronger than the variant 2 (NM_138709.1) (Qiu et al., 2007a).

In addition to having multiple TSS, the hDAB2IP transcript also undergoes alternative splicing of the last exon, affecting the C-terminal sequence of the encoded protein.

In summary, the hDAB2IP gene can encode at least six different protein isoforms; the various TSS generate mRNAs with different start codons, encoding for three possible N-terminal regions. Moreover, each of these variants can undergo alternative splicing of the last exon, generating two possible C-terminal regions (Bellazzo et al., 2017) (Fig. F)

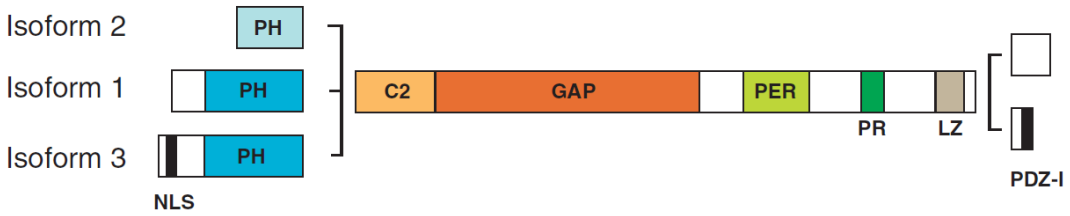


Figure F. hDAB2IP structure and domains. Alternative N- and C-terminal sequence combinations can potentially generate six isoforms (Bellazzo et al., 2017). See text for details.

Very little is known about the differential expression of the various isoforms. One recent study reported that a shorter DAB2IP isoform is specifically transcribed in human umbilical vein endothelial cells (HUVECs) treated with TNF-α (see below).

Among the six predicted isoforms of DAB2IP, the core region with the RasGAP domain is unaltered, while N- and C-terminal regions vary. In particular, DAB2IP isoforms that originate from the most distal promoter have an extended Pleckstrin Homology (PH) domain and contain a Nuclear localization signal (NLS). In contrast, DAB2IP transcripts from internal promoters have a shorter N-terminal region that contains only a reduced PH domain. In addition, the shorter C-terminal sequence generated from alternative splicing of the last exon encodes a potential PDZ-interacting domain of still unknown relevance (Bellazzo et al., 2017).

The two most abundant DAB2IP transcripts have different N-terminal regions; hDAB2IP transcript variant 1 (NM_032552.3) encodes hDAB2IP isoform 1 (NP_115941), a protein of 1132 amino acids, with a molecular weight of approximately 130kDa. The hDAB2IP isoform 2 (NP_619723), encoded by hDAB2IP transcript variant 2 (NM_138709.1), is composed of 1066 amino acids, and has a molecular weight of approximately 110kDa. With respect to isoform 1, hDAB2IP isoform 2 is shorter at the N-terminus and has a reduced PH domain (Fig. G).

Currently, there is only one study reporting evidence for differential functions of DAB2IP isoforms. In HUVEC, stimulation with TNF-α induces expression of a short DAB2IP

isoform, that authors called AIP1B; the shorter protein lacks the N-terminal PH domain and localizes to the mitochondria, where it enhances TNF-α-induced generation of mitochondrial reactive oxygen species (mitoROS) and endothelial cell activation (Z. Li et al., 2020). This work highlights that DAB2IP activities may be isoform dependent, suggesting that a different composition of regulatory domains can impact on DAB2IP function and stability. Currently, most of the published functional studies involved ectopic expression of isoform 2 (NP_619723).

DAB2IP protein domains and interactors

DAB2IP belongs to the SynGAP family of Ras GAPs (Scheffzek & Shivalingaiah, 2019), characterized by the presence of a PH and C2 domains at the N-terminus. In addition, DAB2IP has a peculiar C-terminal region, with additional domains that mediate functional protein interactions; in this way, DAB2IP modulates an intricate cytoplasmic network in response to a variety of extracellular signals. Many interactions have been mapped to specific domains and are listed below.

Nuclear localization signal (NLS)*:* it is an arginine-rich sequence in the N-terminal region of DAB2IP, can mediate interaction with importins (Kalderon et al., 1984).

Pleckstrin homology domain (PH domain): it is a domain of approx. 100aa, that allows DAB2IP binding to phosphatidyl-inositol lipids PIP4 within biological membranes (Wan et al., 2010). In addition, the PH domain is also implicated in protein-protein interactions, driving the recruitment of different protein associated to membrane, their physiological localization and activity (Musacchio et al., 1993).

The PH domain of DAB2IP binds:

- IRE1 is a transmembrane serine threonine kinases/endonucleases that sustain ER stress response. By binding IRE1 through its PH domain DAB2IP promotes activation of apoptotic ASK/JNK axis (D. Luo et al., 2008).
- KIF3A is a kinesin that act in the anterograde protein transport during ciliogenesis. Through PH domain DAB2IP binding stabilize KIF3a that results in a proper primary cilium formation (Lin et al., 2021).

Protein kinase C conserved region 2 (C2 domain): It is a Ca2+ binding motif found in many signaling proteins. This domain displays the remarkable property of binding a

variety of different ligand and substrates such as Ca2+, phospholipids, inositol polyphosphates, and other proteins. Not all proteins containing C2 domains are regulated by Ca2+, suggesting that some C2 domains may play a purely structural role (Nalefski & Falke, 1996).

The C2 domain of DAB2IP binds:

- ASK1 is an upstream activator of JNK and p38 MAPK signaling cascades. The DAB2IP interaction prevents ASK1 binding to 14-3-3 and enhances TNF-induced ASK1-JNK activation (R. Zhang et al., 2003).
- GSK3β is master regulator of canonical Wnt/β-catenin signaling. DAB2IP binding facilitates GSK3β de-phosphorylation and decrease nuclear translocation and transcriptional activity of β-catenin (Xie et al., 2009a).
- VEFGR2 is a regulator of physiological angiogenesis and binding with DAB2IP inhibits auto-phosphorylation of VEGFR2 and counteracts VEGFR-2 mediated angiogenic signals (H. Zhang et al., 2008).
- Missense mutants of the tumor suppressor p53 (mutp53) are frequent in cancer and are often expressed at high levels. Mutp53 binds DAB2IP preventing its interaction with ASK1, inhibiting JNK phosphorylation and enhancing NF-κB activation in response to TNF (Di Minin et al., 2014a).

In addition, the PH and C2 domains of DAB2IP are adjacent, and can cooperate in regulation of cell signaling, resulting in an unique PH-C2 domain. The PH-C2 domain of DAB2IP binds:

- JAK2 is a non-receptor tyrosine kinase involved in JAK/STAT pathway. DAB2IP binding inhibits JAK2, STAT1 and STAT3 phosphorylation and activation upon IFN-y treatment functions, strongly reducing proliferation and migration of vascular smooth muscle cells (VSMC) (Luyang Yu et al., 2011).

***RasGTPase-activator protein (RasGAP domain)*:** it is a well characterized module that promotes the GTPase activity of target proteins, fostering hydrolysis of GTP to GDP (Self & Hall, 1995). The GAP domain of DAB2IP binds:

- Ras is a small GTPases that activate classical mitogenic pathway RAF-MEK-ERK, Phosphatidylinositol-3 kinase (PI3K)-AKT and RALGDS (Simanshu et al., 2017). DAB2IP inactivates RAS after the binding (H. Chen et al., 2002).

- RIP is an activating enzyme of NF-kB and p38 MAPK pathways. After binding to RasGAP domain, RIP promotes DAB2IP phosphorylation is ser-604 and stimulates DAB2IP activation upon TNF treatment (R. Zhang et al., 2007a).
- HIPK1 is a serine/threonine Kinases that act as nuclear co transcriptional repressor. However, after TNF-α stimuli HIPK1 moves into the cytoplasm where binds to AIP1-ASK1 complex. Binding to RasGAP domain of AIP1 releases the 14-3-3 and Trx inhibitor from ASK1. HIPK1 sustains TNF-α induced JNK/p38 activation (Xianghong Li et al., 2005).
- RAB40C is Rab small GTPases that regulates biogenesis of lipid droplets and vesicle transport in oligodendrocytes (Tan et al., 2013) RAB40C binding to DAB2IP need also interaction with a coiled coil structures in the c terminal of DAB2IP (X. Luo et al., 2018).

***Period-like domain (PER domain)*:** It is a conserved region located in the C terminal half of the protein. This domain is important for DAB2IP protein-protein interactions. It associates with:

- TRAF, a signal-transducing protein essential in the bifurcation point in TNF-induced activation of NF-kB and activation of JNK. DAB2IP binding enhances JNK activation, while counteracts NF-kB cascade (H. Zhang et al., 2004).
- 14-3-3, an inhibitory molecule in the TNF induced ASK1-JNK/p38 signaling pathway and the interaction with DAB2IP facilitates dissociation of 14-3-3 from ASK1 (W. Min et al., 2008).
- AKT is a serine/threonine kinase that binds DAB2IP via a short motif in the PER domain. DAB2IP binding of to AKT1 counteracts its activation (Xie et al., 2009b).

Proline rich domain (PR domain): this is a sequence rich in prolines located in the C terminal region. Proline-rich regions constitute a docking site for proteins with SH3 domains (Feller et al., 1994). The PR domain of DAB2IP contains an unusual sequence of ten repeats of proline, and binds:

- c-Src is a non-receptor tyrosine kinase protein involved in AR-mediated cell growth (Wheeler et al., 2009). DAB2IP disrupts the interaction between c-src and AR, and blocks AR activation (K Wu et al., 2014).
- PI3K is a phosphoinositide 3-kinase. DAB2IP binds the SH3 domain in p85 subunit of PI3K. Upon stress stimuli DAB2IP PR domain is critical for PI3K/AKT inhibition and ASK activation, resulting in limited proliferation and enhanced apoptosis (Xie et al., 2009b).
- STAT3 is a transcription factor principally activated in response to cytokines (Tkach et al., 2013). Via PR domain DAB2IP interacts with STAT3 resulting in the suppression of anti- apoptotic STAT3/surviving axis (J. Zhou et al., 2015).
- NOX2 is a super-oxide generating enzyme that produces reactive oxygen species (ROS)(Hervé et al., 2006). DAB2IP via its PR domain binds to the SH3 domain of cytosolic subunit p47phox and prevents formation of an active NOX2 complex. DAB2IP binding to p47phox attenuates ROS production (Zhang et al., 2018).

Leucine zipper. It is a hydrophobic motif, usually found within the DNA-binding domain of transcription factors but it is also involved in the formation of protein dimers (Landschulz et al., 2011). A LZ domain is present in the C-terminal region of DAB2IP, and was shown to mediate interaction with the transcription factor GATA-1. Nuclear DAB2IP was reported to bind and function as a GATA-1 cofactor to coordinate the transcriptional repression of cancer stem cell (CSC) markers such as CD117 in prostate cancer cells (E. J. Yun et al., 2015).

Class-I PDZ binding: this is a short sequence at the C-terminus that matches the consensus of class-I PDZ-binding peptides. These peptides are recognized by PDZ domains of various polarity and signaling proteins. PDZ-containing proteins are generally restricted at the plasma membrane where they mediate formation of protein complexes that may also link with actin filaments (Fanning & Anderson, 1999). Currently there are no evidences of proteins binding DAB2IP in this region.

In addition to those listed above, a number of other proteins have been reported to bind DAB2IP, but the region of interaction has not been mapped in detail.

- In the nucleus DAB2IP interacts with the enzymes DNA methyltransferase 1 (DNMT1) and histone deacetylase (HDAC2). Through formation of this complex DAB2IP prevents DNMT1/HDAC2 binding to DNA and concomitantly sustains expression of oncogenic miR-138. Notably, DAB2IP/ DNMT1/HDAC2 complex limits cancer cell stemness and resistance to therapy (Yun et al., 2017).
- DAB2IP interacts with RanBP2, TRIP12, and RNF40 that are E3 ligases. In addition, DAB2IP associate also to PARP-1, initial protein of DNA damage repair (DDR) pathway. Acting as scaffold protein, DAB2IP sustains PARP1 degradation by RANBP2. Loss of DAB2IP in cancer cells results in upregulated PARP1 that fostering DNA repair allow cell resistance to drug and radio treatment (Yun et al., 2019).
- DAB2IP interacts with PLK1, a serine/threonine-protein kinase, that regulates attachment of spindle microtubule to kinecotore of sister chromatide during mitosis or meiosis. DAB2IP activates PLK1/BubR1 axis and allows a proper chromosome segregation (Lan Yu et al., 2016).
- RASSF1A is a scaffold protein RAS associated that foster pro apoptotic signal (Donninger et al., 2007). RASSF1 binds to DAB2IP and cooperate in mitigation of RAS signaling (Stewart et al., 2020).
- DAB2IP binds to phosphatase PP2a. In this case more DAB2IP domains have been assigned to PP2a binding. In details, in ECs under TNF-α stimulation DAB2IP binds to PP2a via its GAP domain eliciting ASK1 dephosphorylation at pS967. This allows the release of inhibitory 14-3-3 protein and subsequent activation of ASK1-JNK signaling cascade (W. Min et al., 2008). DAB2IP can also bind PP2a via its C2 domain. In this case, DAB2IP/PP2A complex facilitates dephosphorylation of GSK3β at the inhibitory pS9 site, leading to inhibition of β-catenin activity (Xie et al., 2010).
- In Wnt signaling context, DAB2IP also interacts with Axin, a scaffold protein that regulates phosphorylation and stability of β-catenin. Although DAB2IP binds Axin, the functional impact of this interaction has not been defined (Xie et al., 2010).
- DAB2IP interacts with androgen receptor (AR) and inhibits AR signaling via multiple molecular mechanisms: DAB2IP binding retains AR in the cytoplasm, DAB2IP/PP2A complex removes the activating phosphorylation of AR on Ser 81,

and DAB2IP inhibits AR-mediated c-Src activation by preventing AR binding to c-Src (K. Wu et al., 2014).

- DAB2IP binds VEGF3 and prevents its endocytosis and degradation. In this way, DAB2IP reinforces VEGF3/AKT signaling that result in development of angiogenesis and lymphoangiogenesis (Zhou et al., 2014).
- DAB2IP interacts with TNFR1/2, transmembrane receptor of TNF-α, and sustains activation of the ASK1/JNK/p38MAPK pathway in response to TNF (Zhang et al., 2004)(Ji et al., 2012).
- DAB2IP interacts with the amino terminus of the adaptor protein DAB2 (Z. Wang et al., 2002) (Chen et al., 2002). DAB2 acts as clathrin associated sorting protein (CLASP) required for receptor-mediated endocytosis of cargo proteins such as LDL. In addition, DAB2 is involved in endocytosis of integrin b1, E-cadherin and TGF-β receptor (Finkielstein & Capelluto, 2016) (Brüser & Bogdan, 2017). Although DAB2IP has been initially discovered as a protein that interacts with DAB2, functional studies of the impact of DAB2IP on DAB2-dependent endocytosis are surprisingly missing.
- A recent study revealed that Raskol, the Drosophila ortholog of mammalian DAB2IP, interacts with and regulates E-cadherin (DE-cad) in border cells of the egg chamber. By regulating DE-cadherin, Raskol affects cell adhesion, polarity and actin dynamics, so that Raskol mutant cells have a cell migration phenotype (Raza et al., 2019). In addition, also in mammalian epithelial cells and mouse neonatal cardiomyocytes, DAB2IP was identified as associated to cadherins, suggesting a potential role in the biology of Adherens Junctions (Guo et al., 2014)(Li et al., 2019).

Biological activities of DAB2IP

DAB2IP was initially identified in 2002 in rat with the name of DIP1/2. In prostate normal and cancer tissue DIP1/2 interacts with the tumor suppressor DOC-2/DAB2 (differentially expressed in ovarian carcinoma-2/Disable-2) inhibiting RAS activation (Z. Wang et al., 2002). Concomitantly, DAB2IP has been identified as AIP1, ASK1-interacting protein. Playing as a competitor, AIP1 facilitates dissociation of 14-3-3 from ASK1 and it enhances ASK1-JNK pathway activation in response to TNF (Zhang et al., 2003). These two pioneer studies immediately showed the heterogeneous DAB2IP

physiology as a cytoplasmic Ras- GTPase activating protein (GAP) also functioning as a signaling scaffold to modulate the cell's response to multiple extracellular signals.

DAB2IP as a signaling adaptor or scaffold

As described above, DAB2IP interacts with a large number of different proteins. The possibility to form multiple protein-protein interactions on separate domains suggests that DAB2IP can function as a scaffold for other signaling proteins. Notably, in this function DAB2IP can be either an adaptor or a competitor.

For instance, DAB2IP can form a complex that sustain ASK dependent pro apoptotic activity and concomitantly block pro survival PI3K/AKT pathway (Xie et al., 2009). In addition, DAB2IP can complex with IRE1 and sustain pro apoptotic ASK/JNK signaling (Luo et al., 2008) or DAB2IP binding JAK2 blocks STAT1/3 proliferative and migratory activity (Yu et al., 2012).

On the contrary, DAB2IP can also act as a competitor or scavenger: by binding to signaling proteins it can prevent their interaction with upstream activators, or downstream effectors. For instance, DAB2IP limits formation of the multicomplex NOX2 (Zhang et al., 2018), it disrupts the c-SRC/AR complex (Wu et al., 2014) and blocks the DNMT1/HDAC2 complex binding to DNA (Yun et al., 2017).

Importantly, in many such multiprotein complexes DAB2IP impact is not simply dictated by stoichiometry and binding affinity with target proteins; rather, DAB2IP interacts with enzymes that orchestrate fine molecular reactions. For example, DAB2IP can interact with Kinases such as ASK1, GSK3β, and PLK1, but also with phosphates. PP2A is the best characterized DAB2IP interacting phosphatases and is responsible of many DAB2IP functions. Indeed, PP2A sustains ASK1 dephosphorylation at pS967 (W. Min et al., 2008), drives GSK3β dephosphorylation at pS9 (Xie et al., 2010), and causes dephosphorylation of AR at pS81(Wu et al., 2014).

Recently it has been reported that DAB2IP can also interacts with various ubiquitin E3 ligases (RanBP2, TRIP12, and RNF40) (Yun et al., 2017) suggesting that DAB2IP can potentially modulate protein ubiquitination and stability within multi-protein complexes.

DAB2IP as a RasGAP

Ras is a small GTPases protein involved in the signal transduction of external stimuli coming from many cellular receptors including receptor tyrosine kinases (RTKs), G-protein coupled receptors (GPCRs), and integrin family members (Gimple & Wang, 2019). Activated Ras sustains cell proliferation and survival through classical mitogenic cascades of RAF–MEK–ERK and PI3K–AKT respectively. In addition to these Ras canonical pathway, RalGDS-Ral, TIAM1-RAC-PAK and PLC-PKC pathway emerge as RAS dependent pathways, and regulate membrane trafficking, cytoskeleton rearrangement, cell motility and Calcium signaling (Takács et al., 2020).

Ras alternates between GTP-bound and GDP-bound forms that respectively correspond to the active and inactive status, categorizing RAS as a classical binary molecular switch (Bos et al., 2007). Guanine nucleotide exchange factors (GEFs) activate RAS by binding to RAS-GDP and facilitates GDP-GTP exchange. On the contrary, GTPase activating proteins (GAPs) bind to RAS-GTP and stimulate the hydrolysis of GTP to GDP, thereby turning off RAS signaling (Bos et al., 2007). Importantly, point mutations in RAS that stabilize the GTP-bound state, as well as mutations that cause inactivation or loss of RasGAPs, are frequent events in cancer (Olsen et al., 2017).

Through its RasGAP domain, DAB2IP binds and inactivates RasGTP fostering GTP hydrolysis. Evidences came from prostate cancer cell lines where DOC2/DAB2 and DAB2IP were shown to interact and collaborate to promote Ras inactivation and inhibition of MAPK signaling (RAF-MEK-ERK cascade) with concomitant reduction in cell proliferation (Z. Wang et al., 2002). The RasGAP activity is important for tumorigenesis in vivo; in nude mice injected with DAB2IP -/- prostate cancer cells, the reintroduction of a construct expressing wt DAB2IP into the cells significantly suppressed tumor development, while expression of the a point mutant lacking GAP activity had no effect (Min et al., 2010).

A tight regulation of RAS activity is critical for tissue homeostasis, and RasGAPs have a protective role in limiting cell transformation by aberrant RAS activation. Also RASSF proteins, downstream effectors of RAS, can serve as second line of defense against RAS-mediated transformation. Indeed, after aberrant RAS activation, activated RASSF can drive RAS-mutated cells to apoptosis or senescence. Interestingly, RASSF1A can activate the Hippo pathway via MST; however, RASSF1A dependent apoptosis in RAS-mutant

cells is not mediated by YAP sequestration but rather by p53 stabilization and accumulation of RB and Bax proteins (Stewart et al., 2020). Notably, RASSF proteins can act as a scaffold to facilitate Ras interaction with their GAPs. In particular, RASSF1A was shown to bind and sustains DAB2IP protein levels. The RASSF1A/DAB2IP complex reduces Ras-GTP, in a feedback loop whereby a RAS effector can downregulate RAS activity via a GAP. Suppression of DAB2IP in cells lacking RASSF1A caused enhanced growth in vitro and in vivo, supporting the relevance of this feedback regulatory mechanism (Stewart et al., 2020). Thus, DAB2IP acquires a fundamental role in limiting tumor formation by RAS hyperactivation.

Interestingly, via its GAP domain, DAB2IP can modulate other small GTPases. One is RAB40C, that mediates traffic in the perinuclear recycling compartment and also regulate lipid droplet (LD) homeostasis. In detail, RAB40C sustains LD accumulation in GTP dependent manner. In this context, DAB2IP promotes GTP hydrolysis of RAB40C and negatively regulates the effect of RAB40C on LD accumulation. Interestingly, DAB2IP promotes the GTP hydrolysis of RAB40C almost as efficiently as that of H-Ras. Accordingly, the point mutant DAB2IP R385L, lacking GAP function, does not inhibit RAB40C activity (Luo et al., 2018). Thus, by inhibiting RAB40C, DAB2IP modulates the formation of Lipid Droplets.

Another is ARF6, a multifunctional small GTPase that activates lipid modifying enzymes, stimulates actin polymerization and regulates vesicular trafficking. In particular, Arf6 sustains the lipid modifying enzymes phosphatidylinositol 4-phosphate 5-kinase (PIP5K) that transform PIP4 in PIP2. DAB2IP inhibits ARF6 activity reducing the amount of PIP2 in the plasma membrane. Since PIP2 creates a docking site for TLR4-TIRAP-MyD88 complex at the plasma membrane, DAB2IP activity results in the inhibition of LPS-induced NF-kB and MAPK signaling.

Also in this case, DAB2IP(R289L), a mutant defective in Ras-GAP function, restored Arf6-induced NF-kB activity, confirming that DAB2IP GAP activity is essential for this effect (Wan et al., 2010).

Together, these observations reveal that DAB2IP may be a general GAP with ubiquitous activity across at least three subfamilies of small GTPases: Arf, Rab and Ras. This potential activity may have broad implications, so further research in this direction is granted.

DAB2IP expression and function in normal tissues

The hDAB2IP gene is ubiquitously expressed in most tissues and organs, with low level in kidney, liver and some urogenital organs (Z. Wang et al., 2002). According to the public database The Human Protein Atlas the highest DAB2IP expression occurs in brain and muscle tissue, with specific enrichment at level of cerebellum and of skeletal muscle. On the contrary, DAB2IP mRNA is very low in T-cells and totally absent in bone marrow, granulocytes, monocytes, B-cells, NK-cells, and dendritic cells (https://www.proteinatlas.org/ENSG00000136848-DAB2IP/tissue). Data from single cell RNA analysis indicate that DAB2IP is more expressed in epithelial cells than in other cell types within various tissues, and further confirm its total absence in blood cells (https://www.proteinatlas.org/ENSG00000136848-DAB2IP/celltype).

DAB2IP in embryonic development

During development, DAB2IP is expressed in most fetal tissues with a gestational age and tissue-specific pattern, suggesting its potential role in organogenesis and cell differentiation (Liu et al., 2012).

In mammals, DAB2IP knockout (KO) mice are viable and show normal tissue development. DAB2IP-KO mice show normal vascular development even if a dramatic enhancing in inflammatory angiogenesis has been detected. Consistent with this, VEGF-induced neovascularization is greatly increased (Zhang et al., 2008). Intriguingly, DAB2IP KO mice exhibit reduced retinal angiogenesis, delayed developmental lymphangiogenesis in neonatal skin, and show weaker VEGF-C-induced corneal lymphangiogenesis, correlated with reduced expression of VEGFR-3 (Zhou et al., 2014).

