Depletion of *ALMS1* inhibits TGF- β /SMAD3/SNAI1 axis altering normal cell migration capacity and epithelial-mesenchyme transition.

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ABSTRACT:

Background:

ALMS1 is a ubiquitous gene associated with Alström syndrome (ALMS). The main symptoms of ALMS affect multiple organs and tissues, generating at last, multi-organic fibrosis in the lungs, kidneys and liver. TGF- β is one of the main pathways implicated in fibrosis, controlling the cell cycle, apoptosis, cell migration, and epithelial-mesenchymal transition (EMT). Nevertheless, the role of *ALMS1* gene in fibrosis generation and other implicated processes such as cell migration or epithelial-mesenchymal transition via the TGF- β pathway has not been elucidated yet.

Methods:

Initially, we evaluated how depletion of *ALMS1* affects different processes like apoptosis, cell cycle and mitochondrial activity. Then, we performed proteomic profiling with TGF- β stimuli in HeLa *ALMS1* $-/-$ cells and validated the results by examining different EMT biomarkers using qPCR. The expression of these EMT biomarkers was validated in hTERT-BJ-5ta. Finally, we also evaluated the SMAD3 phosphorylation in BJ-5ta model and its cell migration capacity.

Results:

Depletion of *ALMS1* generated apoptosis resistance to thapsigargin (THAP) and C2-Ceramide (C2-C), and G2/M cell cycle arrest in HeLa cells. For mitochondrial activity, results did not show significant differences between *ALMS1* $+/+$ and *ALMS1* $-/-$. Proteomic results showed inhibition of downstream pathways regulated by TGF- β . The protein-coding genes (PCG) were associated with processes like focal adhesion or cell-substrate adherens junction. EMT biomarkers *VIM*, *DSP*, and *SNAI1* showed an opposite pattern to what would be expected when activating the EMT. Finally, inhibition of *SNAI1* was consistent in BJ-5ta model, where a reduced activation of SMAD3 and a decrease in migratory capacity were also observed.

Conclusion:

ALMS1 has a role in controlling the cell cycle and the apoptosis processes. Moreover, the depletion of *ALMS1* inhibits the signal transduction through the TGF- β /SMAD3/SNAI1, which could be affecting the cell migration capacity and EMT.

Keywords: *ALMS1*, Alström syndrome, ciliopathies, primary cilia, TGF- β , epithelial-mesenchymal transition (EMT), fibrosis, cell cycle arrest, apoptosis.

INTRODUCTION:

ALMS1 is a ubiquitous gene with 23 exons located on chromosome 2p13. Mutations in this gene are related to Alström syndrome (ALMS; OMIM #203800), a rare, autosomal and recessive disease with a prevalence of 1-9 cases per 1.000.000 inhabitants and approximately 950 cases reported worldwide (Orphanet). The main symptoms of ALMS affect multiple organs and tissues [1], such as cone-rod retinal dystrophy, dilated cardiomyopathy (DCM) and hypertension [2], hypertriglyceridemia, type II diabetes mellitus (T2D) [3], and hepatic, renal and pulmonary dysfunction caused by multi-organic fibrosis [1], [4], which is the main cause of death in these patients.

The protein encoded by *ALMS1* plays a structural role in the eukaryotic cell centrosomes [5], a microtubule (MT)-nucleating organelle strongly related to primary cilia. The *ALMS1* basal body location [5], [6] has been related to different functions controlled by the primary cilium such as centrosome cohesion, cell cycle control and endosomal trafficking [6]–[9].

Although numerous roles of *ALMS1* have been described at both ciliary and extraciliary levels, the regulation of the pathways underlying these cellular processes remain unknown. Recently, our group has established the relationship between the *ALMS1* gene and the TGF- β signaling pathway [10], which could be related to the development of fibrosis in these patients [11], [12]. TGF- β is a signalling pathway regulated by clathrin-dependent endocytosis (CDE) in the pocket region of primary cilium [13], and coordinates cell cycle, apoptosis and migration processes such as epithelium-mesenchyme transition (EMT) [14]. This pathway acts in close coordination with other signalling cascades such as Wnt, Hippo, Notch, Hedgehog (Hh), mitogen-activated protein kinase (MAPK), and phosphoinositide 3-kinase (PI3K)-Akt, resulting in many cases in complex regulatory networks dependent on the primary cilium [15].

