Enigma proteins regulate YAP mechanotransduction

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JCS Advance Online Article. Posted on 7 November 2018

Abstract

Human cells can sense mechanical stress acting upon Integrin adhesions and respond by sending the YAP (YAP1) and TAZ (WWTR1) transcriptional co-activators to the nucleus to drive TEAD-dependent transcription of target genes. How Integrin signaling activates YAP remains unclear. Here we identify a key role for the Enigma (PDLIM7) and Enigma-like (PDLIM5) family of PDZ and LIM domain containing proteins in Integrin-mediated mechanotransduction. YAP binds to PDLIM5/7 via its C-terminal PDZ binding motif (PBM), which is essential for full nuclear localization and activity of YAP. Accordingly, silencing of PDLIM5/7 expression reduces YAP nuclear localization, tyrosine phosphorylation, and transcriptional activity. The PDLIM5/7 proteins are recruited from the cytoplasm to Integrin adhesions and F-actin stress fibres in response to force by binding directly to the key stress fibre component alpha-actinin. Thus, forces acting on Integrins recruit Enigma family proteins to trigger YAP activation during mechanotransduction.

Introduction

Integrin adhesion to the extracellular matrix is a fundamental mechanism controlling tissue growth and form during normal development (Wickstrom et al., 2011) and in cancer (Hamidi et al., 2016). In addition to providing adhesion to the matrix, Integrins enable cells to sense mechanical forces to activate 'inside-out signaling', which stimulates Integrin binding to matrix ligands, as well as 'outside-in signaling', which activates cytoplasmic mechanotransduction pathways to regulate cell behaviour (Legate et al., 2009; Ross et al., 2013; Sun et al., 2016). In particular, focal adhesion kinase (FAK) and Src family kinases have important roles in Integrin signaling (Avizienyte and Frame, 2005) and in synergy between Integrin and growth factor signaling (Chen et al., 2017). Recent work has unveiled a consensus Integrin adhesome containing a large number of proteins that are likely to be involved in either adhesion or mechanotransduction (Horton et al., 2015).

One crucial downstream effector of Integrin signaling is the Yes-associated protein (YAP or YAP1), originally discovered by virtue of its ability to form a complex with the Src family kinase, Yes (Sudol, 1994). YAP (and its paralog TAZ or WWTR1) was found to be a transcriptional co-activator that is negatively regulated by interaction with 14-3-3 proteins (after serine phosphorylation) and positively regulated by interaction with PDZ domains (via a C-terminal PDZ binding motif or PBM) (Kanai et al., 2000; Yagi et al., 1999). YAP was subsequently shown to function as a co-activator for the TEAD family of DNA binding transcription factors, even though the majority of the YAP protein was localized to the cytoplasm in complex with 14-3-3 proteins (Vassilev et al., 2001). Although YAP is cytoplasmic at high cell density, it can translocate to the nucleus when cells lose contact with

one another and/or spread out across their substrate (Zhao et al., 2007). Importantly, the nuclear localization of YAP was shown to require the presence of the C-terminal PDZ binding motif (Oka and Sudol, 2009). YAP shuttles dynamically between the cytoplasm and nucleus, with its bulk distribution likely to be determined by relative binding to cytoplasmic (e.g.: 14-3-3) versus nuclear (e.g.: TEAD) proteins (Badouel et al., 2009; Ren et al., 2010; Vassilev et al., 2001; Zhang et al., 2008; Zhao et al., 2007), or possibly through regulated nuclear import or export (Ege et al., 2018; Furukawa et al., 2017; Manning et al., 2018).

Culture of cells on micropatterns and different matrix types suggested that the size of the contact cells make upon spreading over their basal substrate, substrate stiffness, and the resulting mechanical tension on F-actin stress fibres are key determinants of YAP subcellular localization in response to cell density (Dupont et al., 2011; Wada et al., 2011). Nevertheless, different groups drew opposite conclusions as to whether mechanical tension on stress fibres signals via the Hippo pathway kinase LATS1/2 to control YAP localization, or via a LATS1/2-independent pathway (Dupont et al., 2011; Meng et al., 2018; Wada et al., 2011). Recent work has confirmed a key role for Integrin adhesion to the extracellular matrix and Integrin signaling via Talin, Rho and Src family kinases as important mechanosensory mechanisms that regulate YAP (Elbediwy et al., 2016; Elosegui-Artola et al., 2016; Kim and Gumbiner, 2015; Tang et al., 2013). Integrin-Src signaling can affect LATS1/2 phosphorylation of YAP (Elbediwy et al., 2016; Kim and Gumbiner, 2015) possibly via direct tyrosine phosphorylation of LATS1 (Si et al., 2017), or via cross-talk with growth factor signaling (Fan et al., 2013; Kim and Gumbiner, 2015). Alternatively, Integrin-Src signaling can also activate YAP via direct tyrosine phosphorylation of YAP in its transcriptional activation domain (Li et al., 2016; Taniguchi et al., 2015). Finally, YAP can also sense mechanical stretching of E-cadherin based adherens junctions (Benham-Pyle et al., 2015), possibly via Ajuba/LIMD1/TRIP6-mediated LATS1/2 inhibition (Dutta et al., 2018; Ibar et al., 2018; Rauskolb et al., 2014), via Hippo kinase (MST1/2) inactivation (Fletcher et al., 2018), or via Src activation at adherens junctions (Gomez et al., 2015; Kim et al., 2011; McLachlan et al., 2007; Roura et al., 1999; Serrels et al., 2011; Shindo et al., 2008; Tsukita et al., 1991). How YAP might be recruited to Integrin (or E-cadherin) adhesions in order to be directly phosphorylated by Src family kinases in response to mechanical force is an important unsolved problem.

Results and Discussion

To identify possible binding partners of YAP, we performed IP-mass spectrometry of GFP tagged human YAP transfected into human HEK293T cells. Aside from many known interactors, which confirm the quality of our IP-mass-spectrometry analysis, we identified

novel interactors including the PDZ and LIM domain containing (PDLIM) family proteins Enigma (PDLIM7) and Enigma-like (PDLIM5) (Fig 1A-C). Note that both Enigma family proteins were identified as members of the Integrin adhesome, although their function remains poorly understood (Horton et al., 2015). We confirmed this interaction by co-IP of GFP-YAP and western blotting for the Enigma proteins PDLIM5 and PDLIM7 (Fig 2A). Importantly, deletion of the YAP PBM motif (YAPΔC) abolished the interaction between YAP and Enigma family proteins, suggesting that Enigma proteins bind to YAP via the PBM motif (Fig 2A). We further find that IP of endogenous YAP also pulls down endogenous PDLIM5 and PDLIM7 (Fig 2B). We confirm previous observations (Oka and Sudol, 2009; Shimomura et al., 2014) that the PBM motif is important to promote YAP nuclear localization by comparison of transfected GFP-YAP with GFP-YAPΔC in sparsely plated human Caco2 epithelial cells (Fig 2C, D).