DAB2IP expression during development has been assessed also in humans. DAB2IP is expressed in female reproductive system at level of early villi, maternal decidua, and term placenta. DAB2IP is also expressed in the trophoblast, the outer layer of blastocyst, and its expression dynamically changes during pregnancy (Shan et al., 2016). The implantation of trophoblast and formation of maternal placenta is a fundamental process in early stage of embryogenesis, this process depends on migration and invasion ability of trophoblast. It has been suggested that DAB2IP may be required for proper trophoblast migration and invasion via MMP2 and MMP9 secretion, as DAB2IP knockdown reduced

expression of the MMPs and blunted migration of an immortalized trophoblast cell line (Shang Li et al., 2017). Indeed, DAB2IP is strongly expressed in trophoblast cells during the first trimester but is reduced at third trimester, reinforcing its possible role in controlling its invasive and migratory features. Of note, reduced DAB2IP expression was observed in pre-eclampsia placentas (Shan et al., 2016) and trophoblast cells from recurrent abortion patients (Shang Li et al., 2017).

DAB2IP in cardiovascular system

DAB2IP is abundantly expressed in vascular endothelial cells (ECs) and may have a crucial role in the prevention of cardiovascular disorder.

First of all, DAB2IP has a strong impact on VEGF mediated vessels formation. Despite DAB2IP KO mice show normal vasculature, VEGF-A stimulation results in augmented ear, cornea retina mouse neovascularization, indicating that DAB2IP limits the VEGF-A/VEGFR2 dependent angiogenesis (H. Zhang et al., 2008). Opposite effect has DAB2IP in vessel formation upon VEGF-C stimulation. Indeed, DAB2IP can sustain lymphoangiogenesis modulating VEGF-C VEGFR3 signaling (Zhou et al., 2014). Importantly, the modulatory activity of DAB2IP limits pathological angiogenesis fostered by cell inflammatory response; by reducing inflammation associated to VEGFA-VGFR2 signaling, DAB2IP controls vascular permeability and angiogenesis (H. Zhang et al., 2008).

For this reason, DAB2IP may prevent cardiovascular disorder. Accordingly, a DAB2IP polymorphism (variant rs7025486 (G/A)) has been associated with increased risk of vascular diseases like, coronary artery disease (CAD)(Harrison et al., 2012) (Bhanushali et al., 2018), pulmonary embolism, myocardial infarction (MI) and peripheral arterial disease (PAD) and abdominal aortic aneurysm (AAA) (Gretarsdottir et al., 2010). Interestingly, the impact of DAB2IP- rs7025486[A] on AAA is sex-dependent, being correlated to a stronger AAA expansion in women (Ye et al., 2017). A more recent epidemiological study revealed that DAB2IP mRNA expression is elevated in AAA patients, and correlates with reduced expression of two well-known DAB2IP inhibitors: EZH2 and mir-363-3p (Legaki et al., 2019).

DAB2IP/AIP1 knock-out mice exhibit enhanced inflammation and cell proliferation with reduced apoptosis. DAB2IP deletion has been reported to foster the progression of Inflammation-Induced Atherosclerosis and Graft Arteriosclerosis (Jiqin Zhang et al., 2015). In details, AIP1 inhibits intimal formation and graft arteriosclerosis (GA) blocking inflammatory response after allograft. Indeed, in VSMCs (vascular smooth muscle cells) AIP1 downregulates IFN-gamma-–JAK2-STAT1/3 signaling and leads to inhibition of cell proliferation and migration. AIP1 deletion enhances IFN-gamma dependent neointima formation in a mouse graft arteriosclerosis model (Yu et al., 2011). In addition, DAB2IP depletion significantly increases the number and size of aortic plaques in the ApoE−/− mice, due to amplification of LDL-induced NF-kB/JNK proinflammatory signaling. Thus, AIP1 suppresses atherosclerosis progression also by limiting hyperlipidemia-induced inflammation and vascular endothelial dysfunction (Q. Huang et al., 2013). Recently, DAB2IP has been involved also in preventing the development of transplant-related arteriosclerosis in a model of mouse aorta transplantation (L. Qin et al., 2019).

Interestingly, the molecular mechanism through which AIP1 prevents vascular remodeling from inflammatory insult (TNF-α) is based on modulation of the NOX2 complex. Indeed, in the endothelium DAB2IP/AIP1 suppresses NOX2-dependent oxidative stress, regulating vascular remodeling and preventing vascular disease (Jiqin Zhang et al., 2018). Interestingly, a recent study reported that chronic inflammation (i.e. TNF-α stimulation) of endothelial cells induces expression of a shorter isoform of DAB2IP, called AIP1B, that localizes to the mitochondria and enhances generation of mitochondrial reactive oxygen species (mitoROS), leading to chronic inflammation and vascular disease (Z. Li et al., 2020).

DAB2IP in nervous system

DAB2IP is highly expressed in neuronal tissues, being detected in several region of adult mouse brain such as hippocampus, midbrain, cerebral cortex and cerebellum (Homayouni et al., 2003). In the mouse brain, DAB2IP activity is related to Reelin signaling, a molecular pathway that controls neuronal differentiation and migration, dendrite maturation, and synaptic plasticity. In particular, in neurons DAB2IP interacts with

Disabled-1 (Dab1), a cytosolic adapter protein of the Reelin dependent lipoprotein receptors ApoER2 and VLDLR. Interacting with DAB1, DAB2IP acts as a downstream effector in the Reelin signaling pathway that influences Ras signaling during brain development (Homayouni et al., 2003).

DAB2IP is also essential in the process of neuronal differentiation from human mesenchymal stem cells (hMSCs), where high levels of DAB2IP maintain the status of hMSCs. Differently from prostate cells, DAB2IP negatively regulates GSK3β activity in 3A6-hMSCs; downregulation of DAB2IP reduced Ser9-phosphorylated GSK3β, causing elevated GSK3β activity. This event resulted in the mesenchymal-to neuroepithelial stem cell transition (MtNeST) and neuronal differentiation, which were accompanied by a reduction of cell proliferation but not apoptosis or cellular senescence (Chang et al., 2013).

DAB2IP is essential also in the maturation of the neuronal system, indeed, high levels of DAB2IP were found in mouse embryonic brain either at in Purkinje cell precursors either in the neuroprogenitor cells of cortical plate from which originate cerebellum and cerebral cortex respectively (Homayouni et al., 2003). In developing of neocortex, DAB2IP is necessary for the migration of late-born principal cortical neurons destined for upper cortical layers, resulting in the transition from the multipolar to the bipolar stage of glial-dependent locomotion (Lee et al., 2012). Intriguingly, DAB2IP activity results in Rap1 mediated multipolar to bipolar transition and integrin signaling terminal somal translocation (Qiao & Homayouni, 2015). Moreover, DAB2IP dependent neuronal migration and positioning is sustained by expression of several major neuronal microtubule associated proteins (MAPs) which are important for neurite growth and stabilization (Lee et al., 2012). Dab2IP is required for early stages of Purkinje cell (PC) dendrite development and formation of cerebellar synaptic structures. The CpG85 site-specific methylation of mDab2ip gene results in a specific Dab2ip transcript variant with a full pleckstrin homology (PH) domain related to synapse formation in the brain (Salami et al., 2015). Indeed, Dab2IP is highly expressed in Purkinje cell soma and dendrites and localized to presynaptic structures. Deficiency of Dab2IP produces a number of cerebellar abnormalities such as a delay in the development of Purkinje cell (PC) dendrites, a decrease in the parallel fiber synaptic marker VGluT1, and an increase in the climbing fiber synaptic marker VGluT2, resulting thus in altered number of synapses in the cerebellum (Qiao et al., 2013).

Given its role in the nervous system, alterations in DAB2IP expression and function may be implicated in neurodegenerative disease and brain cancer. In fact, it has been reported that DAB2IP was elevated in the brain of Alzheimer's disease (AD) Tg2576 mice. Authors suggest that DAB2IP may enhance neuronal degeneration in AD by sustaining Aß-mediated apoptosis of cerebral endothelial cells (H. Wang et al., 2015).

Regarding brain cancer, DAB2IP is certainly implicated in medulloblastoma (MB), where it behaves as a tumor suppressor. In fact, it is downregulated in MB cells and in primary MB tissue. Notably, reduced DAB2IP expression correlates with poor overall survival of MB patients, independently of clinical variables such as age, metastatic stage, and histology. In addition, reduced expression of DAB2IP conveys MB resistance to irradiation-induced cell death (Smits et al., 2012).

DAB2IP in cancer

DAB2IP is a tumor suppressor protein and is frequently downregulated in many cancer types such as prostate (Xie et al., 2010) (J. Min et al., 2010), breast (Ji et al., 2015), lung (Yano et al., 2005), intestine (Dote et al., 2005) (Sun et al., 2018), liver (Qiu et al., 2007a), ovary(Zong et al., 2020) and medulloblastoma (Smits et al., 2012). A tumor suppressive role for DAB2IP has been unveiled also in urothelial carcinoma of the bladder (UCB) (Shen et al., 2014), renal cell carcinoma (RCC) (Jacobs et al., 2015)(Cao et al., 2020), nasopharyngeal carcinoma (B. Wang et al., 2017), esophageal squamous cell carcinoma (ESCC) (Sun et al., 2018), and osteosarcoma (He et al., 2018).

Notably, there is one exception to this established expression trend: DAB2IP levels were significantly higher in cutaneous squamous cell carcinomas (CSCC) compared to benign soft fibroma used as control. In addition, DAB2IP expression was associated with the high degree of malignancy and depth of tumor infiltration (Yuan et al., 2020), suggesting that DAB2IP may behave as an oncogene in this cancer type. Skin is the most superficial organ of the human body, while most tumors arise from internal tissues. Based on this, the authors speculate that the effects of DAB2IP in tumor formation and progression could be organ- or tissue-selective (Yuan et al., 2020). This hypothesis will require further studies.

From a molecular point of view, DAB2IP dampens several signaling cascades associated with cell proliferation, survival, and metastasis (L. Liu et al., 2015) (Bellazzo et al., 2016).

Although DAB2IP is frequently down-regulated in tumors, its loss does not induce cancer formation per se, but strongly supports cell transformation induced by driver mutations in various oncogenic pathways (Bellazzo et al., 2016). In addition, DAB2IP negatively correlates with disease progression, and DAB2IP downregulation sustains not only EMT phenotype but also tumor angiogenesis and tumor-associated premetastatic niche formation (Ji et al., 2015). Indeed, DAB2IP depleted tumors present deeper cell invasion and enhanced lymph node metastasis (J. Min et al., 2010). DAB2IP expression levels also negatively correlate with the immune infiltration of pro tumoral B cells and CD8+ T cells that accelerate unfavorable clinicopathological features and poor prognosis (Cao et al., 2020). Finally, reduced DAB2IP expression leads to the maintenance of cancer stem cells (CSC), that are the principal responsible of tumor recurrence after surgery and chemotherapy (Ji et al., 2015) (Eun Jin Yun et al., 2016) (W. Qin et al., 2018) (Zong et al., 2020). CSCs are more resistant to therapies thanks to their pro survival and self-renewal properties. CSCs activate antiapoptotic signals, drug efflux pumps, and DNA damage repair (DDR) machinery, that strongly protect tumor cell from radiation and anti-tumoral drugs. By reinforcing many of these pathways, loss of DAB2IP acquires a crucial role in chemio and radio resistence (Lan Yu et al., 2016) (Eun Jin Yun et al., 2017)(Eun Jin Yun et al., 2019)(Duan et al., 2020).

In summary, DAB2IP can potentially be used as diagnostic biomarker, and evaluation of its levels in tumor samples may be a good predictor for the therapeutic strategy to approach. In addition, DAB2IP could be a potential target of innovative drugs aimed to restore its physiological expression levels, that could be used in combination with traditional chemio and radio therapy, in order to augment their therapeutic effects (Kaijie Wu et al., 2013).

Differently from other tumor suppressor genes, DAB2IP is rarely mutated or deleted in human cancers; instead, various mechanisms for DAB2IP inactivation have been identified, both at the transcriptional and post-transcriptional level.

Genetic alterations

As already mentioned, the frequency of cancer related mutations in DAB2IP gene is relatively low, indicating that DAB2IP inactivation occurs via other mechanism. However, some genetic alteration in DAB2IP have been described and may be linked to

tumor onset (Bellazzo & Collavin, 2020). For instance, a certain number of missense mutations have been reported in luminal B breast cancers, occurring in functional domains and thus potentially compromising DAB2IP activity (Olsen et al., 2017). In colorectal (CRC) and gastric (GC) cancers with high-microsatellite instability (MSI-H), frameshift mutations in DAB2IP were detected with a frequency of 23.5% and 19.0% respectively (Son et al., 2018). Interestingly, DAB2IP has been described to undergo a gene fusion event in acute myeloid leukemia. Specifically, a translocation t(9;11)(q34;q23) joins intron 9 of the MLL gene to exon 2 of AIP1/DAB2IP, disrupting the PH domain and possibly affecting also DAB2IP expression, since the gene is placed under the MLL promoter. This chromosomal translocation abolishes tumor suppressor DAB2IP function (Von Bergh et al., 2004). There are also two single nucleotide polymorphisms (SNP) in the DAB2IP gene that may be associated with specific cancers; with aggressive prostate cancer in European and African American man (Duggan et al., 2007). and with increased risk and early onset of lung cancer in under 60 non-smoker males with a family history of cancer (Yang et al., 2011). Although the biological relevance of such variants remains to be studied, these two SNPs may be considered for clinical use in diagnosis, screening, and treatment of prostate and lung cancers.

Transcriptional alterations

The best characterized mechanism for DAB2IP downregulation in cancer is through epigenetic silencing.

Methylation of the DAB2IP promoter inactivates DAB2IP expression in many cancers, including prostate, breast, gastrointestine , lung and liver. Compared to normal tissue and cell lines, the various CpG islands of DAB2IP promoter are hypermethylated and DAB2IP transcription is silenced. Accordingly, in tumoral cells expression of hDAB2IP was substantially restored after 5-Aza-dC treatment, a cytosine analog that inhibits DNA methylation by DNA methyltransferases (H. Chen et al., 2003)(Yano et al., 2005)(Qiu et al., 2007).

In addition to promoter methylation, also modifications of the histone core in the nucleosome structure of DAB2IP gene can be altered in cancer. Indeed, both hDAB2IP and mDAB2IP promoters show aberrant histone methylation and acetylation that lead to suppression of DAB2IP levels in tumor cells (H. Chen et al., 2003).

Histone acetylation was also linked to regulation of the hDAB2IP gene in normal prostatic epithelia and prostate cancer (PCa) cells. In normal prostate histone H3 in DAB2IP promoter is acetylated. On the contrary, high level of histone deacetylases (HDACs) in PCa cells resulted in DAB2IP gene deacetylation and concomitant decrease of DAB2IP expression. Accordingly, treatment with trichostatin A (TSA), a histone deacetylase inhibitor, increased the levels of acetyl-histone H3 and restored DAB2IP expression in PCa cells (H. Chen et al., 2003).

Also histone methylation can regulate DAB2IP transcription; specifically, it has been reported that TNF-α-induced changes in histone methylation of specific regions of the gene have been linked to differential expression of DAB2IP variants in endothelial cells (Z. Li et al., 2020).

Regulation of DAB2IP promoter methylation is mainly dependent on the Human enhancer of Zeste homolog (EZH2), a histone lysine N-methyl transferase enzyme that is a component of PRC2 (polycomb-repressive complex-2). EZH2 acts on different histones; in detail, EZh2 methylates Lys 27 and Lys 9 of histone H3 and Lys 26 of histone H1, resulting in the compaction of chromatin, and silencing of related gene. In addition, Ezh2 is able to recruit and cooperate with the histone deacetylase (HDCA) to reinforce gene silencing. In PCa epithelium the Ezh2/HDAC complex targets DAB2IP and blocks its transcription (H. Chen et al., 2005). On the contrary, cells treated with S-adenosyl homocysteine hydrolase inhibitor (DZNep), a potent inhibitor of EZH2 histone methyltransferase activity, induced a delayed increase of DAB2IP (Smits et al., 2012). Accordingly, an inverse correlation between Ezh2 and DAB2IP expression has been identified in many tumors, including prostate (H. Chen et al., 2003) (H. Chen et al., 2005) medulloblastoma (Smits et al., 2012), hepatocellular carcinoma (HCC) (Z. R. Wang et al., 2016) ovarian (Zong et al., 2020) and breast cancer (L. Wang et al., 2013).

Interestingly, DAB2IP repression has been associated to various conditions that modulate EZh2 activity. For instance, in hepatocellular carcinoma (HCC) under chronic infection of Hepatitis B virus (HBV), the tumor suppressor DAB2IP is downregulated at promoter level by chromatin compaction mediated by histone modification H3K9me3 (D. Y. Wang et al., 2016). Also, the loss of BRCA1 in breast cancer cells results in EZH2 recruitment on DAB2IP gene, and its consequent repression (L. Wang et al., 2013).

The inverse relationship between DAB2IP and EZh2 is also maintained in ovarian cancer stem cells (OCSC), where high levels of EZH2 result in H3K27me3 on DAB2IP promoter. EZH2-dependent DAB2IP repression induces a stem like phenotype via activation of Wnt5b/RAC1/c Jun axis. Reciprocally, pharmacological inhibition of EZH2 restored DAB2IP levels and reduced stem features and aggressiveness of ovarian cancer cells (Zong et al., 2020). Finally, low DAB2IP levels mediate by EZh2 inhibition in cancer and CSC correlate significantly with poor overall survival of patients and resistance to radiotherapy (Smits et al., 2012). In this context, DAB2IP could be a prognostic marker to identify patients at higher risk of resistance to radiotherapy; in addition, since high levels of EZH2 precede loss of DAB2IP expression, EZH2 could be used as an early marker to discriminate low and high risks patients (Jacobs et al., 2015).

Post-transcriptional alterations

Post-transcriptional silencing by microRNAs (miRNAs) and long noncoding RNAs (lncRNA and circ-RNA) is another mechanism that contributes to reduce DAB2IP levels in cancer cells. By binding the 3′ UTR region of their complementary mRNAs, miRNAs or can act as inhibitors of translation or can induce target transcripts degradation (Oliveto et al., 2017). Since DAB2IP has a long 3′ UTR sequence, it is a good candidate for a direct silencing by micro-RNAs (miRNAs). A growing number of miRNA have been described to downregulate DAB2IP expression in cancer: miR-32, miR92a-b, miR-138, miR149 -3p, miR 182, miR-328-5p, miR-367, miR-431, miR-566, miR-889, miR-1266 and miR-1307-3p (Bellazzo & Collavin, 2020).

Notably, miRNA mediated downregulation of DAB2IP can occurs also through indirect and more complex regulatory networks. For example, DAB2IP is targeted by miR-889 that is upregulated after arsenite-induced repression of its competitive circRNA-008913 (Xiao et al., 2018). Same regulation occurs for DAB2IP-targeting miR-328-5p, that is upregulated after overexpression of circ-5692 (Z. Liu et al., 2019).

In 2018, the Collavin's group identified miR-149-3p as a negative modulator of DAB2IP (Bellazzo et al., 2018). DAB2IP downregulation by miR-149-3p was shown to amplify NF-κB signaling, resulting in enhanced prostate and breast cancer cell invasiveness, but also enhanced secretion of pro-inflammatory and pro-angiogenic factors (Bellazzo et al., 2018).Interestingly, miR-149-3p is secreted by prostate cancer cells and can induce

DAB2IP downregulation in nearby vascular endothelial cells, promoting their consequent migration and proliferation (Bellazzo et al., 2018). This study therefore described for the first time a mechanism of cell non-autonomous downregulation of DAB2IP.

Post-translational alterations

Little is known about post-translational modifications on DAB2IP and their possible functional impact. Currently, two regulatory phosphorylations have been identified. The first is by RIP-1 kinase on Ser 604. This is a stimulatory modification that helps DAB2IP to acquire an open conformation necessary for complex formation with PI3K-Akt and ASK1. However, pS604 in DAB2IP is not necessary for its RasGAP activity (R. Zhang et al., 2007) (Xie et al., 2010) (J. Min et al., 2010). The second, is by AKT1 kinase on Ser 847. This modification is inhibitory, as it reduces DAB2IP interaction with Ras and TRAF2, leading to Ras-MAPK activation and inhibition of ASK/JNK (Dai et al., 2014). However, there are no strong evidences linking these modifications to DAB2IP tumor suppressive functions in cancer.

DAB2IP activity can also be inhibited by aberrant protein-protein interactions. For instance, DAB2IP is bound by mutant forms of p53 (mutp53) that accumulate in the cytoplasm of cancer cells, preventing its interaction with physiological partners. As a consequence, mutp53 binding to DAB2IP reprograms the cell response to multiple inputs. Through this action, mutp53 induces pro-oncogenic behaviors such as cell proliferation and invasiveness upon insulin-induced AKT1 activation (Valentino et al., 2017), and promotes cell invasiveness and survival by fostering the NF-κB pathway upon inflammation (Di Minin et al., 2014).

DAB2IP protein is generally rather stable, but some studies described its regulation by specific ubiquitin E3 ligases in various cell models. Two potential phospho-degron sequences with homology to consensus Fbw7 substrates are present in the DAB2IP protein; accordingly, Fbw7 complexed with the Cullin-Ring based E3 ligase SCF induced DAB2IP degradation after CK1-mediated phosphorylation. In addition, also Skp2 complexed with SCF, and Smurf1 have been reported to enhance proteasome-mediated DAB2IP degradation (Dai et al., 2014) (Xiaoning Li et al., 2016) (Tsai et al., 2014).

Interestingly, it has been observed that chronic TNF-α stimulation of ECs increases expression of SMURF1, leading to proteolytic degradation of the longer AIP1A isoform, but not the shorter AIP1B isoform (Z. Li et al., 2020); this suggests that a different composition of regulatory domains at the N and C terminal region can not only impact on DAB2IP protein function, but also on its stability.

Extracellular stimuli regulate DAB2IP stability and function

The tumor suppressor DAB2IP modulates many oncogenic pathways through its GAP activity or scaffolding functions, controlling specificity, amplitude and duration of intracellular signalling events in response to many extracellular inputs. However, much less is known about DAB2IP regulation by extracellular stimuli.

First of all, DAB2IP has a fundamental role in modulating the cell's response to inflammatory signals, in particular TNF-α and INF-gamma, and its expression can be modulated by inflammatory cytokines.

In response to TNF-α, DAB2IP limits inflammation and survival by repressing NF-kB signaling, while it concomitantly sustains apoptosis by activating ASK1-JNK/p38 MAPK signaling (R. Zhang et al., 2007). Reciprocally, TNF-α can regulate DAB2IP expression in a cell and context dependent manner. In endothelial cells (ECs), chronic exposure to TNF-α (but also interleukin 1b) promotes transcription of the pro-inflammatory isoform AIP1B, and induces selective degradation of the anti-inflammatory isoform AIP1A (Z. Li et al., 2020). DAB2IP regulation by TNF-α has also been reported in ovarian cancer stem cells (OCSCs), where TNF-α secreted by Dendritic Cells and Cytokine-induced killer (DC-CIK) cells upregulates DAB2IP mRNA and protein, enhancing activation of the ASK1-JNK pathway and promoting OCSC death (W. Qin et al., 2018).

Among cytokines, DAB2IP limits INF-gamma mediated cell proliferation blocking JAK/STAT pathway. Interestingly after 1 hour of INF-gamma treatment, DAB2IP protein increases in vascular smooth muscle cells (VSMCs)(Luyang Yu et al., 2011). On the contrary, IFN-gamma (but also CCL3, CCL5, IGF and FGF7) secreted from infiltrating T cells downregulate DAB2IP in renal carcinoma cells (RCCs). In this context, DAB2IP downregulation by INF-gamma correlates with higher invasivity of RCC (Yeh et al., 2015). Notably, also chronic inflammation induced by cigarette smoke (CSE) was reported to downregulate DAB2IP. Indeed, long-term exposure of 16HBE cells to CSE

correlated with increased levels of EZH2 and H3K27me3, as well as a massive decrease of DAB2IP protein when compared to untreated cells (Anzalone et al., 2019).

DAB2IP also modulates cell responses to hormones, and its expression can be affected by hormonal stimuli. Evidences show that DAB2IP limits dihydrotestosterone-induced proliferation of prostate cancer cells (K. Wu et al., 2014)and estrogen-induced invasion of renal cancer cells (Yeh et al., 2015). Interestingly, conditioned medium of Infiltrated T cells increased estrogen-receptor ERβ expression in RCC cells, with concomitant reduction of DAB2IP protein levels. Although ERβ does not bind directly to DAB2IP promoter, DAB2IP downregulation could be due to ERβ-mediated overexpression of EZH2 (Yeh et al., 2015). DAB2IP can also be regulated by progesterone; during throphoblast implantation DAB2IP is transcribed by SP1 under the control of the circadian gene BMAL1. Stimuli from the hypothalamic-pituitary-gonadal axis foster BMAL1/DAB2IP expression. Notably, upon dysregulated BMAL1 pathway, DAB2IP levels could be restored by dose-dependent administration of progesterone in human trophoblast cells (Shang Li et al., 2017).