Stimulation of the TGF- β pathway can activate two main cascades: the canonical (SMAD-dependent) pathway and the non-canonical (SMAD-independent) pathway [16]. The canonical pathway is coordinated by transcription factors called R-SMAD (SMAD2 and SMAD3) which join co-SMAD (SMAD4) to activate the expression of several genes such as *SNAI1* [17], [18], a key modulator of EMT [19], [20]. This pathway is inhibited by an I-SMAD, SMAD7, which inhibits R-SMAD signalling in a variety of ways, binding to TGF- β receptors or directly in the nucleus, avoiding the binding of the R-Smad-Smad4 complex to DNA [21]. On the other hand, the non-canonical activation pathway includes a wide group of pathways such as p38 MAPK, PI3K/AKT, or Rho GTPases [16].

In this context, the role of *ALMS1* in the regulation of the TGF- β pathway remains unknown. Understanding how the absence of *ALMS1* controls cell migration processes such as EMT through the TGF- β pathway is key to understanding how the multi-organ fibrosis occurs in these patients.

In this article, we studied how the lack of *ALMS1* affects different cell processes such as mitochondrial activity, cell cycle, apoptosis, and signal transduction through the TGF- β pathway. Moreover, we evaluated the expression pattern for different EMT biomarkers after TGF- β pathway stimulation in two independent CRISPR-KO cell models (HeLa and BJ-5TA) for *ALMS1*. Finally, we measured p-SMAD3 activation and migration capacity in the BJ-5ta line in the presence of the TGF- β 1 ligand.

RESULTS:

ALMS1 depletion does not alter mitochondrial basal activity despite generating G2/M cell cycle arrest in HeLa cells:

ALMS1 is a structural centromere protein [5] that controls the structure of the cytoskeleton [22]. Since *ALMS1* depletion causes cell cycle elongation [8], [23] it is expected that mitochondrial activity will also be altered, as both processes are dependent on each other and regulated by cytoskeletal dynamics. [24].

We evaluated the different phases of the cell cycle using the fox synchronous algorithm [25] to determine the Gaussian distribution of the different populations. In the plots, an initial population was defined to isolate cells from debris. Next, DAPI fluorescence (461 nm) and events count (cells) were displayed in the plots, and the algorithm was applied to plots (Figure 1.A). After 24h of incubation, we determined that *ALMS1* $-/-$ genotype in HeLa cells significantly reduced (p-value < 0.05) the percentage of the cell population in S phase from $24.26\% \pm 1.32$ in *ALMS1* $+/+$ to $19.27\% \pm 1.69$ in *ALMS1* $-/-$. The cell cycle arrest was caused by a significant increase in the G2/M population (p-value < 0.01), from $1.6\% \pm 0.62$ in *ALMS1* $+/+$ to $4.88\% \pm 0.80$ (Figure 1.B). The results in the G0/G1 population did not show significant differences between genotypes. Despite this, the basal mitochondrial activity did not show a significant difference between *ALMS1* $+/+$ and *ALMS1* $-/-$ (Figure 1.C).

Figure 1. Cell cycle assay and mitochondrial activity results. **(A)** Cytometry plots with DAPI fluorescence and events count. Colours correspond with each population defined by fox synchronous algorithm. **(B)** Box-plot for each population previously defined in the cytometry plot that represents the percentage of cell cycle populations from parent population. **(C)** Linear regression between relative fluorescence units (RFU) and cell concentration in both genotypes (*ALMS1* +/+ and *ALMS1* -/-). This experiment was carried out in triplicate and repeated 3 times on different days (N=3, n=3). *: p-value < 0.05, **: p-value < 0.01.