To test whether Enigma family proteins are required for YAP to localize to the nucleus upon cellular stretching, we plated human Caco2 cells at moderate density and transfected them with either control or Enigma PDLIM5/7-targeted siRNAs. We find that control siRNAs had no effect, while silencing of Enigma family proteins strongly inhibited nuclear localization of YAP, without affecting cell shape or density (Fig 2E-G). Silencing of Enigma PDLIM5/7 also reduced the Src family kinase-dependent tyrosine 375 and 428 phosphorylation of YAP, similar to loss of the YAP C-terminal PBM, without affecting p-Src levels or localization to focal adhesions (Fig 2H, I; Fig S1). The reduction in YAP transcriptional activity upon siRNA knockdown of Enigma PDLIM5/7 was also comparable to that of deleting the YAP C-terminal PBM in a TEAD-multimer reporter gene assay (Fig 2J). Analysis of the YAP target genes *AREG*, *MYC* and *PCNA* by quantitative-PCR reveals a comparable reduction in target gene expression upon silencing of Enigma PDLIM5/7 (Fig 2K). Furthermore, the rate of cell proliferation, as measured by a pulse of EdU incorporation in cells undergoing S-phase of the cell cycle, is reduced upon silencing of Enigma PDLIM5/7 (Fig 2L). We conclude that Enigma family proteins bind directly to YAP via the C-terminal PBM to promote YAP tyrosine phosphorylation, nuclear localization and transcriptional activation in human cells.

We next examined the subcellular localization of Enigma/PDLIM7 and Enigma-like/PDLIM5. We find that both proteins localize to the cytoplasm in densely cultured cells, similar to YAP (Fig 3A, B). In response to spreading of the cells upon plating at low density, both Enigma family proteins relocalize in part to F-actin stress fibres and focal adhesions, as well as to F-actin fibres at adherens junctions (Fig 3A, B). At the same time, YAP translocates to the nucleus, suggesting that the relocalization of Enigma proteins to sites of mechanical force

sensing could be a trigger for YAP nuclear localization (Fig 3A, B). Accordingly, PDLIM5/7 can both be detected in a complex with the F-actin stress fibre component alpha-actinin (ACTN1; Fig 3C, D). We propose that, upon mechanical stimulation of human cells, Enigma family proteins bind to alpha-actinin on F-actin stress fibres at Integrin focal adhesions in order to promote tyrosine phosphorylation of YAP by Src family kinases and thus YAP activation (Fig 3D). Note that we find similar co-regulation of YAP and Enigma when cells are mechanically stimulated by plating on matrices of varying stiffness (Fig S2). Interestingly, Src family kinases can also be activated at alpha-actinin containing F-actin cables organized by E-cadherin containing adherens junctions (McLachlan et al., 2007), and we are also able to detect some PDLIM5/7 localization with p-Src at adherens junctions as well as at focal adhesions (Fig S3).

We sought to test our hypothesis that Enigma proteins can induce nuclear localization of YAP by promoting direct phosphorylation of YAP by Src family kinases, rather than by an indirect mechanism that requires the phosphorylation of YAP by LATS family kinases. To test this idea, we examined the localization of phospho-mutant YAP5SA, which is unable to be inhibited by canonical Hippo-LATS1/2 kinase signaling. We find that human YAP5SA remained cytoplasmic in densely cultured cells and becomes nuclear in sparsely cultured cells, indicating the requirement for a parallel pathway that regulates YAP nucleo-cytoplasmic translocation independently of canonical Hippo signaling (Fig 4A). Treatment with the Src family kinase inhibitor Dasatinib, or PDLIM5/7 siRNA, reduced nuclear localization of YAP5SA, such that most cells had a comparable level of YAP5SA in both nucleus and cytoplasm – similar to the effect of Src/Fyn/Yes triple siRNA on endogenous YAP localization (Fig 4A-C; Fig S1). We next considered whether direct phosphorylation of YAP on multiple tyrosine residues by Src family kinases (Li et al., 2016) could account for density-dependent regulation of YAP5SA (Fig 4D). Accordingly, we find that mutation of three tyrosine residues to phenylalanine (3YF) in the YAP transcriptional activation domain (TAD) reduces the nuclear localization of YAP5SA, similar to treatment with Dasatinib (Fig 4D-F). Finally, Dasatinib also reduced YAP nuclear localization even in the absence of LATS1/2 induced by either siRNA silencing in densely cultured human epithelial cells (Fig 4G-I) or by double conditional knockout of homozygous floxed *LATS1*$^{fl/fl}$ and *LATS2*$^{fl/fl}$ upon ubiquitous expression of tamoxifen-inducible *Cre-ERt* allele with GFP-YAP in mouse embryonic fibroblasts (Fig 4J,K). These results suggest parallel regulation of YAP by LATS1/2 and Src family kinases in response to cell density, in agreement with recent findings in cholangiocarcinoma cells (Sugihara et al., 2018).

In conclusion, our results indicate that mechanical stress-induced binding of Enigma proteins to alpha-actinin to basal stress fibres provides a platform for YAP to be tyrosine phosphorylated by Src family kinases to promote YAP nuclear localization and full activation of YAP-driven transcription. Further work is necessary to understand how tyrosine phosphorylation promotes YAP localization to the nucleus, although such phosphorylation could promote either nuclear import, as in the case of STAT proteins (Reich, 2013; Reich and Liu, 2006), or interaction with nuclear proteins such as TEADs or SWI/SNF components, which could maintain nuclear localization as well as regulate transcription (Skibinski et al., 2014; Song et al., 2017; Zhu et al., 2015). Importantly, this Enigma-dependent mechanism for regulation of YAP must act in parallel to inhibition of the canonical Hippo pathway by Integrin signaling, via multiple signaling pathways (Elbediwy et al., 2016; Elosegui-Artola et al., 2016; Kim and Gumbiner, 2015; Kissil et al., 2002; Meng et al., 2018; Sabra et al., 2017; Si et al., 2017; Xiao et al., 2002). It must also act parallel to any direct mechanical regulation of the nucleus, which becomes strongly deformed and permeable to small proteins in extremely flattened cells (Elosegui-Artola et al., 2017; Shiu et al., 2018) and, indeed, Enigma proteins are no longer required for nuclear localization of YAP upon such extreme cellular flattening (data not shown). In future, it will be of great interest to investigate with genetically modified mice which of these mechanotransduction pathways operates in different mammalian tissues *in vivo*.

Methods

Plasmids

pEGFP C3-YAP2 and pEGFP C3-YAP-DeltaC plasmids were a gift from Marius Sudol (Addgene plasmids # 19055 and # 21126). pCMV-Flag YAP2 5SA was a gift from Kun-Liang Guan (Addgene plasmid # 27371). pEGFP C3-YAP2 3YF and pCMV-Flag YAP2 5SA 3YF were created using pEGFP C3-YAP2 and pCMV-Flag YAP2 5SA, respectively, by mutating the 3 tyrosine residues in question (Y375F Y391F and Y428F). Note the YAP constructs are generated using mRNA isoform 3. Site directed mutagenesis was performed by Creative Biogene. pEGFP-N1 alpha-actinin 1 was a gift from Carol Otey (Addgene plasmid # 11908). pNL2.2 – 8xTEAD and pRL-CMV Renilla (Promega). All plasmids were transfected using Lipofectamine 3000 (Invitrogen) To constitutively overexpress human PDLIM7, PDLIM7 (isoform 1) ORF was subcloned from the corresponding entry vector (Dharmacon; clone 3562 for PDLIM7 isoform 1) into the destination vector pcDNA-PDEST47 (Invitrogen, 12281010) by recombination using the Gateway LR clonase enzyme mix (Invitrogen,11791).

Human Cell Culture

Human Caco-2 cells and HEK293T (Francis Crick Institute cell services) were grown in conditions as previously described (Elbediwy et al., 2016). All cells are subject to mycoplasma testing.