DAB2IP modulates signaling by growth factor receptors and its expression can be regulated by growth factors. Although no specific study analyzed the role of DAB2IP in the cell's response to EGF stimulation, DAB2IP expression can be modulated by EGF. In a context of early response, DAB2IP protein levels increased after EGF administration, suggesting that DAB2IP may be induced to dampen tyrosine receptor signaling through its inhibitory action on Ras and PI3K. Interestingly, DAB2IP was not induced by EGF after silencing of NF1 or RASA1 in HeLa cells, suggesting that such regulation requires other RasGAPs (Hennig et al., 2016). The mechanism driving DAB2IP accumulation upon EGF treatment is unexplored.

Interestingly, the extracellular concentration of glucose can regulate DAB2IP levels, suggesting the intriguing possibility that the protein may also respond to variations in other metabolites. In endothelial cells grown in low glucose, mRNA and protein levels of DAB2IP are reduced if compared with culture in high glucose. DAB2IP downregulation leads to HIF1-a (hypoxia inducible factor alpha) activation and induction of VEGF (vascular endothelial growth factor). Curiously, high insulin concentration in culture medium reduced DAB2IP levels in high glucose conditions; however, the mechanism involved remains undefined (Shuang Li et al., 2015).

It is possible that oxidative stress also impinges on DAB2IP expression. In fact, hypoxia–reoxygenation (H/R) treatment increased DAB2IP protein levels in the extravillous trophoblast cell line HTR8/SVneo. In this context, DAB2IP repression by reactive oxygen species (ROS) correlates with inhibition of the invasive and migratory abilities of trophoblasts (Shan et al., 2016).

Finally, in addition to soluble factors, also density and composition of the ECM can potentially modulate DAB2IP. In fact, a recent study with colon cancer cell lines demonstrated that soft fibrin matrix mediates DAB2IP suppression. Specifically, DAB2IP protein levels were dramatically decreased in 3D cultures of HT29 cells compared to 2D controls but were restored when matrix stiffness was increased (M. Zhang et al., 2019).

This last observation raises the intriguing hypothesis that DAB2IP expression and function may respond to mechanical inputs from the substrate (ECM) as well as from neighboring cells.

Aim of the thesis

The tumor suppressor DAB2IP/AIP1 is a regulator of multiple cytoplasmic pathways involved in proliferation, survival and apoptosis, and its downregulation in human malignancies supports tumor progression and aggressiveness. In particular, DAB2IP is a negative modulator of oncogenic RAS/MAPK, TNF/NF-kB and PI3K/AKT pathways, thus attenuating cell proliferation, survival, metastasis, and chemoresistance (L. Liu et al., 2015) (Bellazzo et al., 2016).

Interestingly, DAB2IP is ubiquitously expressed in human tissues with the sole exception of blood and immune cells, which organize specific and transitory cell-matrix and cell-cell contacts (Harjunpää et al., 2019). Moreover, recent studies highlighted a possible correlation between DAB2IP and E-cadherin (Brüser & Bogdan, 2017) (Raza et al., 2019). So, it is possible that physical interactions with the surrounding tissue may control DAB2IP expression. In support of this notion, Zhang and collaborators recently reported that growth in a soft fibrin matrix caused DAB2IP suppression in colon cancer cells. Specifically, DAB2IP protein levels were reduced in 3D cultures of HT29 cells compared to 2D controls, but were restored when matrix stiffness was increased (M. Zhang et al., 2019).

Starting from these observations, I hypothesized that the cell's physical interactions with the surrounding tissue could represent a novel extracellular input in regulating the tumor suppressor DAB2IP. The first part of this Thesis is aimed to determine if mechanical signals generated at cell-ECM and cell-cell contact sites can impinge on DAB2IP protein levels via remodeling of the cytoskeleton. In particular I tried to define the specific contribution of Focal Adhesions and Adherens Junctions, respectively sensors of cell attachment to the substrate and cell interaction with other cells.

Considering that DAB2IP can modulate multiple signaling pathways downstream to several receptor and sensors, I also hypothesized that DAB2IP could modulate the cellular response to mechanical inputs. Changes in the physical and structural features of the tissue are sensed by cells at FAs and AJs, and induce remodeling of the actomyosin cytoskeleton to maintain cell shape and function (Humphrey et al., 2014). Importantly, they also modify the cellular transcriptional program, through activation of the co-transcriptional regulators YAP and TAZ (Dobrokhotov et al., 2018). Specifically, formation and contraction of stress fibers, as a result of tensile force generated by ECM

stiffness, favors nuclear localization and transcriptional activity of YAP/TAZ. In contrast, formation and contraction of the circumferential actin belt, coordinated by force generated at Adherens Junction, causes nuclear exclusion of YAP - contributing to the phenomenon of contact inhibition of proliferation (Furukawa et al., 2017). Therefore, since YAP and TAZ are major effectors of mechano-transduction, the second part of this Thesis is aimed to investigate whether DAB2IP can modulate YAP/TAZ activity, in particular in response to mechanical inputs such as contact inhibition.

By exploring if external forces can modulate DAB2IP levels and/or functions, and whether DAB2IP can modulate the cellular response to external forces, this Thesis aims to better define how signal transduction events can interact with mechanical inputs from the cell's environment. This knowledge may have strong implications for a comprehensive definition of the molecular and genetic determinants of cancer aggressiveness.

Results

Part one

The research activity of the thesis focused on regulatory signals that may play a crucial function in dictating DAB2IP levels and function within an epithelial tissue.

To aim this, mechanical inputs from the extracellular environment generated either at cell-ECM and cell-cell contact site have been assessed. I investigated if mechanical cues exerted on different cellular actomyosin structures can modulate DAB2IP levels and functions, with the aim to subsequently identify the underlying molecular mechanism.

Cell interactions with the extracellular matrix can affect DAB2IP protein levels

In silico analysis of tissue expression of human DAB2IP protein performed on web tools such as The Human Protein Atlas (https://www.proteinatlas.org/ENSG00000136848-DAB2IP/tissue) showed that DAB2IP is expressed in adherent cell lines, but limited or completely absent in non-adherent cell lines, such as blood and immune cells. Same results were obtained in the Collavin's laboratory during a project aimed to analyze the expression of various hDAB2IP isoforms in multiple cell lines (not shown).

Blood and immune cells acquire a round shape because they are not caged into a solid and static tissue. Although there are many possible explanations for lack of DAB2IP expression in blood cells, it is tempting to speculate that mechanical inputs from cell-ECM and cell-cell contact within solid tissues could be a crucial extracellular cue in regulating DAB2IP.

To indagate the contribution of ECM and neighboring cells in DAB2IP modulation, all the following experiments have been performed in the non-transformed mammary epithelial cell line MCF10A, with normal expression and function of Focal Adhesions (FAs) and Adherens Junctions (AJs), that act as sensors of cell-ECM and cell-cell contact, respectively.

Initially, due to technical and logistic reasons, I could not monitor formation and maturation of FAs and AJs by using microscopy, so I evaluated the status of cell junctions indirectly by analyzing specific markers. For FAs, I measured the activating phosphorylation in tyrosine 397 of Focal Adhesion Kinase (FAK), which is generally used as a marker of Integrin signaling, although its role in the transduction of mechanical inputs from ECM is still unclear (Pirone et al., 2006). The formation and maturation of AJs has been provisionally monitored by immunoblotting of E-

cadherin protein levels. These results will need to be complemented by immunofluorescence of junctional markers such as Paxillin, Vinculin, E-cadherin, and α/β catenin, to provide more exhaustive information about organization and remodeling of FAs and AJs.

FAs are integrin-based multiprotein structures that form mechanical links between the intracellular actin and the extracellular matrix. In epithelial tissue, FAs serve as anchorage device for stationary cells, ensuring cell morphology and polarity. FAs are also mechanosensitive complexes, that translate mechanical inputs into biochemical signals (Kechagia et al., 2019).

To understand the potential impact of cell-ECM contact in controlling DAB2IP protein levels, I compared MCF10A cells grown attached to a Petri dish or in suspension after detachment from the dish. To this aim, I used two different treatments: Trypsin, that breaks interactions by degrading adhesion proteins, and EDTA, that breaks interactions by chelating Calcium and Magnesium ions (Fig. 1a). The results confirmed the role of cell-ECM contact in DAB2IP modulation. Detached cells acquired a round shape coherent with loss of the canonical cytoskeleton structure of adherent cells (not shown). Concomitantly, loss of cell-ECM contact induced a drastic reduction in activating phosphorylation of FAK, suggesting a parallel reduction in FAs and stress fibers.

When cells were detached from the petri dish, I observed a significant reduction of DAB2IP levels, showing a clear correlation with FAK activity (i.e. phosphorylation). Interestingly, although in some experiments it was not obvious (e.g. blot in Fig. 1a), in most detachment (and re-attachment) experiments I also observed a change in total FAK protein levels (e.g. Fig.1c, Fig.4a), that are reduced in suspended cells.

To monitor Adherent Junctions (AJs), I analyzed E-cadherin; interestingly, detachment by trypsinization, but not EDTA treatment, caused a marked decrease in E-cadh – possibly due to proteolysis of the extracellular domain. Of note, detachment by trypsin had a greater impact on DAB2IP levels, suggesting that E-Cadherin might be directly involved in the mechanisms regulating DAB2IP expression.

To corroborate the potential impact of cell-ECM contact in controlling DAB2IP stability, I decided to asses DAB2IP protein levels during the process of Focal Adhesion formation.

To this aim, I prepared protein lysates at different times after re-plating trypsin-detached MCF10A cells on plastic Petri dishes. Upon detachment, loss of cell-ECM contact resulted in rounded cells (Fig. 1b) with drastic reduction of total and p-Y397 FAK levels. As expected, detached cells also had reduced levels of DAB2IP protein (Fig. 1c). The attachment of suspended cells resulted in rapid formation of focal adhesions. Indeed, 4 hours after seeding on plastic, cells acquired a spread

shape (Fig. 1b) and FAs were active, as suggested by increase of total and phosphorylated FAK. In parallel, there was a progressive increase in DAB2IP protein levels (Fig. 1c), again showing a correlation with FAK activity. Interestingly, also E-cadherin protein levels were modulated during cell detachment and subsequent reattachment on the Petri dish, with a behavior similar to DAB2IP (Fig. 1c). Notably, at early time points during re-attachment, DAB2IP levels were further downregulated, before the marked subsequent accumulation (Fig. 1c). This pattern may be linked to the specific mechanism of DAB2IP regulation, that remains unknown; for instance, it is possible that enhanced DAB2IP degradation continues for some time even when the stimulus is suppressed, generating a "delayed response" when compared to E-cadherin.

In conclusion, these results support the hypothesis that cellular contacts, either with the ECM or with surrounding cells, may be a relevant factor controlling DAB2IP protein levels.

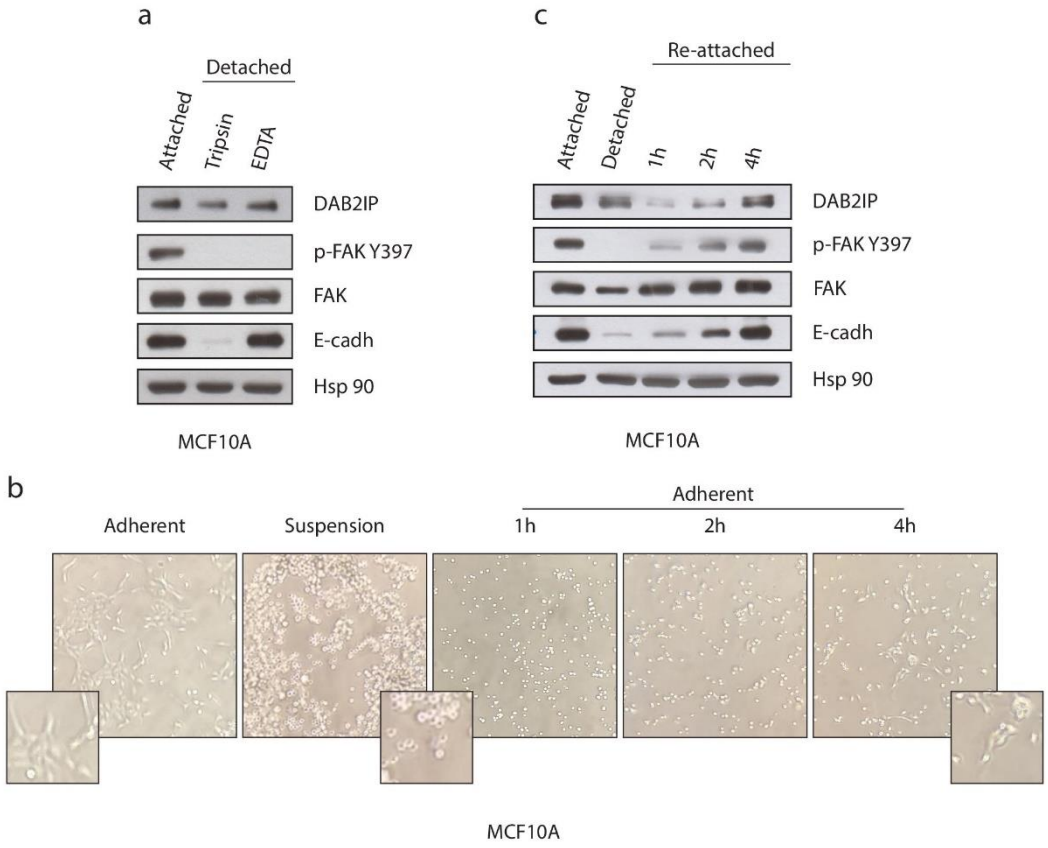


Fig. 1 Cell-ECM interactions sustain DAB2IP levels.
a) Cell detachment decreases DAB2IP protein levels. MCF10A cells were cultured until sub-confluence for 48 hours before being detached by trypsin treatment, or 5mM EDTA as indicated. After 15 minutes adherent and detached cells were collected and lysed. DAB2IP, p-FAK Y397, total FAK and E-cadherin were detected by immunoblotting, with HSP90 as loading control.
b) Representative pictures of mammary epithelial cells at different states of attachment. MCF10A cells were cultured until sub-confluence for 48 hours (Attached). Then, cells were detached by trypsinization as in b). After 15 minutes, cells were seeded again in Petri dishes and photographed at the indicated times (Re-attached).
c) Cell attachment increases DAB2IP protein levels. MCF10A cells were cultured as described in b. At the indicated times, re-attached cells were collected and lysed. DAB2IP, p-FAK Y397, total FAK and E-cadherin were detected by immunoblotting, with HSP90 as loading control.

DAB2IP protein levels are affected by cell density within confluent 2D cultures.

In addition to substrate attachment, it is possible that also cell-cell contact impinges on DAB2IP protein levels. To explore this hypothesis, I plated MCF10A cells at different concentrations, achieving increasing degrees of confluence and cell constriction within monolayers (Fig. 2a). Cell confluency was monitored by counting the number of cells on a given area of substrate (Fig. 2b). Notably, DAB2IP protein levels were strikingly regulated by cell density, increasing progressively from sparse cells to confluent cells, reaching maximum levels when cells formed an hyperconfluent monolayer (Fig.2c). Interestingly, although maturation of AJs has not been properly monitored, the progressive increase in cell density correlated with a gradual increase of E-cadherin protein levels (Fig.2c).

To understand the basis of this regulation, I analyzed DAB2IP mRNA by RT-PCR; I detected a modest but significant difference in DAB2IP mRNA between sparse and dense cells, but no relevant changes between confluent and hyperconfluent cell monolayers. This suggests that growing cells in sparse conditions can possibly affect DAB2IP transcription, but most clearly it shows that high density packing of epithelial cells regulates DAB2IP protein at the post-transcriptional level (Fig 2d).

This experiment also recapitulated the expected cell density-mediated inhibition of YAP/TAZ. In fact, transcription of CYR61 and ANKRD1, two well established YAP/TAZ target genes, was progressively downregulated by increasing cell density (Fig. 2e), with a corresponding reduction of cell proliferation (Fig. 2f).

Epithelial cells cultured at different density change their mechanical properties. Specifically, compared to cells cultured in low density (single cells), cells in high density (monolayer) form fewer stress fibers, but can develop a contracted circumferential actin belt (Furukawa et al., 2017). In a confluent monolayer cells have less space to anchor to the substrate, therefore integrin-mediated focal adhesions, stress fibers, and cell contractility - mainly depending on ECM - are reduced (Aragona et al., 2013).

Although in my experiment cytoskeleton morphology was not analyzed by microscopy, I assessed FAK activity and E-cadherin levels by immunoblotting, as a proxy to monitor perturbations in cell mechanical status. The analysis revealed an increase of E-cadherin in dense cultures compared to sparse cells (Fig. 2c), suggesting a more organized and robust circumferential actin belt. On the contrary, in confluent cells I did not observe the expected reduction of FAK activity, that would indicate fewer stress fibers. However, total E-cadherin levels and FAK activity are not exhaustive parameters to define changes in cell tension; thus, in this experiment, DAB2IP regulation cannot

be associated to mechanical stimuli from cell density. Nonetheless, the increase of DAB2IP levels by cell crowding clearly indicates a regulation based on cell-cell contact, and suggests a possible implication in the phenomenon of contact inhibition of proliferation (CIP).

Intriguingly, cell density sustains DAB2IP protein levels while at the same time it inhibits YAP/TAZ activity; thus, under these conditions, DAB2IP and YAP/TAZ are inversely regulated.

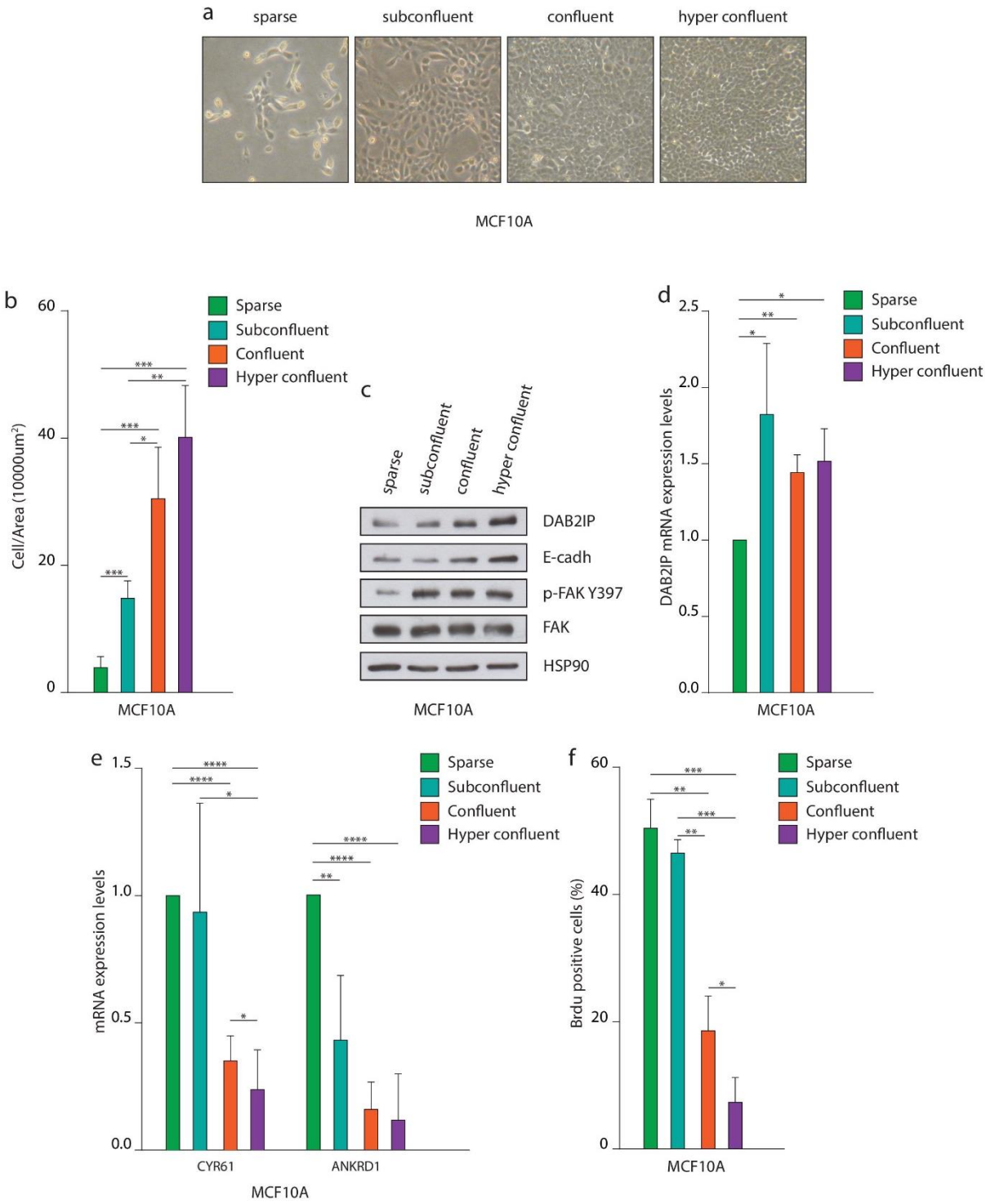


Figure 2. Increasing cell density in monolayers sustains DAB2IP protein levels, while it inhibits YAP/TAZ activity and cell proliferation.

a) Representative images of mammary epithelial cells cultured at different density. MCF10A were plated at different concentrations and photographed after 48 hours (sparse= 2500 cells/cm2; subconfluent= 15000 cells/cm2; confluent= 50000 cells/cm2; hyperconfluent= 75000 cells/cm2).

b) Quantification of mammary epithelial cells density. MCF10A cells were cultured as described in a), and nuclei were labelled with Hoechst for 5 minutes. Graph indicates the number of nuclei in a square area of 10000 um^2 (mean +/- SD; n=4; *P<0.05** P<0.01, *** P<0.001).

c) Higher cell density increases DAB2IP and E-cadherin levels but does not change FAK activity. MCF10A cells were cultured as described in a). DAB2IP, E-cadherin, p-FAK Y397 and total FAK were detected by immunoblotting with HSP90 as loading control.

d) Higher cell density does not affect DAB2IP mRNA levels. MCF10A cells were cultured as described in a). Expression of DAB2IP was measured by RT-qPCR. Values are normalized on H3 (mean ± SD; n = 3; *P<0.05** P<0.01)

e) Higher cell density inhibits transcription of YAP/TAZ target genes. MCF10A cells were cultured as described in a). Expression of CYR61 and ANKRD1 was measured by RT-qPCR. Data were normalized on histone H3 (mean ± SD; n =4; *P<0.05, ** P<0.01, **** P<0.0001).

f) Higher cell density inhibits cell proliferation. MCF10A cells were cultured as described in in a), and labelled with BrdU for 2 hours. Graphs summarize the percentage of BrdU positive nuclei (mean +/- SD; n=3; *P<0.05** P<0.01, *** P<0.001).

Preliminary evidences that DAB2IP protein levels are modulated by substrate stiffness in breast cells

Recently, the stiffness of extracellular matrix has been identified as potential modulator of DAB2IP (M. Zhang et al., 2019). This result suggests that formation and activity of stress fibers generated by cell adhesion to the ECM plays a fundamental role in DAB2IP regulation. However, cytoskeleton contractility not only depends on the ECM, but is also modulated by cell-cell contact. In order to investigate if DAB2IP is regulated by cytoskeleton rearrangement exerted either at cell-ECM and/or cell-cell contact sites, I performed experiments with different conditions of actomyosin tension, trying to distinguish the contraction of stress fibers at Focal Adhesions from contraction of the circumferential actin belt at Adherens Junctions.

Formation of stress fibers can be modulated by culturing cells on a soft or a stiff substrate: on a soft substrate cells become rounded and the cytoskeleton collapses, on a stiff substrate cells spread and develop more anchoring points and stress fibers. Actomyosin tension at cell-cell junctions can be modulated by culturing cells at different degrees of confluency: high cell density results in more adherent junctions and thus cells establish a circumferential actomyosin ring. Moreover, cells grown in high density (monolayer) on a stiff substrate can apparently resemble cells cultured at low density on a soft substrate, since they share reduced stress fibers and cytoplasmic localization of YAP (Pavel et al., 2018) (Panciera et al., 2017); however, they differ for the presence of a contracted circumferential actin belt only in high density conditions. I therefore asked whether DAB2IP is differentially regulated under these specific conditions.