The lack of *ALMS1* in HeLa prevents apoptosis induced by C2-ceramides and Thapsigargin:

Accumulation of cells in G2/M phase triggers apoptosis by different pathways such as caspases [26], [27]. We evaluated whether these cells were sensitive to apoptosis in response to an ER stress-inducing stimulus (Thapsigargin; THAP) [28], and a pro-apoptotic sphingolipid such as C2-ceramide.

As in the cell cycle assay, initial isolation of the cell population of interest was performed and named as P1. In the P1 population, plots were represented with the FL4 (7-AAD) fluorescence in the y-axis and FL1 (Annexin V) fluorescence in the x-axis, both on a logarithmic scale. Thresholds for different populations (viable, early apoptotic, late apoptotic, and necrotic) were defined by a previous compensation analysis using THAP-like positive control of cell death. Finally, experiments were performed by triplicates in the plate (n=3) and in 3 alternative days (N=3).

The results showed a significant difference between the genotypes *ALMS1* $+/+$ and *ALMS1* $-/-$ in viable (44.02% and 69.49%, respectively) and early apoptotic (43.47% and 23.44%) populations in cells stimulated with C2-C (Figure 2.A-B), and viable (49.56% and 71.33%), early apoptotic (18.25% and 8.56%) and late apoptotic (25.09% and 11.93%) populations in cells stimulated with THAP (Figure 2.C-D). These results indicate a resistance to apoptosis for these stimuli due to the lack of the *ALMS1*.

Figure 2. Apoptosis assay by annexin-V IP. **(A)** Cytometry plots for C2-ceramide (C2-C) with Log FL4 channel (IP) vs Log FL1 channel (Annexin-v). **(B)** Stacked-bar graph for cells treated with C2-C in a concentration of 25 μM. Percentages of each population are represented from a parent population previously defined. **(C)** Cytometry plots for thapsigargin (THAP) with Log FL4 channel (IP) vs Log FL1 channel (Annexin-v). **(D)** Stacked-bar graph for cells treated with THAP in a concentration of 100 nM. Percentages of each population are represented from a parent population previously defined. This experiment was carried out in triplicate and repeated 3 times on different days (N=3, n=3). *: adj. p-value < 0.05, **: adj. p-value < 0.01, ***: adj. p-value < 0.001, ****: adj. p-value < 0.0001.

The proteomic profile in HeLa cells reveals *ALMS1* like a regulator of Epithelium Mesenchymal Transition through the TGF-β pathway:

TGF-β is a pathway that plays a key role in the control of cell proliferation and apoptotic processes [14]. Since signal transduction through this pathway occurs via the primary cilium [13], [29], the lack of *ALMS1* could explain the aberrant processes seen above.

We evaluated how *ALMS1* depletion affects the signal transduction through TGF-β pathway. In this bulk analysis, we obtained a total of 86 differentially expressed proteins (DEP) (Figure 3.A), 31 (36%) over-expressed and 55 (64%) under-expressed. From this dataset, a total of 76 proteins were associated with genes (Figure 3.C).

Figure 3. Differential expression analysis for proteomic data in HeLa. **(A)** Heatmap for all proteins (associated and not associated with genes) differentially expressed. Plot shows the expression pattern for the 3 biological replicates of genotype *ALMS1* +/+ (CT) and genotype *ALMS1* -/- (KO). **(B)** Principal components analysis (PCA) for the top 500 variable proteins with all biological replicates for each genotype. **(C)** Volcano-plot showing the top 40 most significant genes associated with proteins. Proteins with no clear gene association were removed from this plot. Significant thresholds: log2 FC > 1.5, adj. p-value < 0.05. This experiment was carried out in triplicate (n=3).