Generation of LATS1/2 MEFs

All experiments were carried out in accordance with the United Kingdom Animal Scientific Procedures Act (1986) and UK Home Office regulations under project license number 70/7926. Mouse embryonic fibroblasts were derived from E14.5 *Lats1*$^{lox/lox}$;*Lats2*$^{lox/lox}$ ((Yi et al., 2016) carrying the *Rosa26-cre-ERT2* (Seibler et al., 2003**)** allele on a mixed background. At passage 4 or 5, 100 000 cells were plated in each well of an 8-well Ibidi chamber slide. 24 hours after plating, pEGFP C3-YAP2 was transfected using Lipofectamine 3000 while simultaneously adding tamoxifen. Transfection was left for a further 48 hours and media was changed 24 hours post tamoxifen treatment before experiment was fixed using standard immunofluorescence protocol.

Co-Immunoprecipitation

HEK293T cells were transfected with the relevant plasmids using Lipofectamine 2000. the sample was then lysed and subjected to co-immunoprecipitation using a GFP Trap Kit containing Lysis Buffer which comprised 10 Mm Tris Ph 7.5, 150 Mm Nacl, 0.5% NP- 40 And 0.5 Mm EDTA (Chromotek). Lysis buffer was supplemented with PhosStop Phosphatase Inhibitor Cocktail Tablets (Roche), Protease Inhibitor Cocktail (Roche), 0.1 M NaF and 1 mM PMSF. Samples were left on ice to solubilise for 10 min, before being centrifuged, pre-cleared and incubated with the GFP Trap- M beads for one hour. IPs were subjected to three washes before being lysed in 2x sample buffer and boiled. For mass spectrometry, proteins were subjected to SDS-PAGE followed by in-gel trypsin digestion. Peptide mixtures were analyzed using a Q-Exactive mass spectrometer connected to a U3000 nanoLC. Raw data was processed with MaxQuant software using an estimated 1% false discovery rate for protein identification and intensity based absolute quantification (iBAQ) for protein quantification. For endogenous IP, Mouse YAP antibody (Santa Cruz, 63.7) was used at a concentration of 3ug and bound to Dynabeads. Caco-2 Lysates were processed with as with overexpression co-IPs shown above.

EDU incorporation assay

Cells were processed for RNAi as described and processed with the Click-iT™ EdU Alexa Fluor™ 555 Imaging Kit (Invitrogen) as manufacturers protocol suggests.

QPCR

Extraction of total RNA from Caco-2 siRNA transfected cells processed as previously described (Elbediwy et al., 2016). Primers were purchased as Quantitect Primers (Qiagen).

Antibodies

Antibodies used in mammalian cell culture include: Mouse GFP (Roche), Mouse Flag (Roche), Rabbit PDLIM5 (Atlas antibodies), Rabbit PDLIM7 (Novus), Rabbit YAP H-125, Mouse YAP (63.7) (Santa Cruz), Mouse alpha actinin1 (Abcam), Rabbit pY418 Src (Life technologies), Rabbit Src (Cell Signalling Technology)

Fixation

Cells were fixed as previously described (Elbediwy et al., 2016). cells were lysed in 2x Sample buffer (Tris glycine SDS containing 1x sample reducing agent (Novex).

Mechanotransduction

Cells were plated on Prime coat substrates (2KPa, 10KPa and 30KPa) (Excellness Biotech) and left for 24 hours before being fixed and processed for immunofluorescence.

siRNA transfection

siRNA transfection experiments were performed using Lipofectamine RNAiMax (Invitrogen) in Optimem and antibiotic free media (Gibco). Caco-2 and HEK293T cells were reverse transfected using a final concentration of 80nM siRNA. The following day another round of siRNA transfection was performed. Cells were left for a total of 72 hours before being either fixed in PFA for immunofluorescence or lysed in sample buffer for immunoblotting. Oligonucleotides used for PDLIM5, PDLIM7, Src, Fyn, Yes and YAP were as a siGenome pool (Dharmacon).

Inhibitor treatments

For Dasatinib experiments, cells were treated with 5uM of the compound for a period of 4 hours (Selleck chemicals).

Microscopy

Images were taken on a Leica SP5 laser-scanning confocal microscope.

Quantification

For YAP localisation, Quantification was scored in three separate categories: N = nuclear; N/C = nuclear and cytoplasmic and C = cytoplasmic. Cells were assessed over three independent experiments counting 500-600 cells per condition from random cellular areas. For fluorescence intensity quantification, images were measured using 6 independent areas of cells and over three independent experiments and processed using ImageJ. Graphs were plotted using Prism.

Author contributions

Conceptualization: A.E., B.J.T.; Methodology: A.E., B.J.T.; Software: A.E.; Validation: A.E. Formal analysis: A.E.; Investigation: A.E., B.J.T., H.V., M.D.C.D.D.L.L; D.S; F.P.S.; Resources: B.J.T.; Data curation: A.E.; Writing - original draft: B.J.T.; Writing - review & editing: A.E., B.J.T.; Visualization: A.E.; Supervision: B.J.T.; Project administration: B.J.T.; Funding acquisition: B.J.T.

Funding

This work was supported by The Francis Crick Institute, which receives its core funding from Cancer Research UK (FC001180), the UK Medical Research Council (FC001180), and the Wellcome Trust (FC001180), as well as a Wellcome Trust Investigator award (102853/B/13/Z).

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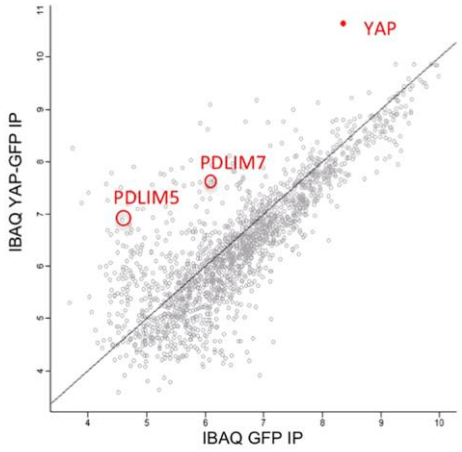
Figures

A



B

List of YAP-associated proteins

Crumbs complex components		
	Lin7	LIN7C
	PALS1	MPP5
	NF2	NF2
	Ezrin	EZR
	Radixin	RDX
	NHERF2	SLC9A3R2
Tight-Junction associated proteins		
	AMOT	AMOT
	AMOTL1	AMOTL1
	AMOTL2	AMOTL2
	ZO-1	TJP1
	ZO-2	TJP2
	ASPP2	TP53BP2
	RASSF8	RASSF8
	PEZ	PTPN14
	MAGI1	MAGI1
Core kinase cassette		
	LATS	LATS1
	MOB1	MOB1
14-3-3 family proteins		
	14 3 3	YWHAB
	14 3 3	YWHAZ
	14 3 3	YWHAQ
	14 3 3	YWHAG
	14 3 3	YWHAE
TEADs, WBP2 & SWI/SNF complex		
	TEAD1	TEAD1
	TEAD3	TEAD3
	TEAD4	TEAD4
	WBP2	WBP2
	SWI/SNF	SMARCE1
	SWI/SNF	SMARCD2
	SWI/SNF	SMARCC2
	SWI/SNF	SMARCB1
	SWI/SNF	SMARCB1
	SWI/SNF	SMARCC1
	SWI/SNF	SMARCD1
	BRG1	SMARCA4
	SWI/SNF	DPF2
SWI/SNF associated proteins		
	BCL7A	BCL7A
	BCL7C	BCL7C
	BRCC3	BRCC3
	BRE	BRE
	ARID1A	ARID1A
	ARID1B	ARID1B
	SSXT	SYT-SSX1
	ACTL6A	BAF53A
Other transcriptional regulators		
	Mediator	ARGLU1
	CBF	CBFA2T2
	DNMT1	DNMT1
	FUBP1	FUBP1
RNA polymerase II complex		
	Pol II	POLR2B
	Pol II	RPAP3
	Pol II	POLR2J2
	Pol II	POLR2C
	Pol II	POLR2H
	Pol II	POLR2E
	Pol II	POLR2A
	Pol II	POLR2L
	Pol II	RPAP2
	Pol II Assoc	WDR92
	Pol II Assoc	UXT
	CDK9	CDK9
	LARP7	LARP7
	GPN-loop	GPN1
Prefoldins		
	Prefoldin	PFDN2
	Prefoldin	PDRG1
Novel Interactors		
	PDLIM5	PDLIM5
	PDLIM7	PDLIM7
	Talin2	TLN2
	CD9	CD9
	TRAF2	TRAF2
	BAG4	BAG4
	NUDT5	NUDT5
	PARP2	PARP2
	c-IAP1	BIRC2