At first, I asked if mechanical inputs modulate DAB2IP in a tumor context different from colorectal cancer as previously shown by Zhang at al. (2019). I started my experiments using a model of triple negative breast cancer. In particular, MDA-MB-231 cells are characterized by a mesenchymal-like morphology; the absence of E-cadherin limits the formation of the actomyosin ring at the level of AJs, and the high turnover of FAs results in dynamic stress fibers. Nonetheless, MDA-MB-231 form cell junctions in monolayer, and thus can be used to monitor DAB2IP regulation by cell-cell contact.

To this aim, I plated MDA-MB-231 cells at low or high cell density, in order to establish a different rate in cell to cell contact, in either a soft or stiff substrate (Fig. 3a). In parallel, to validate if mechanical inputs from cell-ECM and cell-cell contact can also modulate DAB2IP function in normal mammary cells, I plated MCF10A under the same experimental conditions used for MDA-MB-231 (Fig. 3b).

In MDA-MB-231 plated in soft condition, DAB2IP protein levels decreased both in high and low density (Fig. 3a). Notably, DAB2IP protein levels also changed between high- and low-density conditions. Specifically, western blot analysis showed that DAB2IP increases in high cell density, both in soft and in stiff conditions (Fig. 3a).

Similar results were obtained in MCF10A cells plated either on stiff and on soft substrate: DAB2IP protein levels increased in high density, where I could also confirm YAP inhibition (i.e. increase of inhibitory phosphorylation) by cell-cell contact (Fig. 3b).

These experiments have been performed only once and need to be repeated. Nonetheless, DAB2IP downregulation in mammary cells grown on a soft ECM is consistent with previous observations in colon carcinoma cells (M. Zhang et al., 2019). Although preliminary, these data indicate that DAB2IP levels are increased by cell-cell contact independently of the stiffness of the substrate on which cells are cultured. This result suggests that not only cell-ECM interactions (that depend on rigidity of the extracellular matrix) but also cell-cell interactions (that depend on density) contribute to DAB2IP upregulation and/or stability.

Interestingly, DAB2IP levels increase with cell density also in MDA-MB-231, a cell line lacking E-cadherin, where AJs and circumferential actin belt are supposedly less developed than in MCF10A. It is possible that a circumferential actin belt is organized in MDA-MB-231 cells through E-cadherin independent junctions, but to address this hypothesis it will be necessary to analyze adhesion proteins and cytoskeletal markers by immunofluorescence. Thus, although I cannot exclude that DAB2IP is regulated by mechanical tension exerted by the cortical actin ring upon cell-cell junctions, the increase of DAB2IP levels by cell density is also compatible with a molecular signaling that is independent from AJs and reorganization of the actomyosin cytoskeleton.

In summary, my results suggest that DAB2IP modulation by mechanical stimuli from the ECM is a common feature in tumor epithelial cells, presumably sustained by increasing of stress fibers. In addition, they establish that DAB2IP protein levels are increased by cell density in tumor and normal cells, possibly via a mechanism independent of contraction of the acto-myosin cytoskeleton.

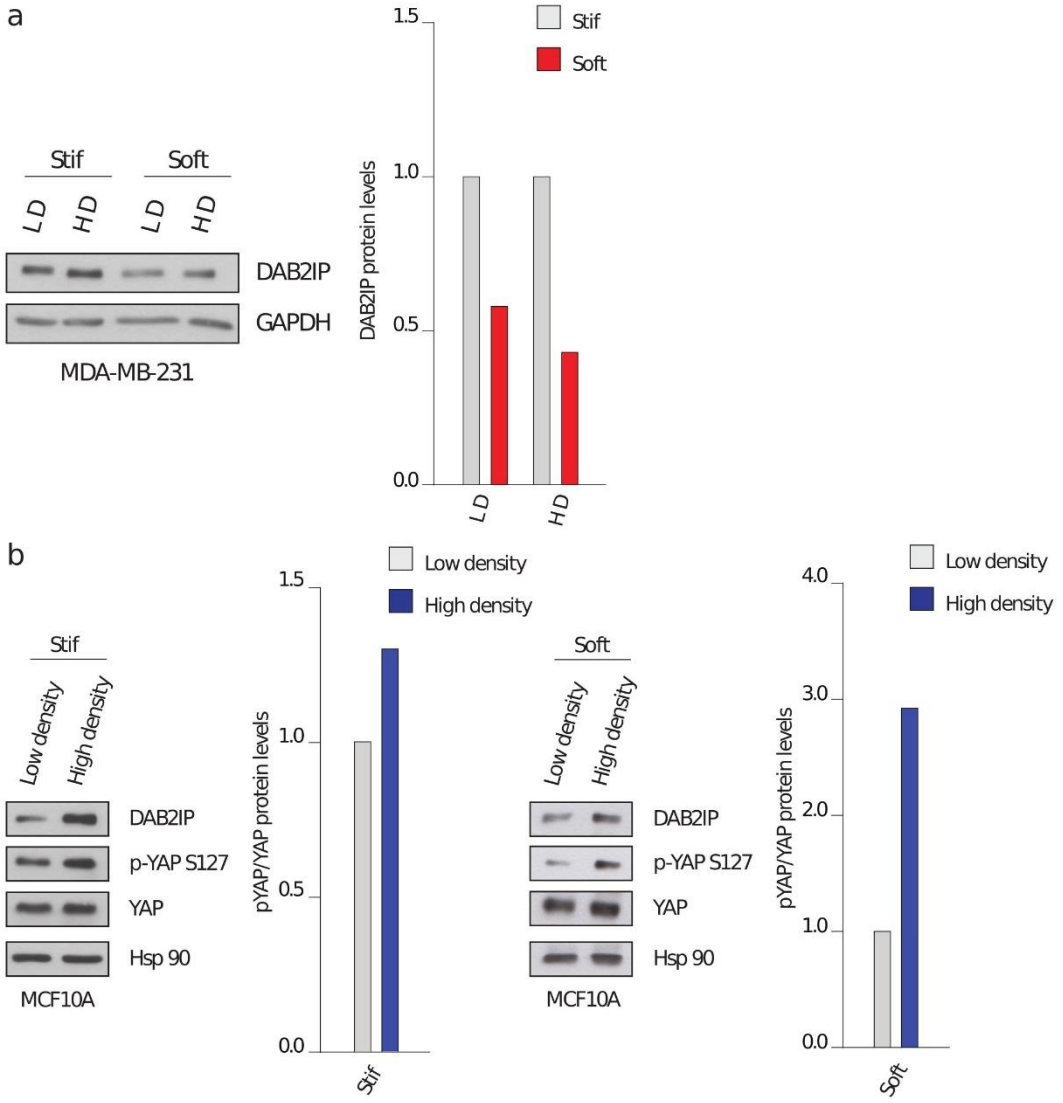


Fig. 3 Both ECM rigidity and cell-cell contact modulate DAB2IP protein levels in normal and tumor cells
a) ECM stiffness and cell-cell contact modulate DAB2IP protein levels in a triple-negative breast cancer cell line. MDA-MB-231 cells were cultured for 24h after seeding at low or high cell density on either soft (0,5 KPa) or stiff (50 KPa) fibronectin-coated hydrogel matrix. DAB2IP levels were measured by immunoblotting, with GAPDH as loading control. DAB2IP protein levels were quantified by densitometry on autoradiography film (performed using ImageJ). Data refer to a single experiment.
b) ECM stiffness and cell-cell contact modulate DAB2IP protein levels in non-tumoral mammary cells. MCF10A cells were cultured for 48h after seeding at low or high density on either soft (0,5 KPa) or stiff (50 KPa) fibronectin-coated hydrogel matrix. DAB2IP, phosphorylated (p-S127) and total YAP have been measured by immunoblotting, with HSP90 as loading control. Protein levels were quantified by densitometry on autoradiography film (performed using ImageJ). Data refer to a single experiment.

Focal Adhesions are involved in DAB2IP regulation

Comparing at first adherent with suspension cells, and then cells cultured on soft vs stiff substrates, I found that alteration of cell-ECM contact impinges on DAB2IP protein levels. In order to define the molecular mechanism involved in such regulation, I started to analyze the contribution of Focal Adhesions and FA-mediated signaling.

Although FAK is not the only mediator of FAs activity upon ECM stimuli, as a first approach I tested the impact of FAK inhibition on DAB2IP levels. To this aim I used PF573228 (PF573), a widely used ATP competitive FAK inhibitor that blocks the activating phosphorylation on Tyr 397. Cells treated with PF573228 are affected by decreased focal adhesion turnover and destabilization of actin filaments, and thus undergo a low tensional state (Slack-Davis et al., 2007).

To investigate the potential impact of Focal Adhesions in controlling DAB2IP levels upon cell-ECM contact, I measured DAB2IP protein in suspended MCF10A cells that were treated with the FAK inhibitor PF573228 before seeding on Petri dishes (Fig. 4a).

Upon detachment, loss of cell-ECM contact induced disruption of focal adhesions, confirmed by reduction of FAK levels and FAK phosphorylation in Tyr397. Detached MCF10A cells had reduced levels of DAB2IP protein, corroborating my previous results. The subsequent reattachment of suspended cells resulted in rapid formation of focal adhesions; at 6 hours from seeding focal adhesions are active, as confirmed by phospho-FAK in control treated cells. As expected, no phospho-FAK was detected at 6 hours in cells treated with PF573228. In parallel to FAK activity, I observed modulation of DAB2IP, with reduced levels of DAB2IP protein in PF573 treated cells compared to untreated controls. This experiment therefore revealed a correlation between FAK activity and DAB2IP levels, suggesting that DAB2IP accumulation upon cell-ECM adhesion could potentially require signaling by FAs.

Next, to test if this regulation is maintained also in attached cells, I treated with PF573228 adherent cells plated at different degrees of confluency. Specifically, I measured DAB2IP levels upon FAK inhibition in sub-confluent MCF10A (Fig. 4b), and in MCF10A cultured at very low or very high cell density; this allowed me to evaluate the impact of FAs signaling with the concomitant contribution of cell-cell contact (Fig. 4c).

Under all culture conditions, PF573228 treatment reduced the levels of DAB2IP protein - even in highly crowded cells, where DAB2IP levels are higher than in other conditions. However, DAB2IP downregulation by FAK inhibition is noticeably less efficient in conditions of high cell density. This result suggests that FAK activity is relevant to sustain DAB2IP levels in sparse cells, while

it is largely dispensable in crowded, confluent cells. Presumably, cell-cell contact can control DAB2IP levels independently of Focal Adhesions or FAK activity.

Notably, PF573228 had no impact on the levels of E-cadherin. When analyzed in MCF10A cells detached and re-attached on Petri dishes, there was an evident correlation between FAK activity and E-cadherin protein levels (Fig. 4a); in contrast, the results obtained with PF753288 clearly demonstrate that FAK inhibition does not perturb E-cadherin expression (Fig. 4 a, b, c).

Finally, to gain some insight on the molecular basis of this regulation, I analyzed DAB2IP mRNA levels by RT-PCR; I detected a reduction of DAB2IP mRNA in confluent MCF10A monolayers (HD) treated with FAK inhibitor, suggesting that FAK signaling may be involved in controlling DAB2IP transcription (Fig. 4d).

Together, these data show that FAK activity directly or indirectly sustains DAB2IP levels, suggesting a novel potential pathway regulating DAB2IP. In this perspective, it should be noted that PF573228 can also inhibit in a less selective manner other kinases such as Pyk2, CDK1/7, and GSK-3β. Thus, even if the role of these kinases in regulating DAB2IP levels is unknown, FAK-independent effects of PF573228 cannot be excluded. To address this possibility, specific experiments, including siRNA-mediated depletion of endogenous FAK, will be necessary.

DAB2IP dependency on FAK activity suggests a possible involvement of FAs in DAB2IP regulation by ECM-cell contact. Moreover, since PF573228 treatment weakly inhibits cytoskeleton contractility, it is also possible that cytoskeleton tension could be the molecular mediator of DAB2IP regulation by cell-ECM contact in a transcriptional and/or post-transcriptional manner. To address this last hypothesis , experiments with a specific and exhaustive modulation of FAs will be necessary.

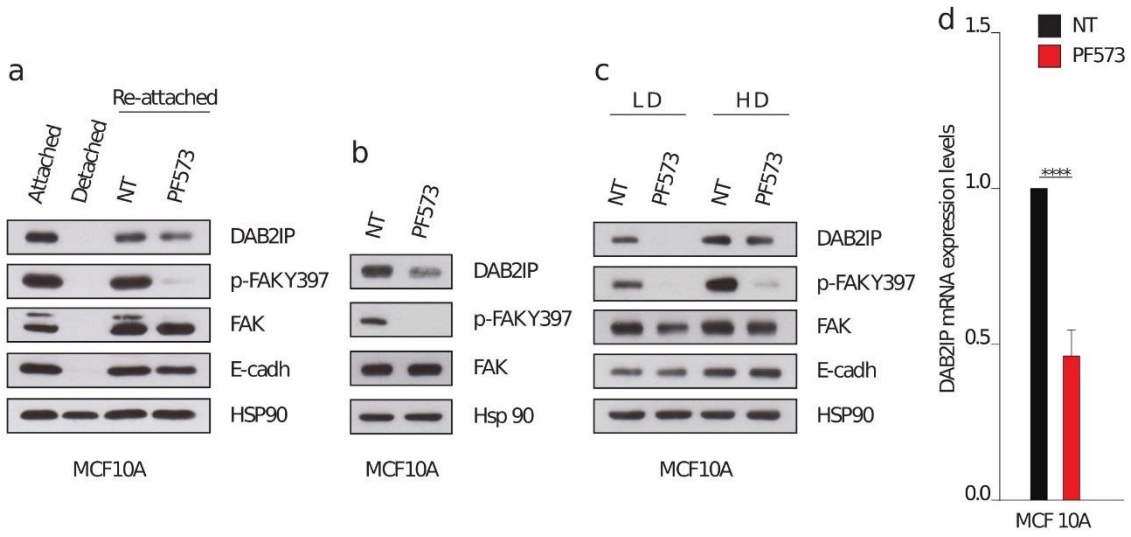


Fig. 4. Focal Adhesion Kinase (FAK) activity sustains DAB2IP protein levels.
a) FAK inhibition decreases DAB2IP levels during cell attachment. MCF10A cells were cultured until sub-confluence for 48 hours and then collected by trypsinization. Before seeding in new Petri dish, detached cells were treated with 10uM PF573228 (PF573). After 6h re-attached cells were collected and lysed. DAB2IP, p-FAK Y397, total FAK and E-cadherin were detected by immunoblotting, with HSP90 as loading control.
b-c) FAK inhibition decreases DAB2IP protein levels in adherent cells. MCF10A were cultured to a sub-confluent state **(b)** or were plated at different concentration (Low density=2500 cells/cm2 and High density=50000 cells/cm2) and cultured for additional 48h **(c)**. Then cells were treated with 10uM PF573228 (PF573) for 6h before lysis. DAB2IP, p-FAK Y397, total FAK and E-cadherin were detected by immunoblotting, with HSP90 as loading control.
d) FAK inhibition decreases DAB2IP mRNA levels. MCF10A cells were cultured in High density as described in c). Expression of DAB2IP was measured by RT-qPCR. Values are normalized on H3 (mean ± SD; n = 4; **** P<0.0001)

Adherens Junctions are involved in DAB2IP regulation

Comparing sparse and crowded cells, I found that high density clearly increases DAB2IP protein levels. In order to define the molecular mechanism involved in such DAB2IP regulation, I started to explore the contribution of adherens junctions and the associated transmembrane protein E-cadherin.

Preliminary data showed that both DAB2IP protein levels and YAP/TAZ activity are regulated by cell density. Starting from these data, I first tested the involvement of AJs in the confluency-dependent regulation of DAB2IP. I treated MCF10A cells plated in high density with EGTA, a chelator of extracellular Ca2+ that breaks the homophilic binding between E-cadherins.

Cells treated with 10 mM EGTA for three hours remained attached to the substratum, but lost adhesion to adjacent cells and acquired a round shape (Fig.5a). This treatment obviously induces a dramatic change of the actomyosin cytoskeleton and corresponding mechanical tension. Then, I washed cells to remove the EGTA and added fresh culture medium in order to reestablish extracellular Ca2+, thus favoring AJs reassembly. Cells acquired a cuboidal shape, suggesting presence of a physiological cytoskeleton, likely characterized by a circumferential actin belt (Fig.5a).

Immunoblotting analysis revealed that disruption of AJs caused a reduction in DAB2IP protein levels, while their reformation resulted in the rescue of DAB2IP levels (Fig.5b). As expected, EGTA treatment also caused a decrease of E-cadherin, dependent on clathrin-mediated endocytosis and degradation (Brüser & Bogdan, 2017).

Interestingly, after EGTA washout, E-cadherin levels were not rescued as efficiently as those of DAB2IP (Fig.5b), suggesting that synthesis rates of DAB2IP and E-cadherin may be different.

Under the same conditions, I verified that functional AJs inhibit YAP/TAZ activity: in fact, transcription of YAP/TAZ target genes CYR61 and ANKRD1 was activated by EGTA treatment, while the reassembly of AJs recovered YAP/TAZ inhibition by cell density (Fig. 5c).

In addition to detaching cadherins, chelation of extracellular Ca2+ by EGTA can have additional effects on cell biology: for instance, it impinges on intracellular Ca2+ signaling with implications for the physiology of endoplasmic reticulum and mitochondria (Clapham, 2007).

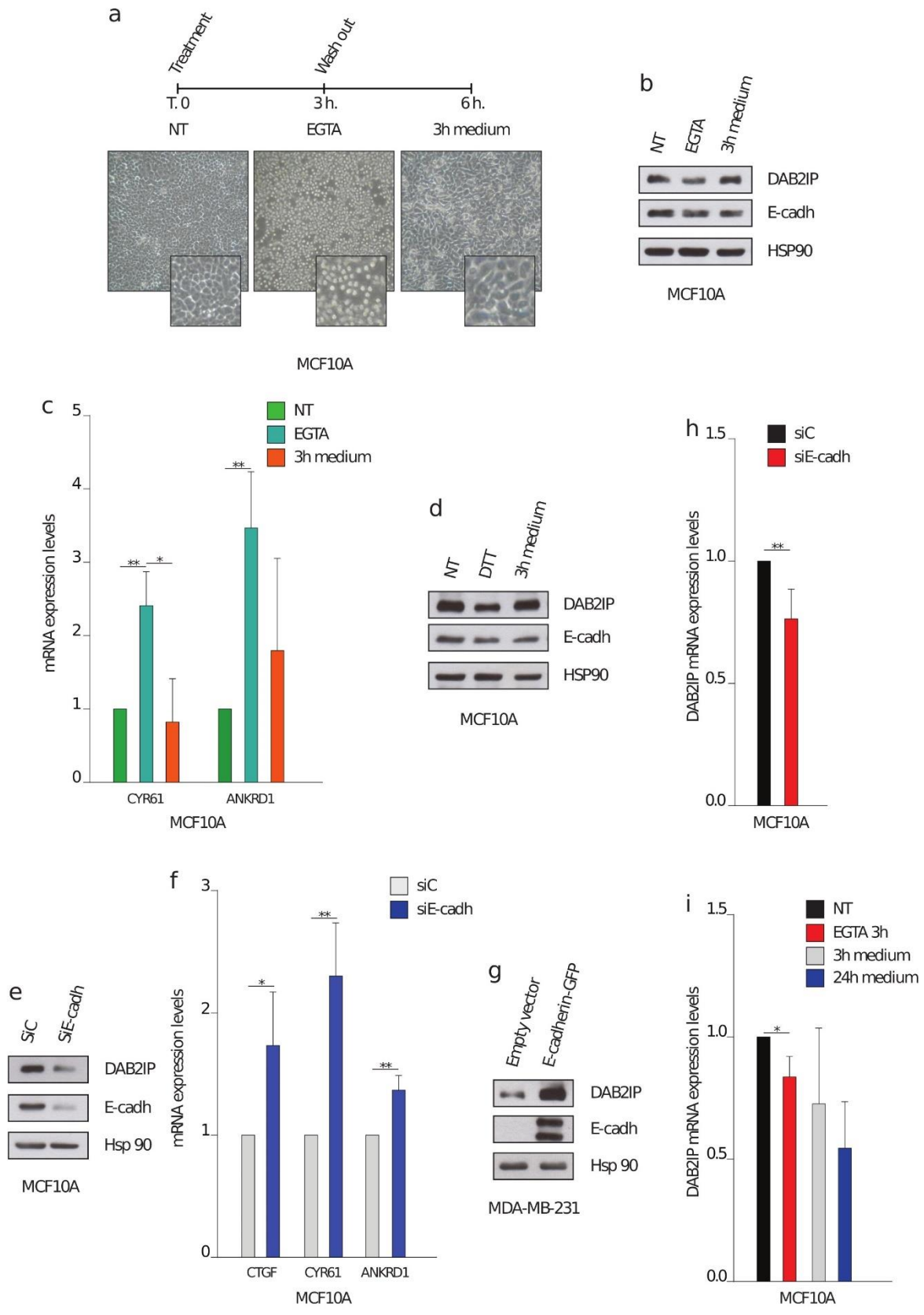
I therefore performed a similar experiment by treating MCF10A cells with DTT, a drug that compromises the functionality of AJs by reducing disulfide bridges. In fact, disulfide bridges within cadherins' structure are essential to make functional cell-cell adhesions (Ozawa & Kemler,

1990). As shown in Figure 5d, cells treated with 10mM DTT for three hours showed a reduction of both DAB2IP and E-cadherin protein levels, that were rescued with the reestablishment of cell adhesions after drug washout (Fig. 5d). Notably, also DTT has additional effects on cell biology, impacting on ER functions and oxidative stress (Cheng et al., 2016). However, results were the same with both treatments, strongly implying that disruption of AJs reduces DAB2IP protein levels. Since these treatments are not expected to strongly affect Focal Adhesions, the data implicate cadherin-dependent cell-cell contacts in DAB2IP regulation.

Since in epithelial cells E-cadherin is the main component of Adherens junctions, I tested the impact of disrupting AJs through E-cadherin silencing. In line with EGTA and DTT experiments, transfection of a siRNA against E-cadherin in MCF10A cells decreased DAB2IP protein levels (Fig. 5e). Accordingly, E-cadherin depletion promoted YAP/TAZ activity, as shown by the increased transcription of target genes CTGF, CYR61 and ANKRD1 (Fig. 5f).

On the contrary, to test the impact of increasing formation of AJs, I transiently transfected an E-cadherin expression construct in MDA-MB-231 cells, a cell model of triple negative breast cancer characterized by total absence of endogenous E-cadherin. Under these conditions, DAB2IP protein levels were upregulated by E-cadherin overexpression, a condition that is expected to increase the number of AJs and stimulate cell-cell interactions (Fig. 5g).

Finally, to establish the molecular basis for the observed DAB2IP protein changes, I analyzed its mRNA expression by RT-PCR. Interestingly, siRNA-mediated depletion of E-cadherin in MCF10A cells caused a reduction in DAB2IP mRNA levels (Fig. 5h) paralleling the observed reduction in protein levels (Fig. 5e). This indicates that DAB2IP transcription could be reduced by long-term depletion of E-cadherin in epithelial cells. In contrast, when AJs are disrupted via EGTA treatment and then re-established by EGTA washout, variations in DAB2IP mRNA are not correlated with fluctuations in DAB2IP protein (Fig. 5b), thus suggesting a post-translational mechanism of regulation. This concept is further supported by the fact that the short time points of the experiment (1-3 hours) do not allow for modest variations in mRNA transcription to be translated in significant changes of protein levels.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Treatment
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b
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DAB2IP
E-cadh
HSP90
MCF10A
c
NT
EGTA
3h medium
mRNA expression levels
CYR61
ANKRD1
MCF10A
d
NT
DTT
3h medium
DAB2IP
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E-cadh
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i
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EGTA 3h
3h medium
24h medium
DAB2IP mRNA expression levels
MCF10A

Figure 5. Cell-cell junctions affect DAB2IP protein levels and YAP/TAZ activity.
a) Adherens junctions are reversibly affected by EGTA. MCF10A cells were cultured for 48h after seeding at high density (50000cells/cm2). Then, high-density cultures were exposed to 10 mM EGTA for 3 hours causing detachment of cell-cell junctions. Fresh medium was added for 3h to reassemble Adherens Junctions. Representative pictures of cells at the indicated time points are shown.
b-c) EGTA treatment reversibly reduces DAB2IP protein levels and activates YAP/TAZ signaling. MCF10A cells were cultured and treated as in a). **b)** DAB2IP and E-cadherin levels were detected by immunoblotting, withHSP90 as a loading control. **c)** Expression of YAP target genes CYR61 and ANKRD1 was measured by RT-qPCR. Data are normalized on histone H3 (mean ± SD; n =3; *P<0.05, ** P<0.01).
d) DTT treatment reversibly reduces DAB2IP protein levels. MCF10A cells were cultured as in a) but were treated with 10 mM DTT for 3 hours. DAB2IP and E-cadherin levels were detected by immunoblotting, with HSP90 as a loading control.
e-f) E-cadherin depletion reduces DAB2IP protein levels and activates YAP/TAZ signaling. MCF10A cells were transfected with the indicated siRNAs (20mM) and cultured for 48h until sub confluent. **e)** DAB2IP and E-cadherin levels were detected by immunoblotting, with HSP90 as a loading control. **f)** Expression of CYR61 and ANKRD1 was measured by RT-qPCR. Data are normalized on histone H3 (mean ± SD; n =3; *P<0.05, ** P<0.01).
g) E-cadherin overexpression increases DAB2IP protein levels. MDA-MB-231 cells were transfected with pCDNA3-E-cadherin-GFP and cultured until a sub-confluence state for 24 hours. DAB2IP and E-cadherin were measured by immunoblotting, with HSP90 as loading control.
h) E-cadherin depletion reduces DAB2IP mRNA levels. MCF10A cells were cultured and transfected as in e). Expression of DAB2IP was measured by RT-qPCR. Data are normalized on histone H3 (mean ± SD; n = 4; ** P<0.01)
i) EGTA treatment reduces DAB2IP mRNA levels. MCF10A cells were cultured and treated as in a). Expression of DAB2IP was measured by RT-qPCR. Data are normalized on histone H3 (mean ± SD; n =3; *P<0.05)

F-actin cytoskeleton integrity and tension regulate DAB2IP protein levels.