Based on the DE of protein-coding genes (PCG) we performed an enrichment analysis obtaining a total of 9 GO terms significantly overrepresented (adj. p-value < 0.05) (Figure 4. A-B). The majority of these PCG were associated with focal adhesion, cell-cell or cell-matrix interactions. This result suggests the implication of *ALMS1* in the control of adhesion processes through the TGF- β pathway. In addition, we calculated the z-score of these GO terms (Figure 4.A), which showed a significant decrease in the ability of these cells to properly migrate or adhere to each other or their matrix.

Finally, genes such as vimentin (*VIM*) and desmoplakin (*DSP*), common biomarkers of EMT, were associated with these GO terms (Figure 4.B). Interestingly, these genes exhibited an expression pattern opposite to that observed for the EMT process.

Figure 4. Enrichment analysis based on Go terms for proteomic data. **(A)** GoCircle-plot that includes significant over-represented GoTerms in a table (ID and Name) and scatter plot with FC of any gene related with each GoTerm. Moreover, this plot includes Z-score which is an estimation of up-regulation or down-regulation in the Goterms. **(B)** Circos-Plot applied to Goterms to relate any Gene name with the corresponding GoTerm name. The plot also shows the FC of each gene and the list is sorted by FC from the most over-expressed (3) up to the most under-expressed (-3).

ALMS1 depletion alters the expression pattern of TGF- β 1-induced EMT markers in HeLa:

VIM is a key player in the regulation of EMT and its deficiency leads to inhibition of this process [30]. On the other hand, *DSP* is a protein involved in cell-cell junctions mediated by desmosomes. It is therefore considered a typical epithelial marker whose decrease is related to the passage of the cell from an epithelial to a mesenchymal state [31].

The overexpression of *DSP*, together with the underexpression of *VIM* upon stimulation of the TGF- β pathway, seems to be two clear signals that EMT might be inhibited when *ALMS1* is removed. We evaluated the expression of the principal EMT biomarkers (*SNAI1*, *CDH2*, *TWIST1*, *ACTA2*, *VIM*, and *DSP*) and other genes related to EMT (*POSTN*, *EDIL3*). We perform stimulations at different times (24, 48 and 72 hours) with rh-TGF- β 1 (2ng/ μ L), obtaining the same expression pattern in the 3 experiments performed (Figure 5.A-C).

Figure 5. Expression validation of different EMT biomarkers with TGF-β stimulation at (A) 24 hours, (B) 48 hours, and (C) 72 hours. (D) Correlation plot between the expression of three EMT markers in the proteomic analysis and the qPCR assay. The expression is represented based on the log2 FC and the R coefficient using Pearson's method. These experiments were performed by triplicate (n=3). *: adj. p-value < 0.05, **: adj. p-value < 0.01.

At 24 hours of stimulation, *POSTN*, *SNAI1*, *VIM*, *ACTA2* and *EDIL3* were found under-expressed, while *TWIST1*, *CDH2* and *DSP* were over-expressed (Figure 5.A). This data confirms the expression pattern previously detected in the proteome analysis related to the genes *VIM*, *DSP* and *EDIL3* (Figure 5.D).

At 48 hours of stimulation, the over-expressed genes *CDH2*, *TWIST1* and *DSP*, in addition to *EDIL3* and *VIM* inhibition, showed significant differences (Figure 5.B). The levels of the rest of the primary under-expressed genes were not significant.

Finally, at 72 hours of stimulation the expression pattern was similar to the previous stimulation times. However, the data dispersion was too high and no significant differences were detected.

The absence of *ALMS1* inhibits TGF-β/SMAD3/*SNAI1* axis in hTERT BJ-5ta cell line and decreases migration capacity:

In order to validate the results obtained in the HeLa model, the TGF-β pathway was stimulated at 24 and 48 hours with the rh-TGF-β1 (2ng/μL) ligand on the CRISPR model in a hTERT BJ-5ta fibroblast line (Figure 6.A-B). In this cell line, inhibition of *VIM* and overexpression of *DSP* did not appear to occur. However, inhibition of *POSTN* and the transcription factor (TF) *SNAI1* seem to

be constant in both cell lines. *ACTA2* also showed decreased levels in BJ-5ta and HeLa but in the latter, the difference was not too noticeable. Finally, we evaluated the SMAD3 phosphorylation levels at 4 different times after TGF- β 1 stimuli (0, 10, 30 and 90 min). We concluded that the lack of *ALMS1* potentially inhibits the axis TGF- β /SMAD3/SNAI1 because phosphorylated SMAD3 levels appear to be reduced in BJ *ALMS1*^{-/-} cells (Figure 6 C,E). This could be affecting the normal cell migration capacity in these cells (Figure 6 D,F).