C

Protein	Uniprot ID	IBAQ YAP IP	IBAQ GFP IP	Confidence Ratio
YAP1	P46937	6.67	0	6.67
TEAD1	P28347	7.61	5.91	1.29
LATS1	O95835	7.37	4.84	1.52
WBP2	Q969T9	8.63	6.04	1.43
PDLIM5	Q96HC4	6.88	4.58	*1.50*
PDLIM7	Q9NR12	7.61	6.10	*1.25*

Figure 1. YAP immunoprecipitation and mass-spectrometry analysis of binding partners.
(A) YAP immunoprecipitation and mass-spectrometry analysis of co-precipitated proteins identifies the Engima family proteins PDLIM5 and PDLIM7 as novel YAP-associated proteins. Axes are log10-transformed values.
(B) List of all YAP-associated proteins from (A).
(C) Comparison of confidence ratios for PDLIM5/7 and known YAP interactors.

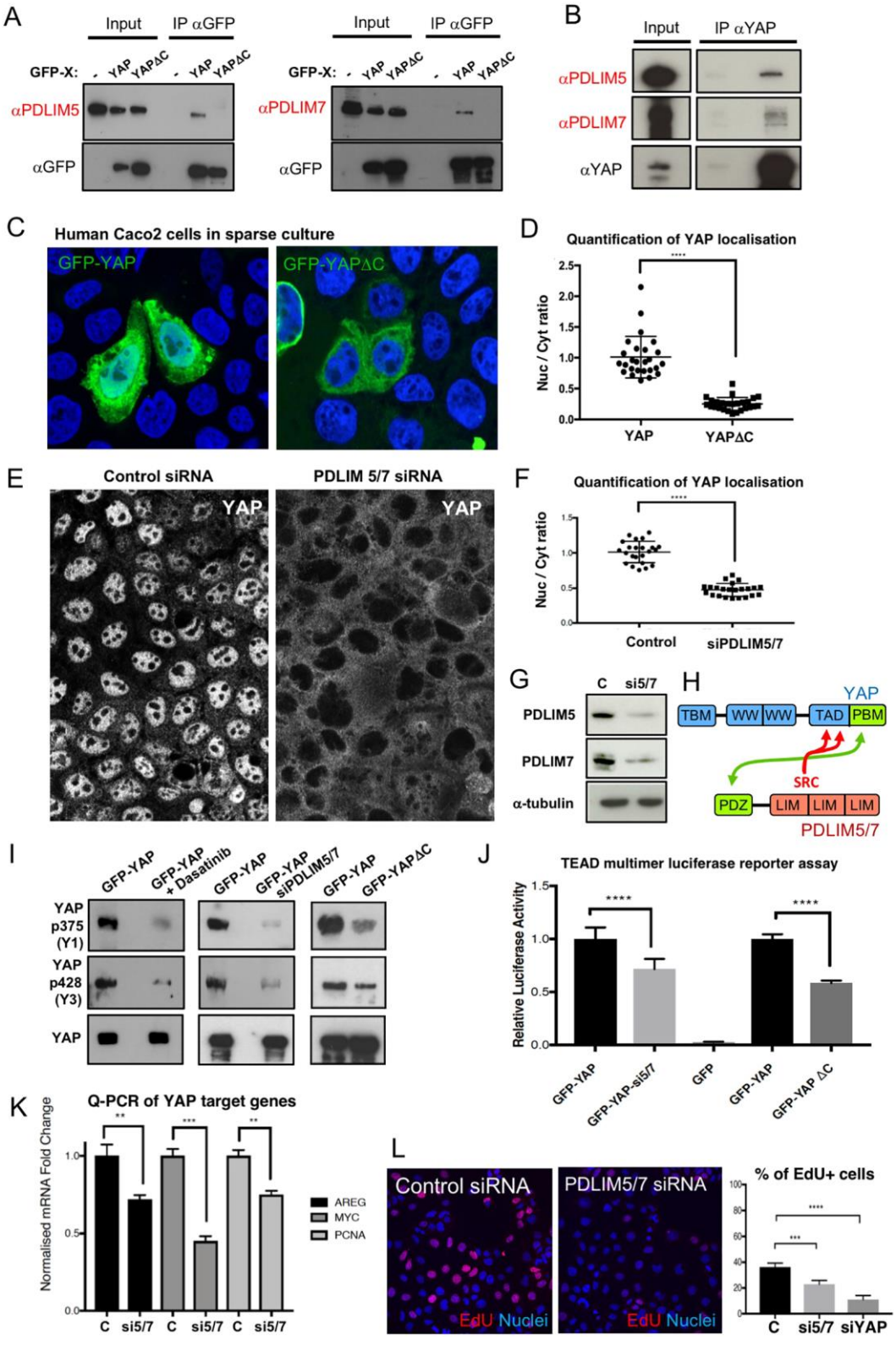


Figure 2. Enigma PDLIM5/7 family proteins bind to the YAP C-terminal PDZ binding motif (PBM) and promote YAP nuclear localization and transcriptional activity.
(A) Confirmation of YAP-PDLIM5 and YAP-PDLIM7 interaction by co-immunoprecipitation of GFP-tagged YAP and immunoblotting with anti-PDLIM5 and anti-PDLIM7 antibodies. Both Enigma family proteins PDLIM5 and PDLIM7 bind to the YAP C-terminal PBM, as deletion of this motif (YAPdeltaC) abolishes the interaction in co-immunoprecipitation experiments. n = 3 biological replicates.