Focal Adhesions and Adherens Junctions are molecular complex that associate the cell inner cytoskeleton with the surrounding environment. Any alteration of cell-ECM and cell-cell contact impinges on the cytoskeleton structure, that reassembles itself to maintain a physiological tension and mechanical homeostasis of the tissue. Similarly, mechanical stimuli generated by the external environment are sensed by cells through changes in the cytoskeleton, that in turn induce a mechanical and/or biochemical signaling response. Mechanical cues perceived at FAs and AJs result in differential responses of the stress fibers and circumferential actin belt, respectively, since these cytoskeletal structures are specifically linked to FAs and AJs.

Previous results indicate that the tumor suppressor DAB2IP can be modulated by cell-ECM and by cell-cell contact.

Regarding cell-ECM contact, experiments done with cell detachment/attachment, and by plating cells on soft/stiff substrates, suggest a correlation between DAB2IP levels and cytoskeletal rearrangements in response to external mechanical cues. Starting from these evidences, I investigated the role of the actin cytoskeleton in DAB2IP regulation.

To this aim, subconfluent MCF10A cells were treated with different inhibitors of cytoskeleton contractility, in order to alter cellular tension. Latrunculin A and Cytochalasin D are natural compounds that destabilize actin filaments inducing low tensional state of cells. Mechanistically, Latrunculin A binds G- actin and prevents the monomers' polymerization (Yarmola et al., 2000). On the contrary, Cytochalasin D acts by capping F-actin and preventing microfilament elongation (May et al., 1998). I also used Blebbistatin, an inhibitor of non-muscle myosin II (NMII) that suppresses actomyosin contraction causing filaments disruption and low cell tensional state (Kovács et al., 2004). As expected, these drugs induce cytoskeleton disassembly in MCF10A cells, as demonstrated by loss of physiological morphology of actin structures and reduced activating phosphorylation in serine 19 of Myosin Light Chain 2 (MLC2) (Fig. 6b). Concomitantly to cytoskeleton collapse, DAB2IP protein levels decreased (Fig 6a).

Same results were obtained in the breast cancer cell line MDA-MB-231, where the effects of cytoskeleton disruption were monitored by reduction of the mechanosensory protein TAZ (Fig 6c).

These results indicate that DAB2IP levels are affected by cytoskeleton activity in normal and cancer breast epithelial cells.

Then, I investigated if DAB2IP regulation mediated by the cytoskeleton depends solely on the specific actomyosin structures generated at FAs, or is also mediated by tension of the circumferential actin belt organized at AJs.

When cultured at low density, MCF10A cells tend to spread and generate more FAs and stress fibers, with no circumferential actin belt; on the contrary, when cultured in high density MCF10A cells build the actomyosin ring at the level of AJs, and greatly reduce stress fibers.

I therefore cultured MCF10A cells at different conditions of confluency, and treated them with Blebbistatin. Immunoblot analysis indicated that Blebbistatin reduces DAB2IP protein levels independently of the confluency status; similarly to the mechanosensor protein TAZ, analyzed as a control (Fig. 6d). However, DAB2IP downregulation in high cell density was weaker as compared to sparse cells. This data recapitulates the regulation of DAB2IP by FAK inhibition, reinforcing the hypothesis that in sparse cells adhesion to the ECM supports DAB2IP levels, presumably via cytoskeletal tension developed at FAs.

In contrast, in crowded cells the contractile F-actin at AJs is dispensable to sustain DAB2IP levels. Thus, in condition of high cell density other mechanisms unrelated to FAK and NMII sustain DAB2IP levels.

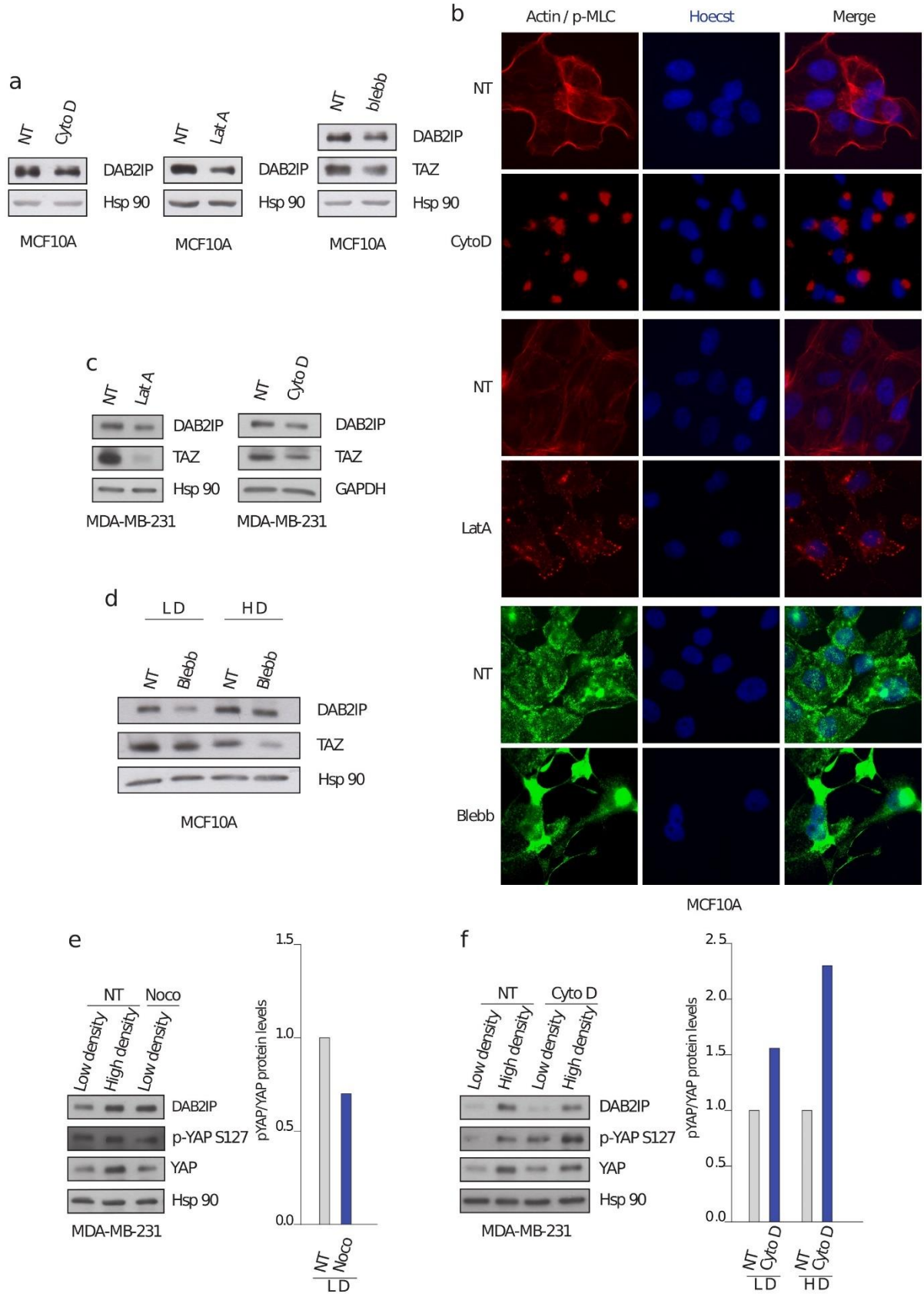
To corroborate these results, I repeated these experiments in the triple-negative breast cancer cell line MDA-MB-231. These cells are characterized by a mesenchymal-like morphology; the absence of E-cadherin limits the formation of the actomyosin ring at the level of AJs, and the high turnover of FAs results in dynamic stress fibers. Thus, compared with the robust and structured cytoskeleton of MCF10A cells, the MDA-MB-231 cytoskeleton is weaker and more flexible (Ong et al., 2020). These cytoskeleton features result in soft cells, with high ability to move (Chiotaki et al., 2014) (Senigagliesi et al., 2019), one of the reasons why MDA-MB-231 are highly aggressive and metastatic.

I previously observed that independently of the stiffness of the substrate on which MDA-MB-231 cells were cultured, high cell density sustains DAB2IP protein levels (Fig. 3a). Thus, in MDA-MB-231 cells, despite the absence of a E-cadherin, DAB2IP is nonetheless accumulated with increasing cell-cell contact. This suggests that an additional mechanism, perhaps impinging on the cytoskeleton, may be functional in DAB2IP regulation.

Starting from these considerations, at first I modulated the cytoskeleton tension driven by cell-ECM contact by treating MDA-MB-231 cells with the microtubule-destabilizing drug Nocodazole (Noco). It has been shown that Nocodazole treatment of sparse cells stimulates stress fibers and

actomyosin cytoskeleton tension (Zhao et al., 2012), as confirmed by YAP activation (indeed, p-YAP protein levels decrease in Noco treated cells). Notably, under these conditions, I observed an increase in DAB2IP protein levels, although not to the same levels observed in the control cells grown at high density (Fig. 6e).

I also treated highly packed MDA-MB-231 cells with Cytochalasin D. In line with previous data, cells cultured in high density have higher DAB2IP protein levels than cells in low density. Notably, Cytochalasin D treatment reduced DAB2IP protein levels in highly packed MDA-MB-231 cells (Fig. 6f). Together these data reinforce the concept that actomyosin activity sustains DAB2IP protein levels, further supporting the hypothesis that DAB2IP may respond to mechanical inputs from the extracellular environment.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Hsp 90
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TAZ
Hsp 90
MCF10A
b
Actin / p-MLC
Hoecst
Merge
NT
CytoD
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LatA
NT
Blebb
MCF10A
c
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Lat A
DAB2IP
TAZ
Hsp 90
MDA-MB-231
NT
Cyto D
DAB2IP
TAZ
GAPDH
MDA-MB-231
d
LD
HD
NT
Blebb
NT
Blebb
DAB2IP
TAZ
Hsp 90
MCF10A
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Noco
Low density
High density
Low density
DAB2IP
p-YAP S127
YAP
Hsp 90
MDA-MB-231
pYAP/YAP protein levels
1.5
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Noco
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Cyto D
Low density
High density
Low density
High density
DAB2IP
p-YAP S127
YAP
Hsp 90
MDA-MB-231
pYAP/YAP protein levels
2.5
2.0
1.5
1.0
0.5
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NT
Cyto D
LD
NT
Cyto D
HD

Fig. 6 Disruption of the actin cytoskeleton affects DAB2IP protein levels.
a) Cytochalasin D, Latrunculin A and Blebbistain decrease DAB2IP in non-tumoral mammary epithelial cells. MCF10A cells were cultured for 48hours to a sub-confluent state. Then cells were exposed to 10 uM Cytochalasin D (Cyto D) for 4hours or to 0,5uM Latrunculin A (Lat A) for 4hours 50uM or to Blebbistatin (Blebb) for additional 24hours. DAB2IP has been detected by immunoblotting, with HSP90 as loading control.
b) Cytochalasin D, Latrunculin A and Blebbistain disrupt the actin cytoskeleton in MCF10A cells. Representative images of cells treated as in a). Cells were stained with an antibody against p-MLC2 ser19 (green) or F-actin was visualized by rhodamine phalloidin (Red). DNA was labelled with Hoechst (blue).
c) Cytochalasin D and Latrunculin A decrease DAB2IP and TAZ expression in breast cancer cells. MDA-MB-231 cells were cultured for 24h to a sub-confluent state. Then cells were exposed to 1uM Cytochalasin D (Cyto D) or 0,5uM Latrunculin A (Lat A) for 4hours. DAB2IP and TAZ have been detected by immunoblotting, with HSP90 or GAPDH as loading control.
d) Blebbistatin decreases DAB2IP levels principally in sparse cells. MCF10A were cultured for 48 hours after seeding at low or high density (2500 cells/cm2 and 50000 cells/cm2 respectively). Then cells were exposed to 50 uM Blebbistatin (Blebb) for additional 24hours. DAB2IP and TAZ were detected by immunoblotting, with HSP90 as loading control.
e-f) Loss of actomyosin contraction affects DAB2IP protein levels in breast cancer cells. MDA-MB-231 cells were cultured for 24 hours at low or high cell density. **e)** Only low density cells were exposed to 3uM Nocodazole (Noco) for 2 hours. **f)** Both low and high density cells were exposed to 1uM Cytochalasin D (Cyto D) for 4 hours. The protein levels of DAB2IP, phosphorylated (p-S127) and total YAP have been measured by immunoblotting, with HSP90 as loading control. Protein bands were quantified and normalized to HSP90 by densitometry of autoradiography film (histograms on the right of western blots).

Part two

The tumor suppressor DAB2IP can modulate the cellular response to a variety of extracellular stimuli such as growth factors, hormones, and cytokines. However, nothing is known about its possible role in modulating cellular responses to mechanical inputs. Having observed that DAB2IP levels respond to mechanical signals from the ECM and from neighboring cells, I asked if DAB2IP may in fact modulate the cell's response to such inputs. Mechanical stimuli exerted at Adherens Junctions and Focal Adhesions impinge on the activity of transcription factors YAP and TAZ (Mugahid et al., 2018) (Nardone et al., 2017). In this second part of my Thesis, I investigated the role of DAB2IP as a potential modulator of the cell's physical properties related to YAP/TAZ signaling.

Preliminary evidence that DAB2IP may modulate the cell's mechanical properties

To define if DAB2IP modulates the mechanical properties of the cell, I first asked if DAB2IP manipulation can impinge on cell morphology. To this aim, I used the non-tumorigenic epithelial MCF10A cell line characterized by luminal ductal features such as basolateral polarization and formation of cell domes in sub-confluent cultures (Fig. 7a). Notably, MCF10A cells depleted for DAB2IP change their structure: they fail to be packaged into a defined cluster, tend to be more spread on the culturing surface, and slightly acquire a spindle-like shape (long and thin). When cultured at very high density, DAB2IP-depleted MCF10A cells reproducibly showed a reduction of the number of cells presents on a given area of substrate (Fig. 7c); this could be due to a change of cell shape, or to a change of size (i.e. hypertrophy). DAB2IP depleted cells have an increased nuclear area (Fig. 7b), which may indicate an increased cell size (Mugahid et al., 2020). However, a larger nuclear area may also reflect a modification of the shape of the nuclear envelope, triggered by cytoskeletal rearrangements. Specific experiments will be required to evaluate if DAB2IP knockdown increases the average size of the cells, or changes the way cells rearrange their cytoskeleton in high density monolayers.

Next, I asked if DAB2IP levels may impact on Adherens Junctions and/or Focal Adhesions, the anchoring structures that sense and modulate extracellular mechanical inputs.

As a first approach, I monitored expression of junctional proteins by immunoblotting. In DAB2IP knockdown cells I detected no significant changes of E-cadherin levels (AJ marker), but a marked increase in FAK levels and activity, as assessed by phosphorylation in tyrosine 397 (Fig. 7d). In particular, DAB2IP depletion strongly increased FAK protein levels, suggesting a specific effect on expression or stability of this kinase. protein. Although I did not detect changes in E-cadherin

levels, I cannot exclude that DAB2IP depletion may affect subcellular localization of the protein and/or maturation of AJs. Specific immunofluorescence assays will be necessary to quantify number, size, and localization of cell junction complexes at the plasma membrane.

Despite all the above limitations, these results highlight a correlation between DAB2IP levels, cell packing at confluency, and FAK phosphorylation, suggesting that DAB2IP may contribute to modulate cell shape – possibly via regulation of Focal Adhesion signaling.

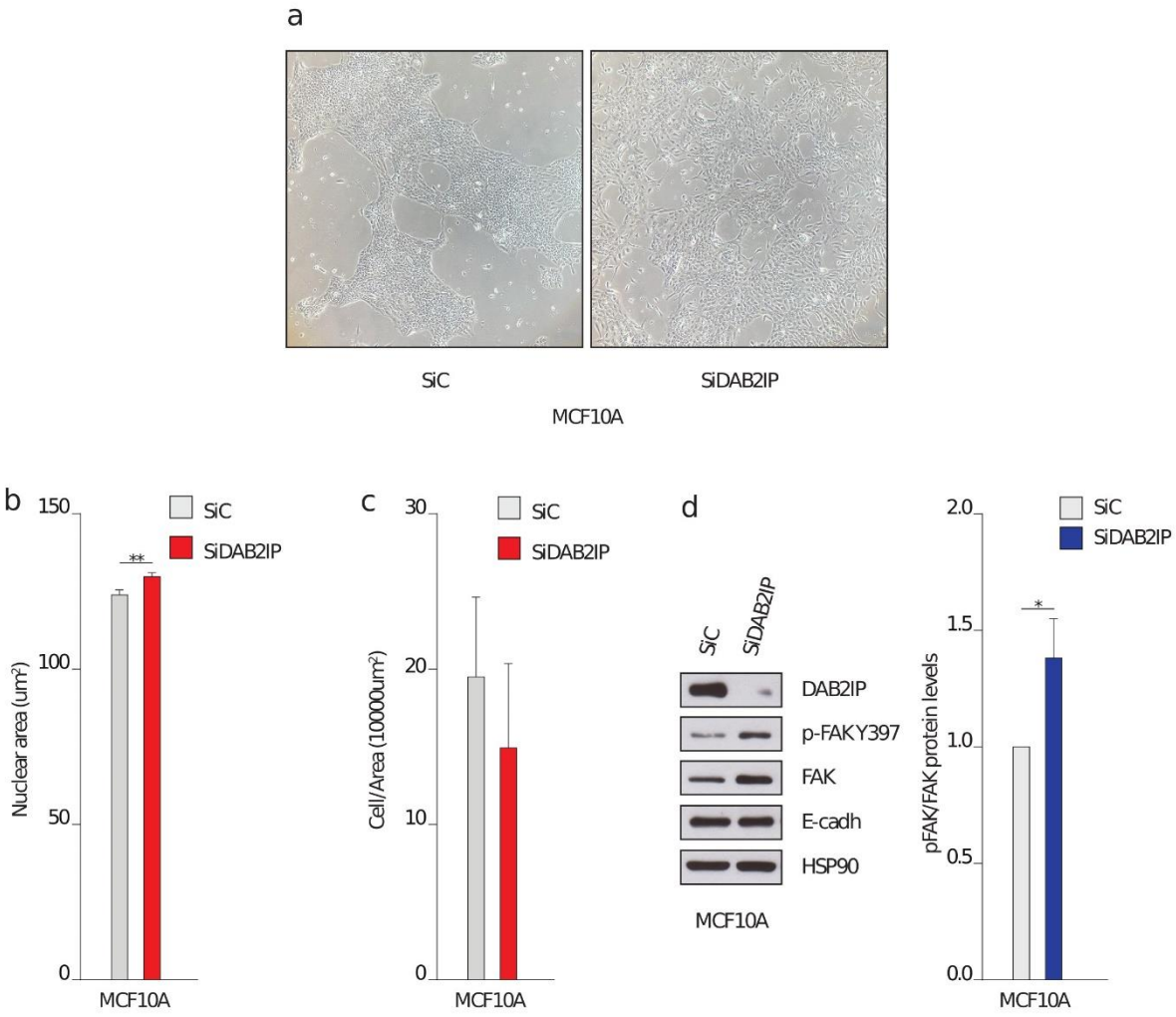
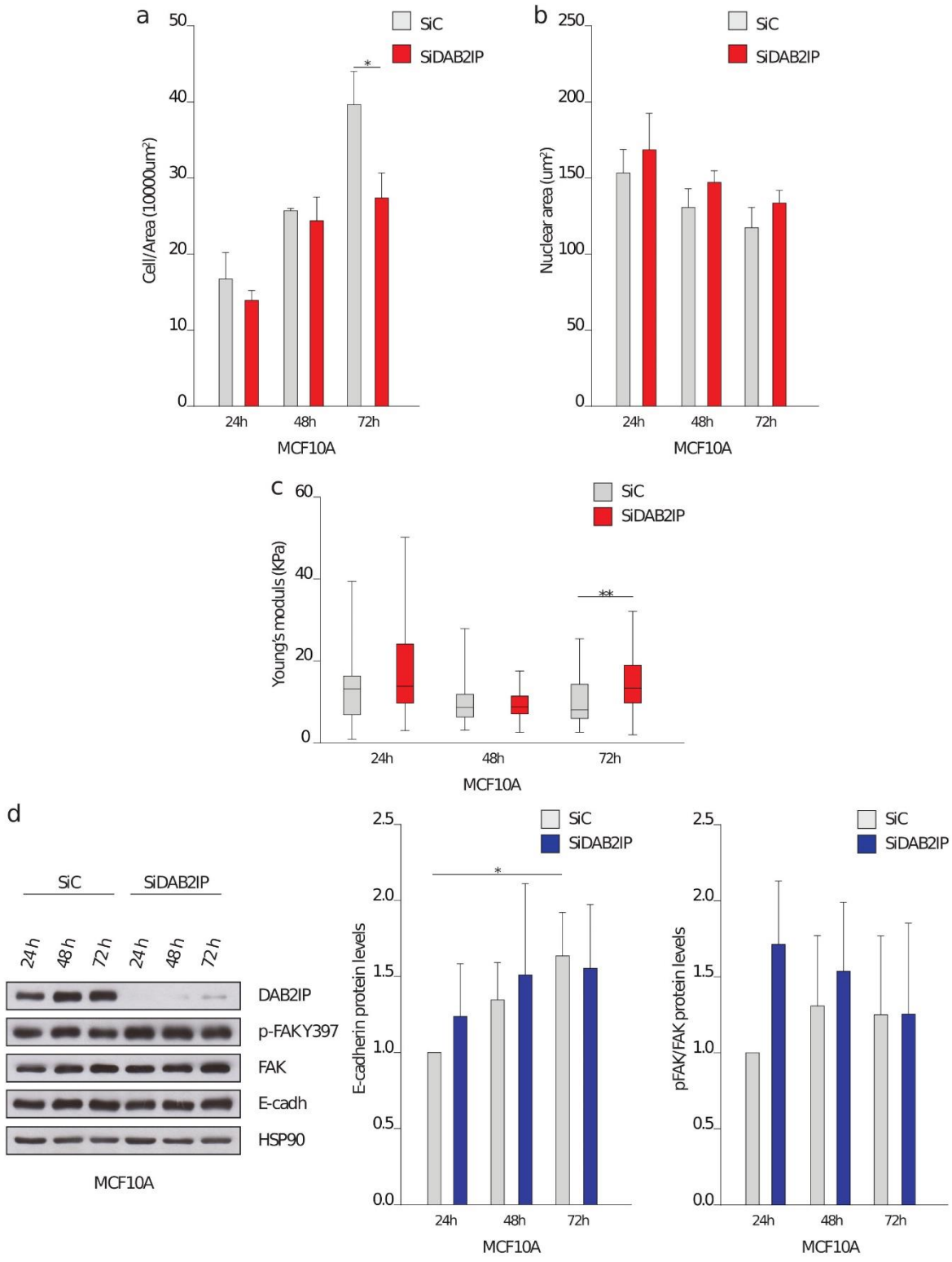


Figure 7. DAB2IP depletion affects cell morphology, apparent size, stiffness, and FAK activity.
a) DAB2IP depletion affects the shape of mammary epithelial cells. MCF10A cells were transfected with the indicated siRNAs (20uM) and cultured for 48h until a sub confluent state. Representative pictures of cells are shown.
b) DAB2IP depletion increases nuclear size. MCF10A cells were cultured as in a). Nuclei were labelled with Hoechst. Nuclear areas were quantified using ImageJ. Graph indicates the average nuclear area of a population of cells present in a square area of 10000 um^2. (mean +/- SD; n=3; ** P<0.01).
c) DAB2IP depletion affects cell density. MCF10A cells were cultured as in a). Nuclei were labelled with Hoechst and counted. Graph indicates the average number of nuclei in a square area of 10000 um^2 (mean +/- SD; n=3)
d) DAB2IP depletion increases FAK Tyr397-phosphorylation but does not affect E-cadherin protein levels. MCF10A cells were cultured as in a). DAB2IP, phosphorylated (p-Y397) and total FAK and E-cadherin have been measured by immunoblotting, with HSP90 as loading control. Protein bands were quantified and normalized to HSP90 by densitometry of autoradiography film (histogram on the right; mean +/- SD; n=3; *P<0.05).