(B) Endogenous YAP co-immunoprecipitates with PDLIM5 and PDLIM7. n = 3
(C) Delection of the C-terminal PBM (YAPΔC) reduces nuclear localization of GFP-tagged YAP in human Caco2 cells plated at low density. n = 3 biological replicates.
(D) Quantification of YAP localization in (C). (**** $p < 0.001$).
(E) Silencing of both PDLIM5/7 expression in human Caco2 cells strongly reduces nuclear localization of YAP even though cells remain spread out due to sparse plating conditions. n = 6 biological replicates.
(F) Quantification of YAP localization in (E). (**** $p < 0.001$)
(G) Depletion of Enigma PDLIM5 and PDLIM7 expression levels by siRNA treatment in (E). n = 3 biological replicates.
(H) Schematic diagram of the interaction between the YAP PBM and the PDZ domain of Enigma PDLIM5/7 proteins. Proximity of Src phosphorylation sites to the PBM is shown. n = 3 biological replicates.
(I) Silencing of PDLIM5/7 in human Caco2 cells reduces tyrosine phosphorylation of YAP. (n = 3 biological replicates, **** $p < 0.001$).
(J) Silencing of PDLIM5/7 in human Caco2 cells reduces YAP-driven TEAD transcriptional activity, as measured by a TEAD multimer luciferase reporter assay (relative to a Renilla luciferase control). Similar results were obtained upon deletion of the YAP C-terminal PBM. (*** $p < 0.005$, ** $p < 0.01$)
(K) Silencing of PDLIM5/7 in human Caco2 cells reduces expression of YAP-target genes *AREG*, *MYC* and *PCNA*. (n = 3, *** $p < 0.005$, ** $p < 0.01$)
(L) Silencing of PDLIM5/7 in human Caco2 cells reduces the rate of cell proliferation, as measured by a pulse of EdU incorporation. Upon loss of PDLIM5/7, proliferation slows by approximately half compared with control cells, which is comparable in magnitude to that achieved by silencing of YAP itself. (**** $P < 0.001$, *** $P < 0.005$)

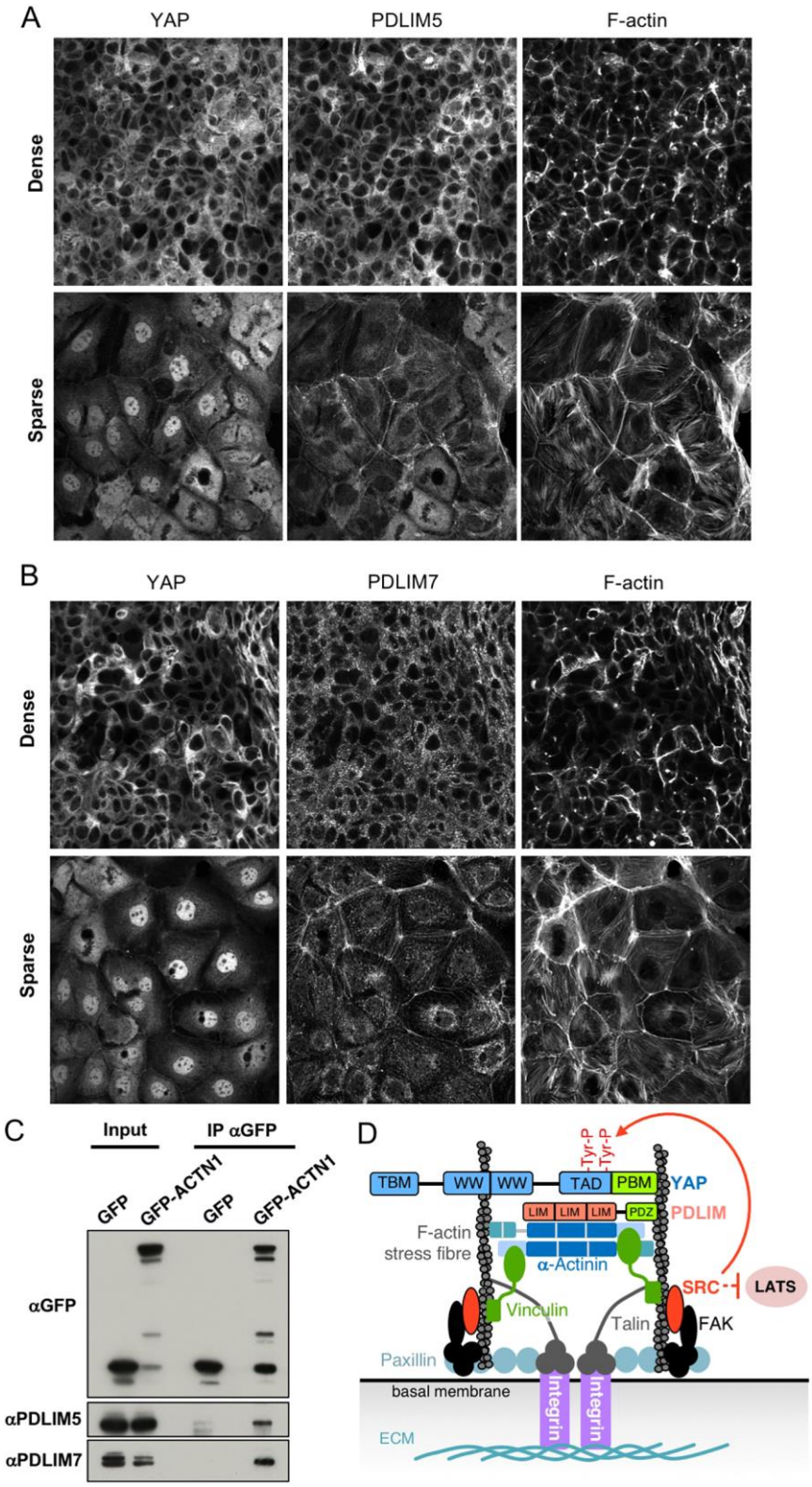


Figure 3. Enigma PDLIM5/7 proteins relocalize from the cytoplasm in dense cells to basal stress fibres in sparse cells and bind directly to the stress fibre component alpha-actinin.
(A) Enigma PDLIM5 localizes to the cytoplasm in dense cells but translocates in part to F-actin stress fibres and adherens junctions in sparsely plated cells.
(B) Enigma PDLIM7 localizes to the cytoplasm in dense cells but translocates in part to F-actin stress fibres and adherens junctions in sparsely plated cells.

(C) Co-immunoprecipitation of Enigma PDLIM5/7 proteins with GFP-tagged α-actinin from human Caco2 cells.
(D) Schematic diagram of YAP recruitment via Enigma PDLIM5/7 proteins to Integrin-Src signaling complexes to sense mechanical forces basally.

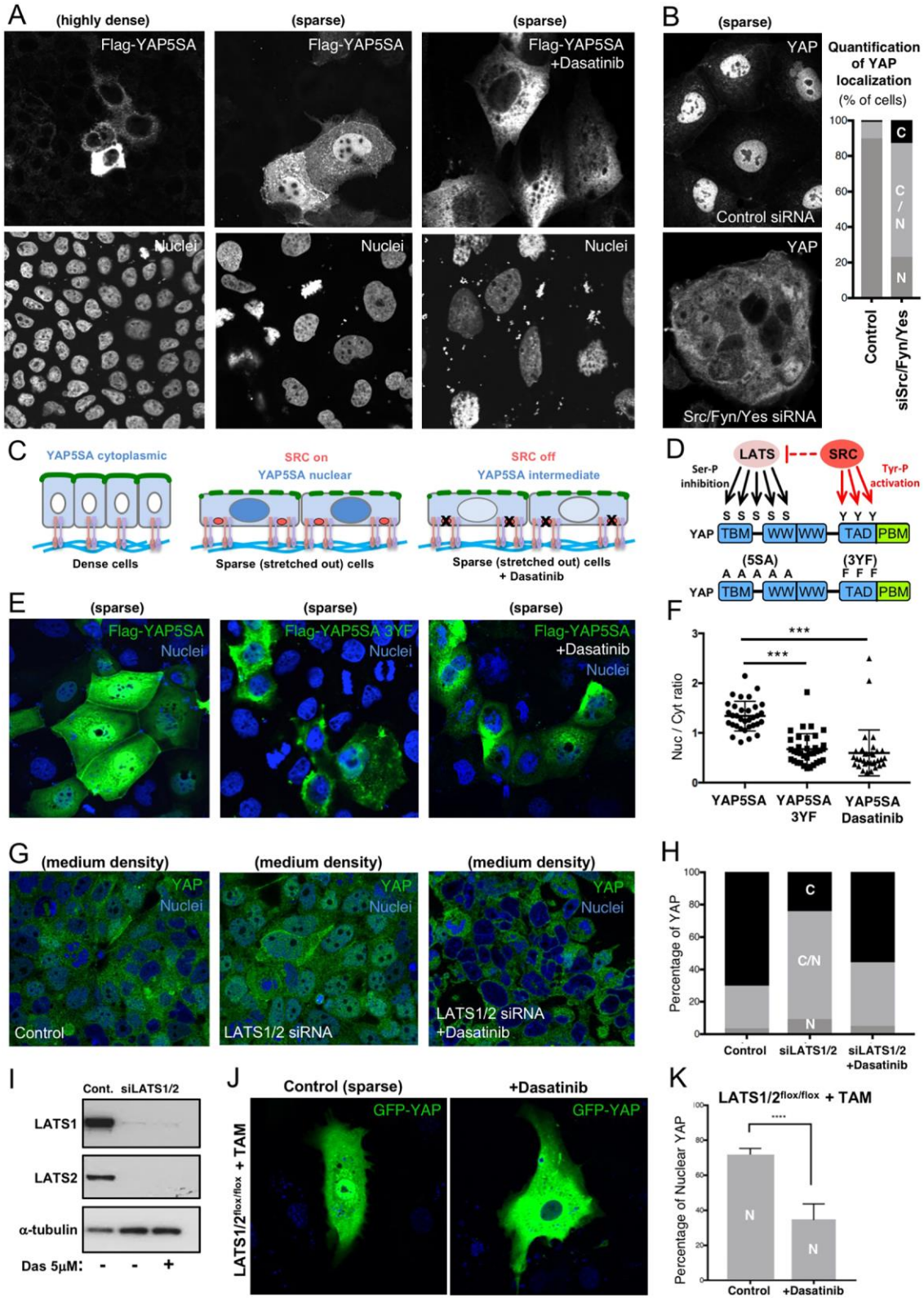


Figure 4. Mechanical control of YAP can occur independently of canonical Hippo-LATS phosphorylation on serine residues and involves Src family kinase phosphorylation on tyrosine residues.

(A) Mutation of 5 serine residues in YAP (5SA) to render it independent of LATS kinase phosphorylation fails to induce constitutive nuclear localization in human Caco2 cells. Flag-tagged

YAP5SA responds to changes in cell density, becoming cytoplasmic in highly dense cultures and nuclear in sparse cultures with spread-out cells. The nuclear localization of YAP5SA is dependent on Src family kinases, as it is reduced upon treatment with the Src inhibitor Dasatinib. n = 4 biological replicates.
(B) Silencing of Src/Fyn/Yes kinases by triple siRNA causes a reduction in YAP nuclear localization. Quantitation is shown on the right. n = 3
(C) Schematic diagram of YAP5SA subcellular localization (blue) in response to cell density and upon treatment with Dasatinib in (A). Basal Integrin attachments to the ECM are shown.
(D) Schematic diagram of YAP phosphorylation by LATS1/2 and Src family kinases and of Ser-to-Ala and Tyr-to-Phe mutations.
(E) Flag-tagged YAP5SA nuclear localization depends upon 3 tyrosine residues in its transcriptional activation domain (TAD), whose mutation to Phenylalanine (3YF) reduces nuclear localization in a manner similar to treatment with Dasatinib. n = 3 biological replicates.
(F) Quantitation of (E).
(G) YAP immunostaining (green) of human Caco2 epithelial cells at medium density transfected with either scrambled control siRNAs or LATS1/2 siRNAs in the presence or absence of the Src family kinase inhibitor Dasatinib. DAPI marks nuclei (blue). n = 3 biological replicates.
(H) Quantitation of (G).
(I) LATS1/2 siRNAs effectively reduce LATS1 and LATS2 protein levels, as in (G).
(J) LATS1/2 double floxed MEFs transfected with Cre-ERt and YAP before treatment with Tamoxifen and dasatinib to induce deletion of both LATS1 and LATS2 genes.
(K) Quantitation of YAP nuclear localization in (J). **** $p < 0.001$. n = 2 biological replicates.

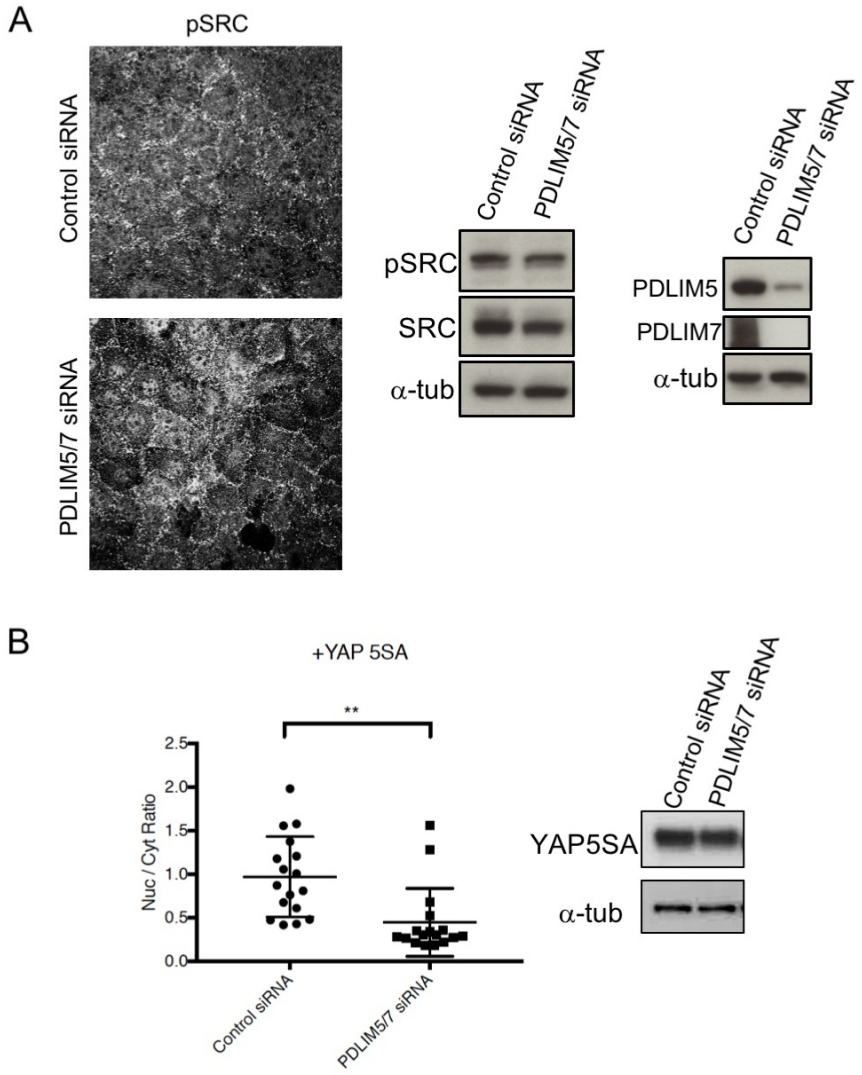


Figure S1. Enigma PDLIM5/7 family proteins are not required to regulate Src protein levels or Src auto-phosphorylation, but are required for Src to promote normal nuclear localization of YAP5SA.