Starting from the evidences that packaging of cells into a confluent 2D culture strongly modifies cell's morphology (Fig. 2a) with concomitant increase in DAB2IP protein levels (Fig. 2c), I analyzed the impact of DAB2IP depletion in MCF10A undergoing a progressive enhancement of cell-cell contact.

To this aim, MCF10A cells were cultured into a confluent monolayer and let grow for additional days in order to achieve even higher degrees of cell density. After 72h hours, MCF10A cells are highly packed (Fig 8a) and display a reduced nuclear area (Fig. 8b). In addition to morphological changes, I asked if cell constriction into a confluent monolayer also affects the mechanical elasticity of these cells. In collaboration with prof. Casalis at Elettra-Sincrotrone in Trieste, we used atomic force microscopy (AFM) to monitor the elastic modulus of confluent cells with or without DAB2IP knockdown. We found that the stiffness of MCF10A was progressively reduced concomitantly to the increase of cell packing (Fig. 8c). In line with my previous results, the increase in cell crowding was paralleled by an increase of E-cadherin and DAB2IP protein levels (Fig. 8d). Interestingly, Focal Adhesion activity displayed a peculiar pattern: FAK protein levels increased progressively over time, while its activating phosphorylation on Tyr397 increased initially but then remained stable, confirming that FAK signaling is eventually blunted in hyperdense monolayer cultures (Fig. 8d).

Under these conditions, siRNA mediated DAB2IP knockdown affected the morphological and physical features of MCF10A. Specifically, upon DAB2IP silencing, the average number of cells (i.e. nuclei) per area was reduced, with concomitant increase in nuclear size (Fig. 8a-b). Regarding the elastic modulus, DAB2IP knockdown cells were tendentially stiffer than controls (Fig. 8c). Interestingly, DAB2IP knockdown had no detectable impact on confluency-dependent E-cadherin accumulation, but affected FAK activity, since phospho-FAK was significantly increased at early times during high density culture (Fig. 8d).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40
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SiDAB2IP
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200
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24h
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c
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d
SiC
SiDAB2IP
24h
48h
72h
24h
48h
72h
DAB2IP
p-FAKY397
FAK
E-cadh
HSP90
MCF10A
E-cadherin protein levels
2.5
2.0
1.5
1.0
0.5
0.0
SiC
SiDAB2IP
*
24h
48h
72h
MCF10A
pFAK/FAK protein levels
2.5
2.0
1.5
1.0
0.5
0.0
SiC
SiDAB2IP
24h
48h
72h
MCF10A

Figure 8. DAB2IP depletion affects cell morphology, size, stiffness, and FAK activity upon different degrees of cell density.
a) DAB2IP depletion affects cell density in monolayers. MCF10A cells were transfected with the indicated siRNAs (20mM) for 24h. Then they were plated at the concentration of 50000 cells/cm2 and analyzed at the indicated times. Nuclei were labelled with Hoechst and counted. Graph indicates the average number of nuclei in a square area of 10000 um^2 (mean +/- SD; n=3; *P<0.05)
b) DAB2IP depletion increases nuclear size. MCF10A cells were cultured and stained as in a). Nuclear areas were quantified using ImageJ. Graph indicates the average nuclear area of a population of cells present in a square area of 10000 um^2. (mean +/- SD; n=3).
c) DAB2IP depletion affects cell stiffness. MCF10A cells were cultured as in a). Cell rigidity was measured using atomic force microscopy (AFM) as detailed in the methods section. At least 30 cells were measured per sample. Data refer to a single experiment.
d) DAB2IP depletion affects FAK Tyr397 phosphorylation. MCF10A cells were cultured as in a). DAB2IP, phosphorylated (p-Y397) and total FAK, and E-cadherin have been measured by immunoblotting, with HSP90 as loading control. Protein bands were quantified and normalized to HSP90 by densitometry of autoradiography film (histogram on the right). E-cadherin (mean +/- SD; n=3; *P<0.05). p-Y397 FAK/FAK (mean +/- SD; n=2).

DAB2IP modulates cellular properties related to YAP/TAZ activity

Since DAB2IP and YAP/TAZ are inversely regulated by cell-cell contact (Fig. 2c), and DAB2IP depletion appears to modulate some physical properties of the cells under high density conditions, I asked if DAB2IP could be involved in YAP/TAZ inhibition by cell-cell contact. To this aim, I analyzed MCF10A cells cultured as a confluent monolayer (high density) as compared to cells cultured under sparse conditions (with minimal points of cell-cell contact).

In high density, MCF10A are subjected to contact inhibition of proliferation (CIP), and DNA replication is efficiently blocked (Fig. 9a). In response to crowding, MCF10A cells also change their physical features, reducing their size, nuclear area, and elastic features (Fig. 9b-d). Under the same conditions, siRNA mediated DAB2IP knockdown slightly but reproducibly increased proliferation of confluent cells, suggesting a less efficient CIP (Fig. 9a). DAB2IP depletion also reduced the number of cells (i.e. nuclei) per area under conditions of full confluency of the monolayer (i.e. lack of empty space between cells); this suggests an increase in average cell size, that was indeed accompanied by an increase in average nuclear area (Fig. 9b-c). Exploratory AFM measurements suggested that such changes in morphological features may correlate with an average increase in cell stiffness (Fig. 9d).

In control silenced cells, as expected, high confluency caused the inhibition of YAP/TAZ activity, revealed by YAP cytoplasmic localization and reduced transcription of YAP/TAZ target genes. Notably, together with the cell's morphology phenotypes, DAB2IP depletion in MCF10A monolayer also promoted YAP shuttling into the nucleus and concomitant transcription of YAP/TAZ target genes (Fig. 10a-c). Notably, ANKRD1 is strongly induced by DAB2IP depletion even in low density cultures, masking the statistical significance of its upregulation in high density; it is possible that transcription of this gene is regulated by a parallel DAB2IP-dependent pathway.

Interestingly, when cells were cultured at low density, DAB2IP depletion did not produce significant effects; not in terms of cell proliferation, nor in cellular shape or YAP/TAZ activity. This experiment therefore suggests that DAB2IP may somehow modulate YAP/TAZ activity specifically in confluent cells.

To explore the possible mechanism linking DAB2IP levels to YAP/TAZ activation, I found no significant changes in YAP mRNA upon DAB2IP depletion (not shown). A quantitative analysis of phospho-specific immunoblots revealed reduced Ser127 phosphorylation of YAP in DAB2IP depleted cells at high cell density (Fig. 10d). Since YAP is retained in the cytoplasm via LATS1/2-mediated inhibitory phosphorylation on Ser127 (Zhao et al., 2007), these data suggest that in

hyper-confluent cells DAB2IP could limit YAP activation and transcriptional activity by favoring its inhibitory phosphorylation.

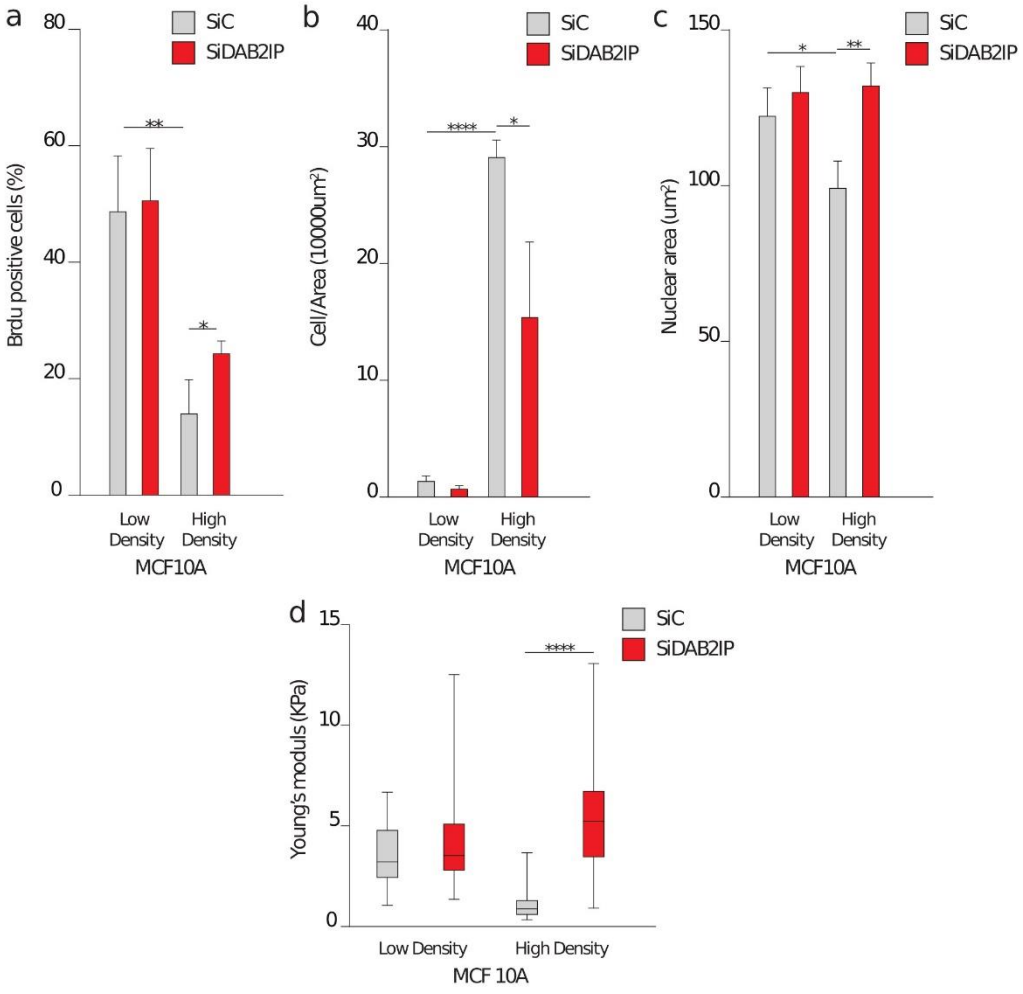

Figure 9. DAB2IP depletion in conditions of high cell density affects cell proliferation, size, and possibly stiffness.
a) DAB2IP depletion increases proliferation of non-transformed breast epithelial cells arrested by contact inhibition. MCF10A were transfected with indicated siRNAs for 24 hours and, cultured for additional 48 hours after seeding at low or high density (2500 cells/cm2 and 50000 cells/cm2 respectively). Then, cells were incubated with BrdU for 2 hours before immunofluorescence analysis. Graphs summarize the percentage of BrdU positive nuclei (mean ± SD; n =3; *P<0.05, ** P<0.01).
b) DAB2IP depletion affects cell number in a confluent monolayer. MCF10A cells were cultured as in a). Nuclei were labelled with Hoechst and counted. Graph indicates the average number of nuclei in a square area of 10000 um^2 (mean +/- SD; n=3; *P<0.05, **** P<0.0001).
c) DAB2IP depletion increases nuclear size in a confluent monolayer. MCF10A cells were cultured and labeled as in a). Nuclear areas were quantified using ImageJ. Graph indicates the average nuclear area of a population of cells present in a square area of 10000 (mean +/- SD; n=3; *P<0.05, ** P<0.01).
d) DAB2IP depletion increases cell stiffness in a confluent monolayer. MCF10A cells were cultured as in a). Cell rigidity was measured using atomic force microscopy (AFM) as detailed in the methods section. At least 30 cells were measured per sample. Data refer to a single experiment.

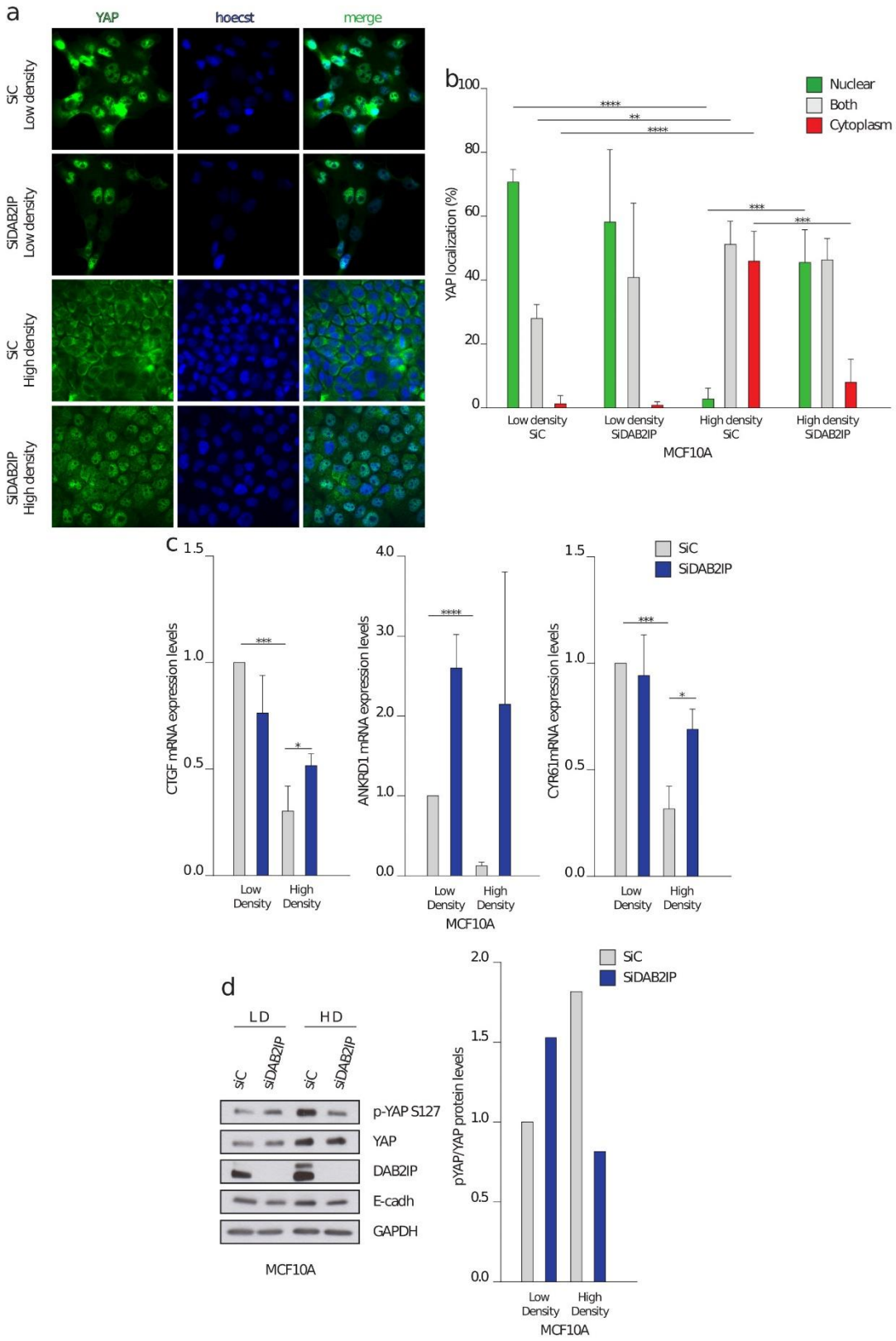
a
YAP
hoecst
merge
SiC Low density
SiDAB2IP Low density
SiC High density
SiDAB2IP High density
b
Nuclear
Both
Cytoplasm
YAP localization (%)
Low density SiC
Low density SiDAB2IP
High density SiC
High density SiDAB2IP
MCF10A
c
SiC
SiDAB2IP
CTGF mRNA expression levels
ANKRD1 mRNA expression levels
CYR61mRNA expression levels
Low Density
High Density
MCF10A
d
LD
HD
siC
siDAB2IP
p-YAP S127
YAP
DAB2IP
E-cadh
GAPDH
MCF10A
pYAP/YAP protein levels

Figure 10. DAB2IP depletion prevents YAP/TAZ inhibition in conditions of high cell density.
a, b) DAB2IP depletion increases YAP nuclear localization in a confluent monolayer. MCF10A cells were cultured as in a). The cellular distribution of YAP was analyzed by immunofluorescence. **a)** representative images of YAP protein (green) localization by immunofluorescence**; b)** quantification of the subcellular localization of YAP in nucleus, cytoplasm, or both cell compartments (mean ± SD; n =4; ** P<0.01, ***P<0.001, ****P<0.0001).
c) DAB2IP depletion increases expression of YAP/TAZ target genes in a confluent monolayer. MCF10A cells were cultured as in a). Expression of CTGF, CYR61 and ANKRD1 was measured by RT-qPCR. Data were normalized on histone H3 (mean ± SD; n =3; *P<0.05, ***P<0.001, ****P<0.0001).
d) DAB2IP depletion reduces YAP Ser127 phosphorylation in a confluent monolayer. MCF10A cells were cultured as in a). DAB2IP, E-cadherin, phosphorylated (p-S127) and total YAP have been measured by immunoblotting, with HSP90 as loading control. Protein bands were quantified and normalized to HSP90 by densitometry of autoradiography film (histogram on the right).

DAB2IP inhibits YAP/TAZ activity through its GAP domain

Since DAB2IP depletion appears to stimulate YAP/TAZ in cells grown at high density, a condition that normally restrains YAP/TAZ activity, I asked if DAB2IP overexpression would inhibit YAP/TAZ in cells grown under conditions in which they are normally active. Since transient transfection of MCF10A cells is technically difficult, I generated monoclonal cell lines stably expressing wild-type hDAB2IP, or the mutant hDAB2IP R385L, bearing a single aminoacid change in the Ras-GAP domain that impairs its GAP activity. The corresponding expression plasmids were kindly provided by dr. Sidney Yu (X. Luo et al., 2018), and encode the hDAB2IP transcriptional variant 1 (NM_032552.3) in fusion with an N-terminal Myc-TAG.

MCF10A cell lines overexpressing Myc-hDAB2IP or Myc-hDAB2IP R385L were cultured to sub-confluency, a condition that allows YAP/TAZ nuclear localization and transcriptional activity. Immunofluorescence analysis revealed that hDAB2IP overexpression reduced YAP nuclear localization and increased YAP cytoplasmic retention (Fig. 11a, b). Notably, overexpression of DAB2IP R385L had no significant effects on YAP localization, suggesting that DAB2IP inhibitory action on YAP is dependent on its GAP activity.

To reinforce these observations, I tested YAP/TAZ co-transcriptional activity by performing luciferase assays with the 8xGTIIC-Lux vector, a synthetic YAP/TAZ responsive luciferase reporter (Dupont et al., 2011). Using this assay, I found that hDAB2IP overexpressing cells have reduced LUC expression as compared to controls (Fig. 11c). In line with these results, I found that also endogenous YAP/TAZ target genes CTGF, CYR61 and ANKRD1 are less expressed in DAB2IP overexpressing cells (Fig. 11d). As with YAP nuclear localization, luciferase assays and RT-PCR experiments confirmed that the hDAB2IP R385L mutant does not inhibit YAP/TAZ activity (Fig. 11c, d).

Finally, I analyzed YAP phosphorylation by immunoblotting. I found that phosphorylation on Ser127 of YAP is increased in DAB2IP overexpressing cells, thus suggesting that DAB2IP supports a canonical Hippo pathway-mediated inhibition of YAP. In line with previous assays, the hDAB2IP R385L mutant did not increase S127 YAP phosphorylation (Fig. 11e). Together, these data indicate that DAB2IP can possibly inhibit YAP/TAZ activity, likely through its Ras-GAP function.

To corroborate the above results, I analyzed the behavior of DAB2IP-overexpressing MCF10A cells cultured at different degrees of confluency. I monitored YAP/TAZ activity by immunofluorescence analysis of YAP, and RT-PCR of endogenous YAP/TAZ target genes. DAB2IP overexpression and cell density were controlled respectively by immunoblotting (Fig.

12a) and by microscopy, counting the number of cells (i.e. nuclei) per area (Fig. 12b). The experiments confirmed that in low density conditions, hDAB2IP overexpression reduces YAP nuclear localization and expression of YAP/TAZ target genes CYR61 and ANKRD1 (Fig. 12c-d). Interestingly, even in high cell density conditions – when YAP/TAZ activity is minimal – hDAB2IP overexpression reduced YAP nuclear localization and transcriptional activity; these results imply that in high density monolayers there are sufficient mechanical inputs to support YAP/TAZ activation, and suggest that DAB2IP may be a potent inhibitor of these signals.

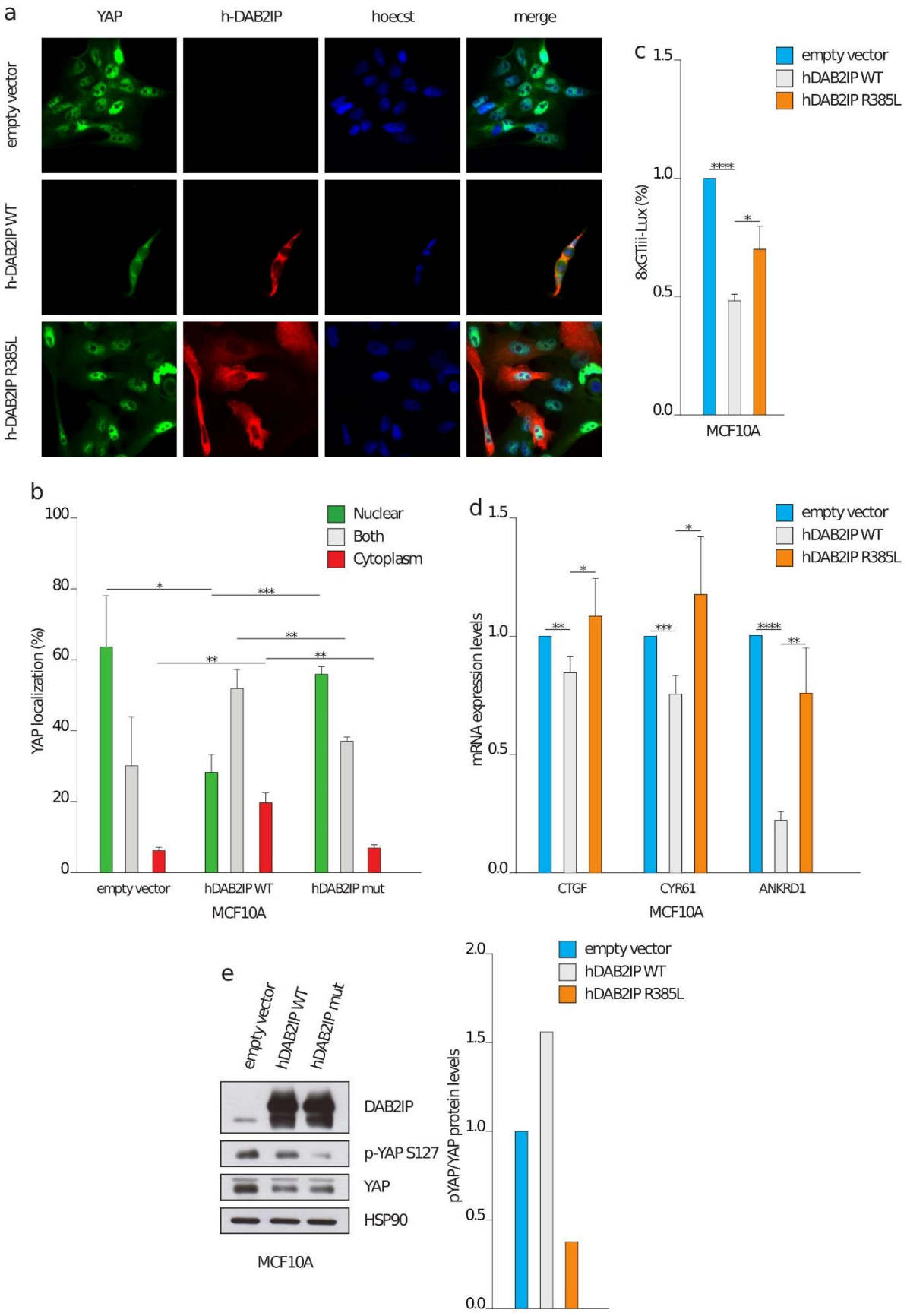

Figure 11. hDAB2IP overexpression inhibits YAP/TAZ activity via its RAS GTPase function.
a, b) Stable expression of wt hDAB2IP, but not its Ras-GAP mutant, induces YAP cytoplasmic retention. MCF10A cells stably expressing Myc-tagged hDAB2IP or hDAB2IP R385L, or a control empty vector, were cultured for 48 hours until sub confluent. YAP localization was detected by immunofluorescence. **a)** representative images of cells stained with antibodies against

DAB2IP (red) and YAP (green). Nuclei were stained with Hoechst (blue). **b)** Quantification of the subcellular distribution of YAP, indicating the percentage of cells with YAP staining only in the nucleus, only in the cytoplasm, or in both compartments (mean ± SD; n =3; *P<0.05, ** P<0.01, ***P<0.001).

c, d) Stable expression of hDAB2IP, but not its Ras-GAP mutant, inhibits YAP/TAZ transcriptional activity. MCF10A clones were cultured as in a). **c)** Cells were transfected with the 8xGTIIC-Lux reporter for 24hours, and YAP/TAZ activity was measured by Dual luciferase assay 24 hours (mean ± SD; n=3; *P<0.05, ****P<0.0001). **d)** Expression of endogenous CTGF, CYR61 and ANKRD1 was measured by RT-qPCR. Data were normalized on histone H3 (mean ± SD; n=3; *P<0.05, ** P<0.01, ***P<0.001, ****P<0.0001).

e) Stable expression of hDAB2IP, but not its Ras-GAP mutant, increases YAP Ser127 phosphorylation. MCF10A clones were cultured as in a). DAB2IP, phosphorylated (p-S127) and total YAP were detected by immunoblotting, with HSP90 as loading control. Protein bands were quantified and normalized to HSP90 by densitometry of autoradiography film (histogram on the right).