(A) Silencing of both PDLIM5/7 expression in human Caco2 cells has no effect on levels of pSRC both by Immunofluorescence and Immunoblot. Note silencing efficiency is displayed on Immunoblot. (B) Silencing of PDLIM5/7 and expressing YAP5SA results in a significant decrease in the nuclear localisation of YAP as shown on graph. Protein levels of YAP5SA are unaffected on Immunoblot. ** $p < 0.01$ $n = 3$.

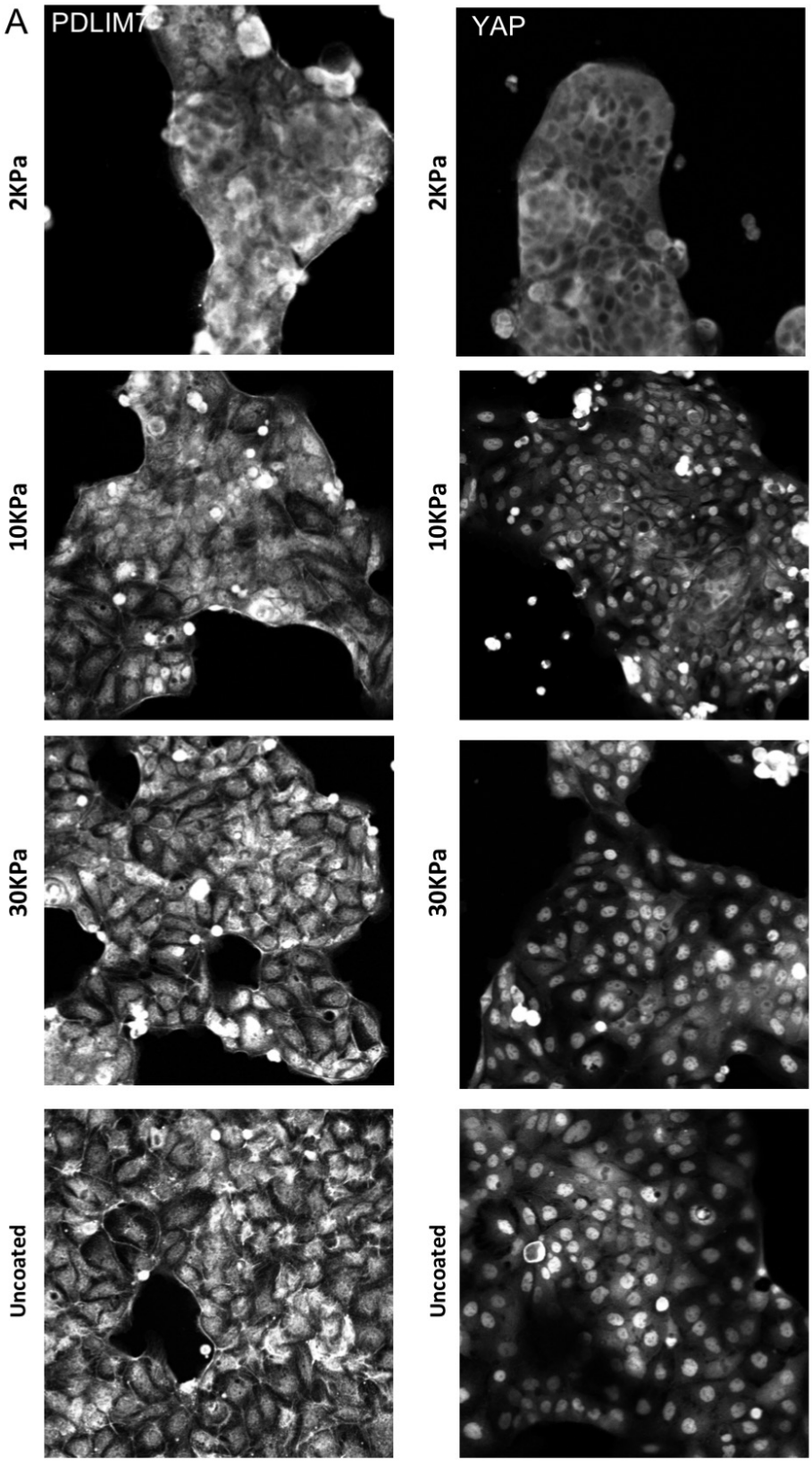


Figure S2: PDLIM7 and YAP subcellular localization are regulated by substrate stiffness.

(A) Localisation of YAP and PDLIM7 changes as hydrogel substrate stiffness increases.