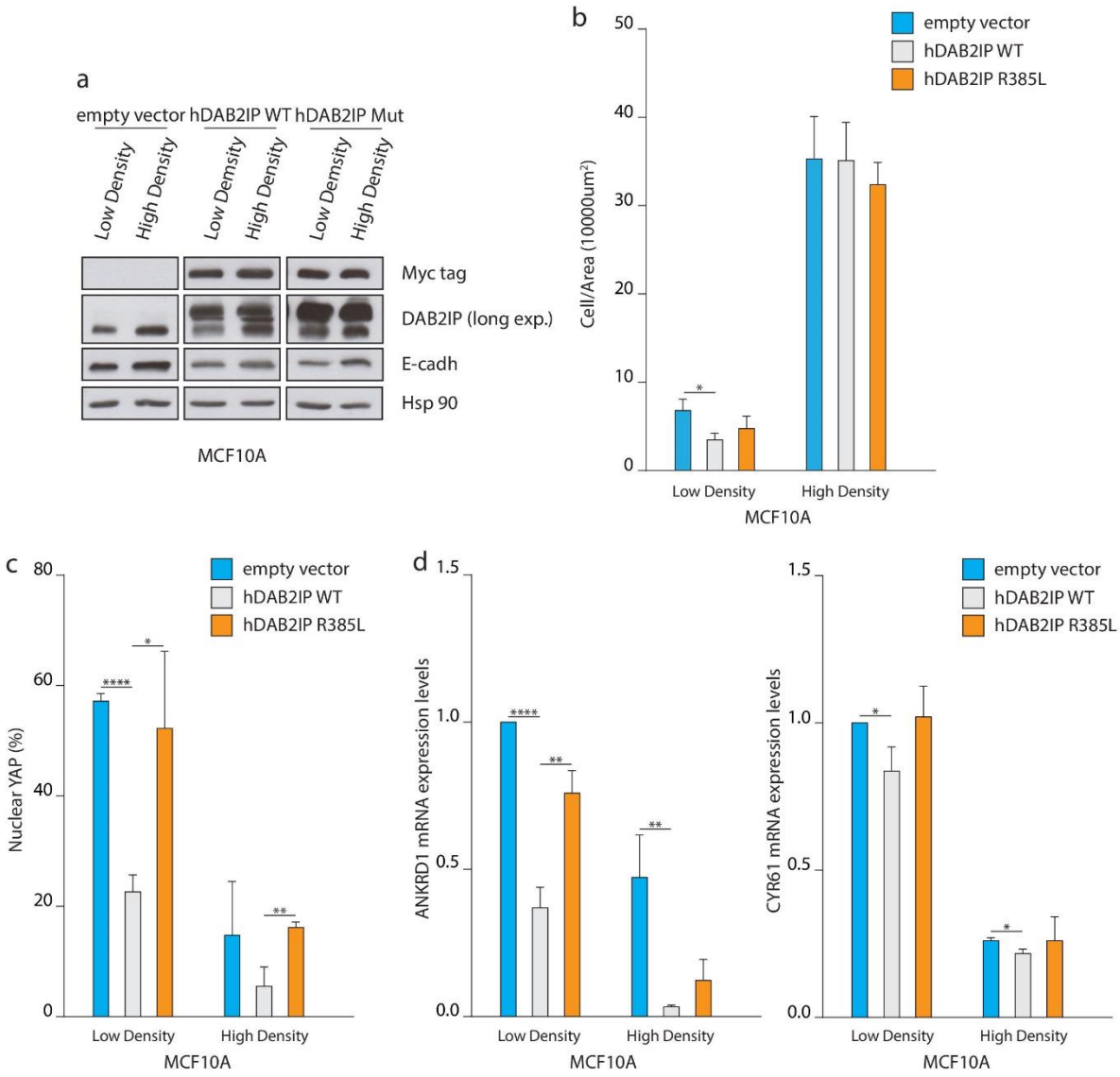

Figure 12. hDAB2IP overexpression inhibits YAP/TAZ activity independently of cell-cell contact.

a-d) MCF10A clones were cultured for 48 hours after seeding at low or high density (2500 cells/cm2 and 50000 cells/cm2 respectively). **a)** Expression of Myc-hDAB2IP WT, Myc-hDAB2IP R385L and E-cadherin were checked by western blot with HSP90 as a loading control. **b)** Nuclei were labelled with Hoechst. Graph indicates the average number of nuclei in a square area of 10000 um2 (mean +/- SD; n=3; *P<0.05, *** P<0.001, ****P<0.0001). **c)** YAP localization was analyzed by immunofluorescence as in Fig. 11. Graph quantifies the percentage of cells with YAP staining in the nucleus (mean ± SD; n =3; *P<0.05, ** P<0.01, ****P<0.0001). **d)** Expression of endogenous CTGF, CYR61 and ANKRD1 was measured by RT-qPCR. Data were normalized on histone H3 (mean ± SD; n=3; *P<0.05, ** P<0.01, ***P<0.001, ****P<0.0001).

DAB2IP inhibits YAP/TAZ independently of Adherens Junctions

My data suggest that DAB2IP is a negative regulator of YAP/TAZ activity; in particular, siRNA depletion experiments in mammary epithelial cells indicate that DAB2IP contributes to the inhibition of YAP/TAZ activity in confluent monolayers. Whether this activity depends on the presence of functional cell-cell junctions, or more generally on cytoskeletal tension, remains to be defined.

Since confluent monolayers are characterized by formation of Adherens Junctions (AJ), and DAB2IP and YAP/TAZ are inversely regulated by cell confluency, I asked if DAB2IP may be involved in YAP/TAZ inhibition by AJs. I therefore analyzed YAP/TAZ activity upon AJ disruption by EGTA in confluent MCF10A cells, with or without DAB2IP silencing.

Efficient disruption and reassembly of AJs were monitored visually by phase contrast microscopy as in Fig. 5a (data not shown), and biochemically by E-cadherin immunoblotting (Fig. 12b). As expected, EGTA efficiently separated cells in the monolayer, with concomitant E-cadherin downregulation. Cell junctions reformed promptly after EGTA washout and addition of fresh medium; however, E-cadherin levels returned to normal (similar to those of untreated controls) only after 24 hours, confirming that more than 3 hours are necessary for a complete recovery of E-cadherin protein expression under these conditions (Fig. 5b).

In this experiment, I found that EGTA treatment modulates YAP/TAZ target genes independently of DAB2IP. Specifically, CYR61 and ANKRD1 transcripts promptly increased after EGTA treatment, and rapidly decreased after restoration of cell junctions, both in control and DAB2IP knockdown cells (Fig. 13a). In parallel, DAB2IP depletion significantly increased expression of CYR61 and ANKRD1 both under basal conditions and after EGTA treatment, further confirming its inhibitory role on YAP/TAZ activity. Notably, in DAB2IP depleted cells, even long term (24h) re-establishment of cell junctions did not repress expression of YAP/TAZ target genes to the basal low levels of high-density control cells (Fig. 13a).

I performed a similar experiment using the hDAB2IP expressing MCF10A stable cell line (Fig. 13d). I found that CTGF, CYR61 and ANKRD1 were induced after AJs disruption both in control and in DAB2IP-overexpressing cells, suggesting that loss of AJ-mediated inhibitory signals is not compensated by DAB2IP ectopic expression. However, in line with depletion data, cells expressing DAB2IP showed reduced expression levels of YAP/TAZ target genes, both under basal conditions and after EGTA treatment (Fig. 13c).

Together, these experiments support the hypothesis that DAB2IP contributes to maintain YAP/TAZ inactive in high cell density conditions. Clearly, DAB2IP is not a major player in such regulation; this is not surprising, since multiple mechanisms are involved in YAP/TAZ inhibition by E-cadherin adhesion complexes (Dobrokhotov et al., 2018). Rather, the data suggest that DAB2IP may act as an additional regulator, acting either independently or modulating some of the already characterized signaling pathways that control YAP/TAZ activity.

DAB2IP inhibits YAP/TAZ activity independently of FAK signaling

To try to define the possible mechanism for YAP/TAZ inhibition by DAB2IP, I looked at mechanical signaling exerted at the level of Focal Adhesions (Lachowski et al., 2018). In fact, I found that DAB2IP depletion increased FAK protein levels and activating phosphorylation (Fig. 8d), which suggests that DAB2IP may limit FA signaling in high density epithelial cells. I therefore asked if DAB2IP can regulate YAP/TAZ through inhibition of FAK signaling.

To this aim, I analyzed the effects of DAB2IP depletion in confluent MCF10A monolayers with or without FAK inhibition by the specific drug PF573228.

Despite YAP/TAZ activity is sensibly blunted in high-density MCF10A cells, inhibition of FAK signaling further reduced the mRNA levels of some YAP/TAZ target genes, in particular ANKRD1 (Fig.14a). In line with previous data, siRNA mediated DAB2IP depletion enhanced YAP/TAZ activity and FAK activation. However, under these conditions, treatment with PF573228 had no impact on YAP/TAZ activation by DAB2IP-knockdown, as assessed by target genes transcription and reduced YAP phosphorylation (Fig.14a-b). Notably, CTGF and CYR61 expression was actually enhanced by concomitant DAB2IP depletion and FAK inhibition. Together, these data argue against the hypothesis that DAB2IP can modulate YAP/TAZ by inhibiting FAK. However, PF573228 does not inhibit basal YAP/TAZ activity, and CTGF, CYR61 and ANKRD1 are not exclusive YAP/TAZ target genes; therefore, to corroborate this hypothesis, exhaustive data on additional target genes and YAP/TAZ localization will have to be provided by additional experiments.

Intriguingly, since DAB2IP levels depend on FAK activity (see Fig. 4), these data suggest that FAs can sustain the inhibitory role of DAB2IP on YAP/TAZ signaling, at least for some target genes.

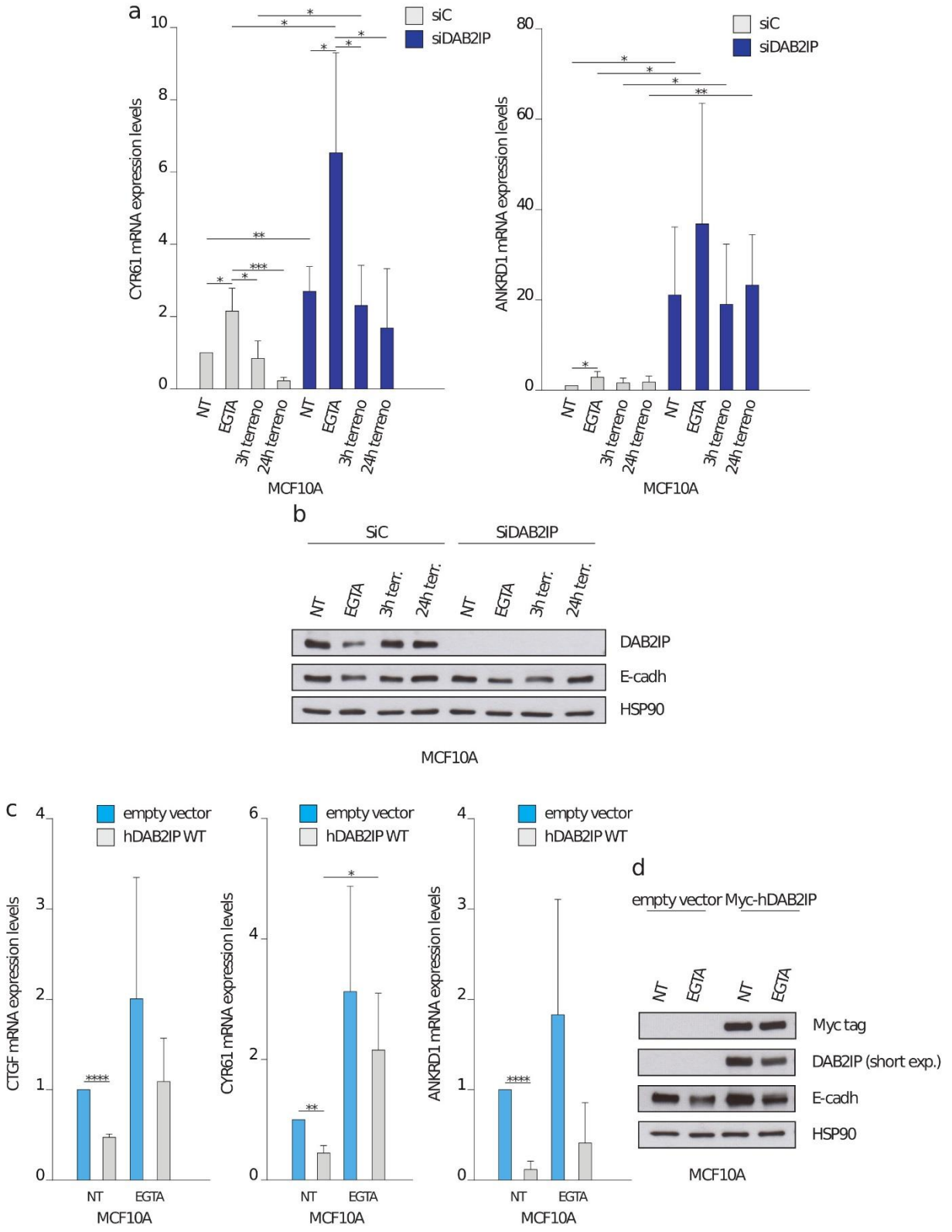

Figure 13. DAB2IP contributes to YAP/TAZ inhibition independently of cell-cell contact.
a-b) Depletion of DAB2IP increases YAP/TAZ target genes expression during AJ disruption/reformation. MCF10A cells were silenced for DAB2IP for 24 hours and cultured for additional 48 hours after seeding at high density (50000 cells/cm2). Cells were treated with 10 mM EGTA for 3 hours. Then EGTA was washed out, and fresh medium was added for 3 and 24 hours. **a)** Expression of CYR61 and ANKRD1 was measured by RT-qPCR. Data were normalized on histone H3 (mean ± SD; n =4; *P<0.05, ** P<0.01, *** P<0.001). **b)** endogenous DAB2IP and E-cadherin proteins were analyzed by western blot, with HSP90 as loading control.

c-d) Overexpression of hDAB2IP reduces YAP/TAZ target genes expression before and after AJ disruption. MCF10A cells stably expressing hDAB2IP or a control empty vector, were cultured for 48 hours after seeding at high density (50000 cells/cm2). Cells were treated with 10 mM EGTA for 3 hours. **c)** Expression of CTGF, CYR61 and ANKRD1 was measured by RT-qPCR. Data were normalized on histone H3 (mean ± SD; n =3; *P<0.05, ** P<0.01, **** P<0.0001). **d**) Exogenous hDAB2IP (detected with anti-tag and anti-DAB2IP antibodies) and endogenous E-cadherin proteins were analyzed by western blot, with HSP90 as loading control.

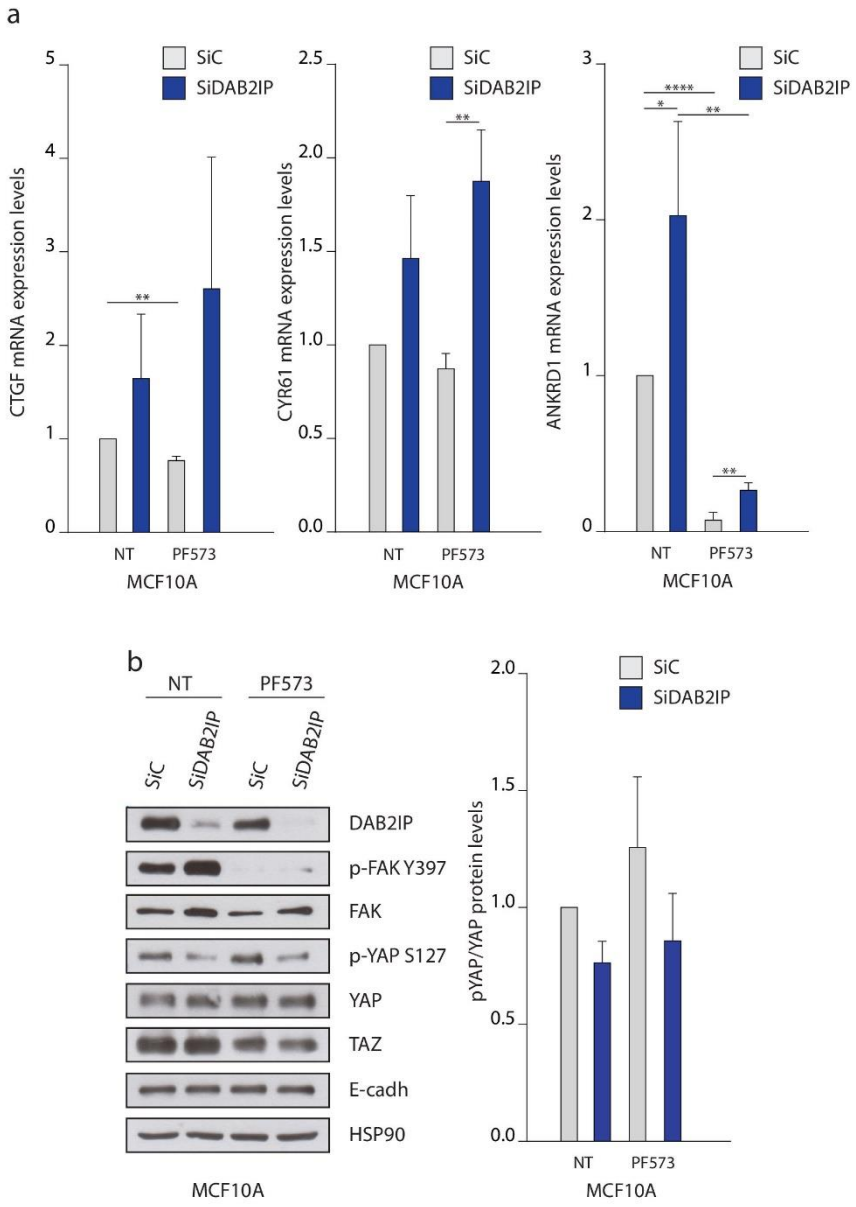

Figure 14. DAB2IP contributes to YAP/TAZ inhibition independently of FAK signaling.
a-b) Depletion of DAB2IP increases YAP/TAZ target genes expression. MCF10A cells were silenced for DAB2IP for 24 hours and cultured for additional 48 hours after seeding at high density (50000 cells/cm2). Then cells were treated with 10uM PF573228 (PF573) for 6h before lysis. **a)** Expression of CTGF, CYR61 and ANKRD1 was measured by RT-qPCR. Data were normalized on histone H3 (mean ± SD; n =3; *P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001). **b)** Expression of DAB2IP, TAZ, E-cadherin, phosphorylated (p-S127) and total YAP, and phosphorylated (p-Y397) and total FAK have been measured by immunoblotting, with HSP90 as loading control. Protein bands were quantified and normalized to HSP90 by densitometry of autoradiography film (histogram on the right). Phosphorylated (p-S127) YAP (mean +/- SD; n=2).

Discussion

Tissue homeostasis is the result of a fine and highly regulated crosstalk between epithelial cells and their environment, including other epithelial and stromal cells. Cells constantly perceive and transduce extracellular signals into biological responses, with the aim to resolve insults and restore physiology. An aberrant communication between cells and their microenvironment leads in general to pathology, and in particular to cancer. Indeed, cancer arises from genetic and epigenetic changes within tumor cells, and the concomitant stimulation from an aberrant surrounding environment. In other words, the crosstalk between tumor cells and the extracellular microenvironment is the major determinant of cancer initiation and progression.

Among the multiple external stimuli that affect cell behavior, it is becoming increasingly evident that also mechanical forces from the extracellular environment have an important role in tumorigenesis, metastasis, and chemoresistance. In particular, it has been established that changes in the extracellular matrix (ECM) can foster epithelial to mesenchymal transition (EMT), leading to cell migration and invasion. Moreover, ECM stiffening sustains genomic alterations, eliciting cell heterogeneity and clonogenicity. Finally, a rigid ECM has an indirect effect by limiting the delivery of anti-cancer drugs to the tumor (Deville & Cordes, 2019).

It is also well accepted that cancer cells take advantage of specific molecular mechanisms to perform an optimal adaptive response necessary to survive the stringent microenvironmental conditions of a growing tumor. This involves signaling proteins and modulators that drive a dynamic interplay between the exterior and the interior of cells. Among such molecules, this thesis is focused on the tumor suppressor DAB2IP.

Various studies have demonstrated that DAB2IP modulates the cell response to various soluble microenvironmental signals such as inflammatory cytokines (Di Minin et al., 2014), growth factors (Wang et al., 2002) (Zhou et al., 2014) and hormones (Valentino et al., 2017). Biochemically, DAB2IP has been shown to modulate pathways involved in the unfolded protein response (UPR), epithelial to mesenchymal transition (EMT), apoptosis, K-Ras, p38MAP kinase, and STAT signaling; therefore, loss of DAB2IP fosters tumorigenesis, metastasis, and chemoresistance. Notably, conditional DAB2IP depletion in endothelial cells generates a pro-oncogenic niche that favors tumour growth and dissemination (Ji et al., 2015). The Collavin group has unpublished evidences that cancer cells can downregulate DAB2IP in neighbouring non-tumoral cells, and this suggests that DAB2IP can modulate the reciprocal exchange of information between cancer and stromal cells.

Given these premises, it is of interest to fully understand the upstream signals that can regulate DAB2IP levels and functions in cancer and non-cancer cells.

This Thesis presents experimental evidence that DAB2IP is regulated by mechanical inputs from the tissue microenvironment, and that upon these stimuli DAB2IP can modulate cellular responses limiting activation of the YAP/TAZ transcription factors. These observations highlight a novel means of regulation and possibly a novel biological function for DAB2IP, both with obvious tumor suppressive implications.

I found that external mechanical inputs such as ECM stiffness and cell-cell contact can modulate DAB2IP. Cell detachment and re-seeding upon a solid substrate modulate DAB2IP protein levels, indicating that cell attachment sustains DAB2IP expression, probably via Focal Adhesions (FAs) activity (Fig1, 4). Moreover, also the topology and rigidity of the substratum can alter DAB2IP expression, since growing cells in a soft ECM reduces DAB2IP protein levels (Fig. 3). Notably, also the cell interaction with neighboring cells dynamically affects DAB2IP protein levels, that are reduced when cells are grown in low density conditions (Fig. 2). In this perspective, E-cadherin, the main component of Adherens Junctions, is involved in DAB2IP regulation by cell-cell contact (Fig. 5).

Thus, the physical interaction with the surrounding environment is an important regulator of DAB2IP levels. This implies that alterations of the mechanical properties of the ECM, or loss of integrity of the epithelium, may affect DAB2IP expression with consequences for the biological response of the cells to inflammation, growth factors, or other stimuli.

Clearly, these observations raise a number of questions.

Although I proved the involvement of AJs in DAB2IP regulation by cell contact, if DAB2IP stability depends on the number of AJs per cell, or on the strength of homophilic binding between membrane E-cadherins, remains to be defined. In addition, since the mRNA levels do not change significantly with cell density, it is likely that AJs modulate DAB2IP protein stability. Specifically, in epithelial cells, through a still undefined mechanism, cell-cell interactions could stabilize DAB2IP protein, which in contrast is degraded when cell-cell contacts are limited or absent. Specific experiments need to be done in order to understand the molecular mechanism through which AJs may control DAB2IP protein levels, and the biochemical basis for DAB2IP turnover in low or high density culture conditions.

One possible connection between DAB2IP and E-cadherin may be mediated by the DAB2IP-interacting protein DAB2. In fact, DAB2 is involved in E-cadherin turnover acting as adaptor for

clathrin-mediated endocytosis; once internalized by clathrin-dependent endocytosis, E-cadherin can be recycled and sorted back to the plasma membrane or sequestered in multivesicular endosomes that finally fuse with lysosomes causing its degradation (Brüser and Bogdan, 2017). Given that E-cadherin and DAB2IP display a similar behavior upon EGTA-mediated AJs disassembly, and they both bind DAB2, it is possible that they are degraded via a similar mechanism. It will therefore be interesting to test if DAB2IP downregulation in conditions of low cell density is mediated by the ubiquitin-proteasome pathway or by lysosomal proteolysis.

My experiments also indicate that focal adhesion kinase (FAK) activity sustains DAB2IP protein levels during cell attachment to the substrate; however, the contribution of FAs in DAB2IP regulation remains to be demonstrated. Indeed, FAK is a kinase related to Integrin signaling, activated by mechanical inputs from the ECM, but its activity is strongly regulated also by integrin-independent signals, such as cytokine receptors, growth factor receptors, and G protein-coupled receptors (GPCR) (Yoon et al., 2015). Thus, to determine whether DAB2IP is actually regulated by FAs, it will be necessary to perform additional experiments with controlled assembly of integrin complexes – for instance by siRNA-mediated ITGA and/or ITGB silencing or by culturing cells on fibronectin with or without RGD peptide.

Differently to what observed with AJs, that impinge exclusively on post transcriptional regulation, my data suggest that FAK activity sustains DAB2IP mRNA expression (Fig. 4d). Interestingly, kinase-inhibited FAK can go to the nucleus, and potentially regulate gene expression to affect cancer progression (Yoon et al., 2015). In particular, nuclear FAK binds to methyl CpG- binding protein 2 (MBD2) and enhances heterochromatin remodeling to promote gene expression. Since DAB2IP expression is regulated by CpG methylation, it could in fact be a target for FAK transcriptional activity. This is pure speculation, but may be tested with appropriate experiments.

In addition to transcriptional upregulation, DAB2IP protein stability could also be affected by formation and clustering of FAs. In fact, similar to AJs, integrin turnover is mediated by the adaptor DAB2 in a clathrin-dependent manner (Kechagia et al., 2019).

Using various actomyosin inhibitors, I also found that integrity and contractility of the actin cytoskeleton can affect DAB2IP protein levels, although the functional link with ECM stiffness and/or cell-cell contact remains to be defined. In general, formation of FAs or AJs implies mechanical tension of linked microfilaments; and I observed that cytoskeleton drugs tendentially reduce DAB2IP levels, suggesting that the protein is indeed regulated by actin-myosin tension. Possible molecular mediators of such regulation remain unexplored.

My data indicate that DAB2IP protein is more expressed in culture settings that partially resemble the physiological condition for epithelial cells: a confluent 2D monolayer. Here, cells establish an apico-basal polarization and a structured cytoskeleton with a defined circumferential actin belt and essentially no stress fibers. In this perspective, it will be interesting to analyze DAB2IP expression and function in mammary epithelial cells grown in 3D structures, or in organoids, as these recapitulate even more physiological epithelial conditions.

In contrast, DAB2IP levels are reduced when epithelial cells are sparse and can stretch on the substrate, rearranging their circumferential actin belt to form stress-fibers.

Nonetheless, I found that actin drugs reduce DAB2IP levels principally in sparse cells with minimum variation in confluent cells. This evidence suggests a double and distinct regulation of DAB2IP at Focal Adhesions and Adherens Junction. In detail, I speculate that when cells are sparse, cell-ECM stimuli positively sustain DAB2IP levels via the integrity and tension of F-actin. On the contrary, in a monolayer of confluent cells DAB2IP levels depend mainly on the integrity of cell-cell junctions, with actomyosin tension being almost irrelevant. This concept is supported by the observation that DAB2IP levels are reduced when cells are grown on a soft substrate – a condition that breaks cytoskeletal tension. Similarly, DAB2IP protein accumulates to maximal levels in very high density cultures – a condition that is considered similar to growth on a soft substrate, with low cytoskeleton tension, but that implies a high number of cell-cell junctions. It is possible that epithelial cells grown at high density regulate DAB2IP in a cytoskeleton-independent manner, possibly through mechanisms associated to change in cell polarity or shape.

A limited number of studies highlighted a possible connection of DAB2IP with the cytoskeleton. A recent paper reported that Raskol, the DAB2IP ortholog in *Drosophila melanogaster*, colocalizes with the cortical actin in border cells (BC) of developing Drosophila egg chambers (Raza et al., 2019). More recently, DAB2IP has been reported to localize on microtubules in the cytoplasm but also in the primary cilium (Stewart et al., 2020) (Lin et al., 2021). It is therefore possible that DAB2IP levels could be modulated by mechanisms that directly involve its interaction with the cytoskeleton.

In the second part of this Thesis, I studied the possible role of DAB2IP in orchestrating cell's morphology and modulating cellular responses to mechanical inputs. I have collected preliminary evidence that DAB2IP depleted cells have a different behavior when reaching confluency, they are more resistant to tight packing with respect to controls, and tend to have a larger nucleus. Explorative analysis based on AFM also suggests that DAB2IP depleted cells fail to reduce their elastic modulus when grown to high density. These data await necessary validation by additional

experiments, but if they are confirmed they may reveal a novel role for DAB2IP in modulating cytoskeletal responses under conditions of high density.

By performing depletion or overexpression experiments, I found that DAB2IP can influence the activity of YAP, a key transcriptional mediator of mechanical inputs. In particular, data indicate that DAB2IP may contribute to YAP/TAZ inhibition in cells growing at high density.

Together these results suggest that DAB2IP knockdown makes cells larger, or anyway more resistant to confluency-induced shrinkage, so that after the same time in culture they maintain a higher cell-ECM adhesion area, thus enabling YAP/TAZ activity.

In this model, DAB2IP would be required to achieve tighter cell packing upon confluency, thus inhibiting YAP/TAZ via limiting cell spreading on the ECM.

Experiments with EGTA-induced AJ disassembly suggest that DAB2IP is not a major effector of YAP inhibition by Adherent Junctions, although AJ mediated YAP/TAZ inhibition is clearly reinforced by the action of DAB2IP. Moreover, despite DAB2IP levels are positively modulated by cell attachment to the substrate, experiments with the FAK inhibitor PF573228 suggest that DAB2IP does not inhibit YAP by interfering with FA signaling.

Concerning mechanism, I found that DAB2IP regulation of YAP involves mostly its nucleo-cytoplasmic localization, with insignificant effects on its mRNA or protein levels. Notably, YAP phosphorylation in Ser127 is increased by DAB2IP overexpression, suggesting that DAB2IP may sustain the Hippo pathway. To test this hypothesis, specific experiments need to be performed to evaluate the role of selected components of the pathway (e.g. LATS1/2, NF2, other kinases) in DAB2IP-induced YAP cytoplasmic sequestration.

In addition, a functional GAP domain is necessary for DAB2IP to promote YAP cytoplasmic retention, suggesting that this biological function involves inhibition of Ras or other cellular GTPases. This is also an important point that may help define the molecular axis linking DAB2IP to YAP (and perhaps TAZ) regulation in epithelial cells.

In fact, in addition to the Hippo cascade, cadherin-mediated cell-cell junctions can modulate multiple signaling pathways, including Tyrosine kinase receptors, PI3K/AKT, Rho GTPase, and Wnt/β-catenin (Kourtidis et al., 2017), that affect YAP/TAZ activity. Intriguingly, DAB2IP negatively modulates most of those pathways.

First, through its Ras-GAP domain DAB2IP promotes Ras inactivation and inhibition of MAPK signaling (Z. Wang et al., 2002) and PI3K/AKT pathway (Bellazzo et al., 2017). In addition,

DAB2IP can bind and inhibit directly PI3K and AKT (Xie et al., 2009). Recently, Azad et al. found that several RTKs can inactivate LAT1/2 through PI3K/AKT and MAPK signaling pathways: as a result, YAP/TAZ nuclear localization is increased, leading to cell proliferation, migration, and transformation (Azad et al., 2020). Moreover, studies in brain cancer cell lines with mutant p53 showed that the activation of AKT2 facilitates YAP/TAZ nuclear localization through the activation of WIP (Escoll et al., 2017). It is therefore possible that DAB2IP down-modulates YAP/TAZ thanks to its inhibitory activity on MAPKs and PI3K/AKT pathways.

Second, both DAB2IP and YAP physically bind to the 14-3-3 protein. DAB2IP binding to 14-3-3 is essential to drive the response to TNF-α toward apoptosis (R. Zhang et al., 2007). Instead, YAP interaction with 14-3-3 is mediated by its phospho-Ser127 and is essential for YAP anchoring to α-catenin, which hides Ser127 from phosphatase PP2A, allowing stable YAP sequestration at AJs (Schlegelmilch et al., 2011). Potentially 14-3-3 protein, located at AJs through α-catenin binding, could mediate DAB2IP-YAP interaction, allowing DAB2IP-induced inactivation of YAP. Future experiments will be designed to test this hypothesis.

Finally, both DAB2IP and YAP/TAZ have a role in the β-catenin destruction complex. DAB2IP binds GSK3β and recruits PP2A, that activates GSK3β, thus leading to β-catenin phosphorylation and subsequent degradation (Xie et al., 2010). Notably, the destruction complex is fully functional only in the presence of YAP/TAZ, which associate to Axin and recruit the β-TrCP ubiquitin ligase (Azzolin et al., 2014). Thus, DAB2IP, through GSK3β activation, could facilitate the formation of β-catenin destruction complex, which serves as a cytoplasmic anchor for YAP/TAZ, preventing their nuclear translocation. Clearly, also this hypothesis will require appropriate testing in future experiments.

In conclusion, tumor formation and progression are a dynamic process where genomic alterations cooperate together with loss of tensional homeostasis within the tissue. The proper tensional homeostasis of adult tissue can be perturbed by oncogenic mutations in the mechano-signaling of cells. Reciprocally, oncogenic mutation can be preceded and even sustained by alterations of the extracellular environment such as stiffening of the ECM by fibrosis. Thus, crosstalk between mutated cells and aberrant extracellular environment promotes a feed-forward loop that leads to tumor evolution (Northey et al., 2017).

From this Thesis, DAB2IP is emerging as a sensor and transducer of mechanical forces. Thus, could these properties make DAB2IP a limiting factor of cancer onset and progression?

The tumor suppressor DAB2IP is frequently downregulated in human malignancies, however, its loss does not induce cancer formation per se, but strongly supports cell transformation induced by driver mutations in various oncogenic pathways. In contrast, YAP and TAZ are frequently hyperactivated in cancer, leading to proliferation, invasion, metastasis, and poor prognosis (Dobrokhotov et al., 2018). I collected evidences that in non-transformed mammary epithelial cells DAB2IP can dampen YAP/TAZ activity, and may contribute to YAP/TAZ inhibition in response to cell-cell contact in high density culture conditions.

Mechanical inputs converge on YAP and TAZ modulation, and this axis has a major role in cancer development and aggressiveness, especially in conditions of a stiffening extracellular environment. Interestingly, DAB2IP is rarely mutated or deleted in cancers; rather, it is repressed or inactivated by various mechanisms that can theoretically be targeted to restore physiological levels of the protein (Bellazzo et al., 2017). Due to its activity on multiple pathways, restoration or activation of DAB2IP function in cancer cells would dampen pro-oncogenic signals originating from a variety of different genetic mutations. Based on my observations, it is possible that DAB2IP restoration in cancer cells would also mitigate oncogenic responses to mechanical stimuli from a stiffening tumoral ECM, and in particular YAP/TAZ hyperactivation, limiting tumor aggressiveness and chemoresistance, and potentially opening to novel therapeutic possibilities.

Materials and Methods

Cell lines and culture condition

The following cell lines were used: triple-negative breast cancer MDA-MB-231, breast epithelial MCF10A, and stably transduced pLPC, pLPCMyc-hDAB2IP, and pLPC-Myc-hDAB2IP R385L MCF10A clones. MDA-MB-231 were cultured in DMEM (Sigma) supplemented with 10% FBS (ECS0180L, Euroclone), and antibiotics (DE17-602E, Lonza). MCF10A were cultured in DMEM/Ham's F12 medium (Euroclone) supplemented with 5% Horse Serum (ThermoFisher Life Tech), 1% Penicillin/Streptomicin (Euroclone), 10 μg/ml Insulin (Sigma), 0,5 μg/ml Hydrocortison (Sigma), and 20 ng/ml Epidermal Growth Factor (Cell).

Preparation of fibronectin-coated hydrogel matrix

50 or 0.5 kPa Easy Coat hydrogels (Cell guidance system) were coated with 10 μg/ml fibronectin (Sigma). Cells on fibronectin-coated hydrogel matrix were cultured for 24 hours.

Cell silencing

Cells were cultured for 48 hours, and then transfected with 20 μM siRNA oligonucleotides, using Lipofectamine® RNAiMax (Invitrogen) following manufacturer's instructions. siRNAs used are listed in Table 1.

siRNA	**Sequence**	**Purchased from**
Control siRNA	Unknown	All star negative control (1027281, Qiagen)
siDAB2IP A	GGAGCGCAACAGUUACCUG	Eurofins MWG
siDAB2IP B	GGUGAAGGACUUCCUGACA	Eurofins MWG
siE-cadherin	GCAGAAAUUAUUGGGCUCUUU	Eurofins MWG

Table 1 siRNAs

For DAB2IP silencing we used a mixture of siDAB2IP A and siDAB2IP B.

After 6 hours of transfection, cells medium was changed. In all experiments cells were silenced at least for 48 hours, in order to obtain proper silencing of the target gene.

Transient and stable transfection, retrovirus production, and infection

MDA-MB-231 (E-cadherin null) cells were transfected with Lipofectamine® LTX (Invitrogen) following manufacturer's instructions. pcDNA3 E-cadherin-GFP has been transfected at concentration of 1 ug/1*10^5 cells cultured.

For retroviruses production, low confluent HEK 293GP packaging cells were transfected by calcium phosphate precipitation with pLPC, pLPC-myc-hDAB2IP and pLPC-myc-hDAB2IP R385L. After 48–72 hours, the virus-containing medium was filtered and added to MCF10A cells. Cells were selected with puromycin (0.5 μg/ml) and kept under selection for the entire experiment.

To obtain monoclonal cell lines, cells with retroviruses pLPC, pLPCMyc-hDAB2IP, and pLPC-Myc-hDAB2IP R385L were plated at increasing dilutions in 96-well plates, to obtain less than one cell per well (average 0,3 cells/well). Among all the colonies obtained, three monoclonal MCF10A lines have been selected on the base of DAB2IP protein level detected by immunoblot and immunostaining.

Drug treatments

Trypsin (Sigma), that breaks interactions by degrading adhesion proteins, and EDTA (Sigma), that breaks interactions by chelating Calcium and Magnesium ions, were used for detaching cells. Cells were treated with Trypsin and 0.5 mM EDTA for 15 minutes.

EGTA (Sigma), a chelator of extracellular Ca2+ that breaks the homophilic binding between E-cadherins, and DTT (Sigma), a drug that reduces disulfide bridges compromising E-cadherin structure were used to break adherent junctions. Cells were treated with 10 mM EGTA/DTT for 3 hours.

Cytoskeleton drugs: Latrunculin A that binds G-actin and prevents the monomers' polymerization, has been used 0.5 uM for 4h. Cytochalasin D that acts by capping F-actin and preventing actin elongation, was used at 10 uM for 4h. Blebbistatin, inhibitor of myosin II that suppresses actomyosin contraction causing filaments disruption and low cell tensional state, has been used at 50 uM for 24 hours. PF573228, an ATP-competitive inhibitor of Focal Adhesion Kinase (FAK)

that decrease focal adhesion turnover and destabilize actin filaments, was used at 10 uM for 6 hours.

BrdU incorporation assay

Cells were plated on coverslips and cultured for 48 hours before being labeled by adding 20 μM BrdU (B5002 Sigma) for 2 hours. After BrdU incorporation, cells were washed with PBS and fixed in 4% PFA for 15 minutes. After permeabilization with 0,1% Triton x-100 at room temperature for 4 minutes, DNA was denaturated with 50mM NaOH for 1 minute. BrdU was detected by immunofluorescence using a specific anti-Bromo-deoxyuridine monoclonal antibody (clone BU-1-Amersham), incubated for 2 hours at 37°C, followed by anti-mouse Alexa Fluor® 594 conjugated secondary antibody (Life Technologies), incubated at 37°C for 45 minutes. Nuclei were stained with Hoechst 33342 (incubation for 5 minutes, Life Technologies), then each coverslip was prepared for microscopic examination by applying the mounting medium ProLong Gold antifade reagent (Life Thecnologies). Images were captured using a Leica DM4000B epifluorescence microscope at 10X magnification. Proliferation was scored by counting BrdU-positive cells over total cell nuclei, in 20 random microscope fields.

AFM Force Spectroscopy

Force spectroscopy analysis of cells was carried out by using a Smena AFM (NT-MDT Co., Moscow, Russia) mounted on an inverted fluorescence microscope (Nikon Eclipse Ti-U). MCF10A cells were cultured on glass and fixed with PFA 4% for 20 minutes by shaking. To visualize nuclei, cells were stained with Hoechst 33342 (Life Technologies) in PBS for 5-10 minutes. Then, cells were analyzed with the AFM in contact mode in liquid solution (PBS). For the measurement, silicon spherical tip with a diameter of 20 μm (Tip: Nova Scan cantilever, k = 0.064 N/m) was used, in order to collect the global stiffness of each cell. A force (SetPoint) of < 1 nN, a Gain of 1 or 2, an indentation rate of 2 μm/sec (low enough to avoid hydrodynamic effects) and an indentation of -500 nm (~10% of the total cell height) were used. For each sample, 30-60 cells were analyzed. Elastic modulus values (E), in kPa, were determined by fitting the obtained force-displacement curves with the Hertz model by using AtomicJ® software.

Immunofluorescence

Cells cultured on coverslips, were fixed in 4% PFA solution for 15 minutes and permeabilized with 0.1% Triton X- 100 for 10 minutes. Then, after incubation for 30 minutes with a blocking solution (3% FBS in PBS), cells were incubated with primary antibodies (Table 2) diluted in PBS-FBS 3% for 2 hours at 37°C. Primary antibodies were recognized by fluorescent secondary antibodies (anti-mouse Alexa Fluor® 488/568 and anti-rabbit Alexa Fluor® 488/568 conjugated secondary antibodies, Life Technologies), incubated for 1 hour. Together with secondary antibodies, Fluorescein phalloidin (Thermo Fisher Scientific), a high-affinity F-actin probe conjugated to the green fluorescent dye fluorescein (FITC), was used to label actin filaments. Nuclei were labeled with Hoechst 33342 (incubation for 5-10 minutes, Life Technologies), then each coverslip was prepared for microscopic examination by applying the mounting medium ProLong Gold antifade reagent (Life Technologies). Images were captured using a Leica DM4000B epifluorescence microscope at 40X magnification. To determine YAP subcellular localization, images were analyzed counting the percentage of cells with YAP into the nucleus, cytoplasm, or both cell compartments over total cell nuclei, in 20 random microscope fields. Nuclear areas were obtained from analysis of epifluorescence images with ImageJ® software: the total epifluorescence of nuclei labelled with Hoechst 33342 has been normalized for the cell number present in each frame. For each sample, 20 random microscope fields were analyzed.

TARGET	**ANTIBODY**	**DILUITION**
DAB2IP	A302-440 A BETHYL	1:200
YAP	Sc-101199 Santa cruz	1:200
p-MLC S19	S3675 Cell Signalling	1:200

Table 2 IF antibodies

Luciferase reporter assay

Cells cultured for 48 hours were transiently transfected with 250 ng/cm2 of pGL3-8xGTIIC-Lux reporter using Lipofectamine® LTX (Invitrogen), following manufacturer's instructions. pGL3-8xGTIIC-Lux is a reporter with Luciferase gene (P. pyralis) under the control of a TEAD responsive promoter (Dupont et al., 2011). Together with reporter, also 25 ng/cm2 of CMV-Renilla were transfected in order to normalize for transfection efficiency. After 24 hours of

transfection, cells were lysed in Passive Lysis Buffer (Promega) and frozen at -20°C. The following day, lysates were analyzed using Dual Luciferase Reporter Assay System Kit (Promega, E1910), on a Turner Design luminometer (Promega). Each sample was transfected in triplicate and each experiment was repeated three times independently.

Western Blot analysis and Antibodies

Total cell extracts were prepared in RIPA buffer without SDS (150mM NaCl, 50mM Tris-HCl pH8, 1mM EDTA, 1% NP-40, 0,5% Na-deoxycholate) supplemented with 1 mM PMSF, and 10μg/ml CLAP (proteases inhibitors), 5 mM NaF, and 1 mM Na3VO4 (phosphatases inhibitors). Protein concentration was determined with Bio-Rad Protein Assay Reagent (#500-0006, Bio-Rad) and lysates were diluted to the same concentration with RIPA. Lysates were resolved by SDS/PAGE and transferred to nitrocellulose (Millipore). Western blot analysis was performed according to standard procedures using the primary antibodies listed in Table 3.

TARGET	**ANTIBODY**
DAB2IP	A302-440 A BETHYL
E-cadherin	BD610182 BD Bioscience
FAK	sc-558 Santa Cruz
P-FAK	8556s Cell Signalling
YAP	sc-101199 Santa Cruz
P-YAP S127	4911s Cell Signaling
HSP90	sc-13119 Santa Cruz
GAPDH	sc-32233 Santa Cruz

Table 3 WB antibodies

Anti-mouse and anti-rabbit IgG HRPO-conjugated (Sigma) were used as secondary antibodies. Bands were detected on autoradiographic film (GE Healthcare) using Liteablot Extent chemiluminescent substrate (Euroclone) and Pierce™ ECL Western Blotting Substrate (ThermoFisher Life Tech).

RNA extraction and RT-qPCR

Total RNA was extracted using EuroGOLD TriFast (Euroclone) following manufacturer's instructions. Purified RNA samples were quantified using a Nanodrop Spectrophotometer. For RNA expression analysis, 0.5 μg of total RNA (100ng/μl) was reverse-transcribed using the iScript™ Advanced cDNA Synthesis Kit (Biorad). A CFX Connect™ Real-Time PCR System (Biorad) was used using Itaq UniversSYBR Green (Biorad), following manufacturer's instructions. Primers used are listed in Table 4.

TARGET		SEQUENCE
ANKRD1	Forward	5'-CAC TTC TAG CCC ACC CTG TGA-3'
	Reverse	5'-CCA CAG GTT CCG TAA TGA TTT-3'
CTGF	Forward	5'-AGG AGT GGG TGT GTG ACG A-3'
	Reverse	5'-CCA GGC AGT TGG CTC TAA TC-3'
CYR61	Forward	5'-AGC CTC GCA TCC TAT ACA ACC-3'
	Reverse	5'-TTC TTT CAC AAG GCG GCA CTC-3'
DAB2IP	Forward	5'-CAC ATC ACC AAC CAC TAC-3'
	Reverse	5'-TCC ACC TCT GAC ATC ATC-3'
H3	Forward	5'-GAA GAA ACC TCA TCG TTA CAG GCC TGG T-3'
	Reverse	5'-CTG CAA AGC ACC AAT AGC TGC ACT CTG GAA- 3'

Table 4 RT-qPCR Primers

Statistical analysis

In all graphs data are expressed as mean ± SD of at least three independent experiments, except when otherwise indicated. Differences were analyzed by Student's t test using Prism 6 (GraphPad). P-values < 0.05 were considered significant.

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