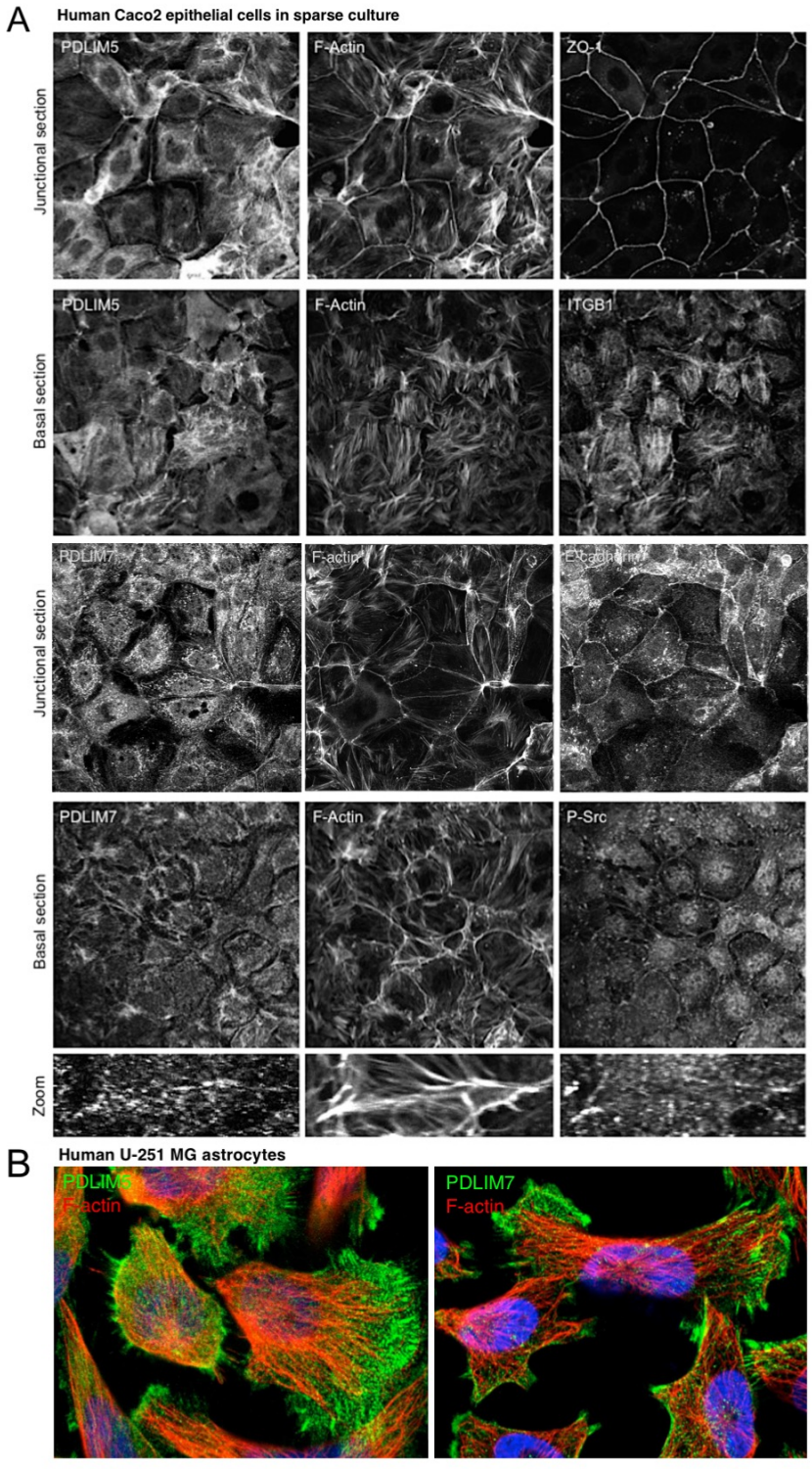
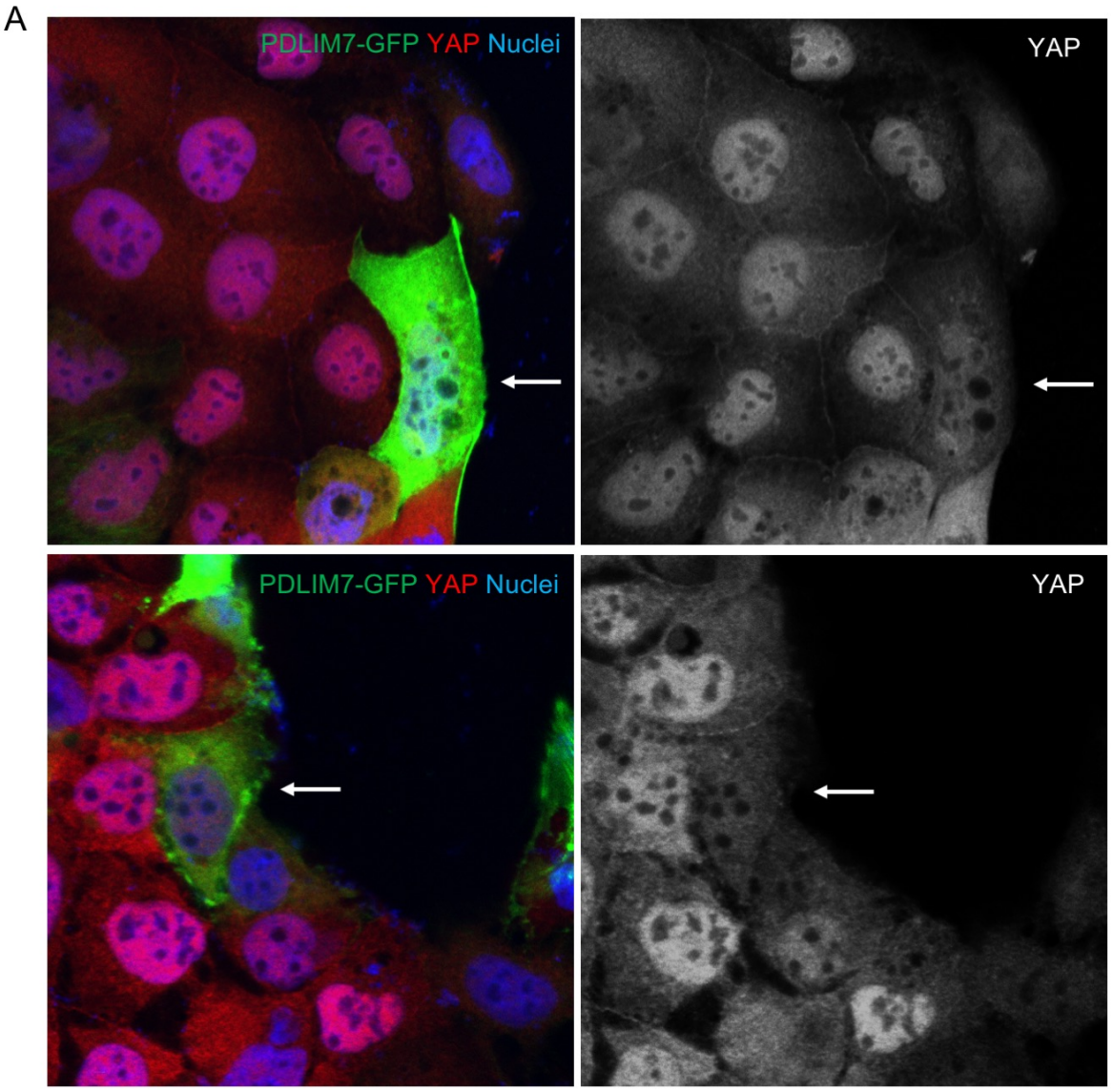


Figure S3. Localisation of Enigma PDLIM5/7 family proteins to F-actin at both Integrin adhesions and adherens junctions.

(A) Caco2 epithelial cells showing junctional and basal planes of PDLIM5 and PDLIM7 showing basal localisation of the proteins to F-actin, Integrin-beta1 and pSRC and junctional localisation to F-actin, ZO-1 and E-cadherin.

(B) U-251 astrocytes showing basal planes of PDLIM5 and PDLIM7 localizing to F-actin and focal adhesions. Adherens junctions do not form between astroctyes. Data were mined from www.proteinatlas.org.



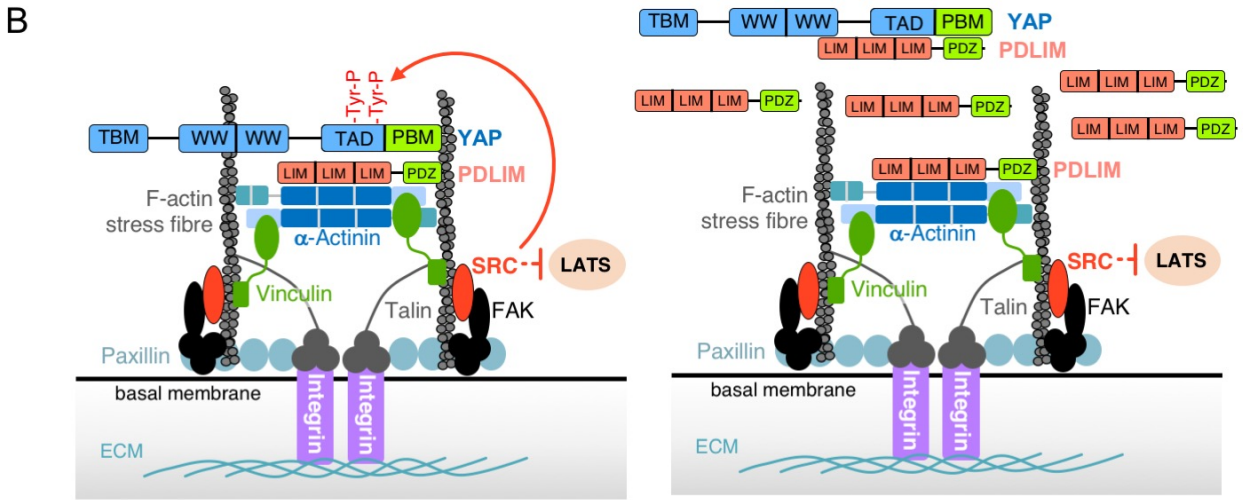


Figure S4. Overexpression of Enigman PDLIM7 causes a moderate reduction in nuclear YAP.

(A) Single flattened cells at the edge of colonies transfected with PDLIM7-GFP reveal a reduced level of YAP in the nucleus despite strong mechanical stimulation.

(B) Schematic diagram showing the possible effect of PDLIM7 overexpression in preventing YAP phosphorylation at Integrin adhesions, which is then predicted to reduce YAP nuclear localisation.