Figure 6. Expression levels of different EMT biomarkers, pSMAD3/SMAD3 and cell migration capacity in *ALMS1* ^{-/-} BJ-5ta cell line. TGF- β stimulation was performed during 24 hours **(A)** and 48 hours **(B)** by triplicate (n=3). *: adj. p-value < 0.05, **: adj. p-value < 0.01. **(C)** Representative western-blot of p-SMAD3 and SMAD3 levels after 0, 10, 20 and 90 min of stimulation with TGF- β 1 (2 ng/ μ L) ligand and their corresponding loading controls (coomassie). **(D)** Representative images of cell migration of BJ-5TA cells at 0, 24 and 48 hours in the presence of 2 ng/ μ L TGF- β 1. **(E)** Quantification of band intensity in WB for p-SMAD3 and SMAD3 at time 0 and after 30 min stimulation with TGF- β 1. (n=3). *: adj. p-value < 0.05, **: adj. p-value < 0.01. **(F)** Quantification of the cell area of the image and how it decreased after 24 and 48 hours of incubation (n=3). *: p-value < 0.05, **: p-value < 0.01.

DISCUSSION:

In this study, we reported new insights to understand the role of *ALMS1* in cell function. *ALMS1* has a clear relation with cell cycle, apoptosis regulation, and cell migration and adhesion processes such as EMT.

Cell cycle arrest in the G2/M phase (Figure 1. A-B), also described in *ALMS1*-knockdown cardiomyocytes [23], seems to be a cell process triggered by *ALMS1* depletion. Several studies have already linked cell cycle arrest in the G2/M phase to the TGF- β pathway and the development of fibrosis [32]–[34]. This fact supports the involvement of the TGF- β pathway in ALMS.

Normally, cell cycle arrest in both the G2/M and G0/G1 phases is often accompanied by the activation of apoptotic processes [24], [35]–[37]. The depletion of *ALMS1* seems to generate apoptosis resistance against THAP and C2-C in HeLa cells (Figure 2), which is in accordance with that reported by Zulato et al., [8]. THAP is a non-competitive inhibitor for Ca²⁺-ATPases located in the endoplasmic reticulum (SERCA). This, inhibits Ca²⁺ reflux to the lumen of the endoplasmic reticulum, generating endoplasmic reticulum stress (ER stress) [38]. At the same time, ER stress activates a signalling network called Unfolded Protein Response (UPR) which alleviates this stress and promotes cell survival, restoring ER homeostasis [39]. At this point, *ALMS1* depletion could be acting at two levels: inhibiting THAP action (ER stress) or over-activating UPR and generating apoptosis resistance. Because of the role of *ALMS1* in the centrosome and its relation with endocytic trafficking [22], its depletion could probably alter the structure of the membrane, inhibiting the signalling transduction of ligands like THAP. THAP also induces apoptosis impairing cytoskeleton dynamics and disorganization of F-actin via mTORC1 inhibition in the mTORC1-RhoA-Limk-Cofilin-1 axis in A549 cells [40]. *ALMS1* depletion already causes disorganization of the F-actin cytoskeleton [22] but does not lead to cell death. Probably, the mTOR pathway through mTORC1 was inhibited in *ALMS1* ^{-/-} cells, but in this case, other mechanisms could prevent cells from reaching apoptotic stages. This would be supported by the inhibition of

LAMTOR3 (Figure 3.B), a protein involved in the formation of the Ragulator complex that activates mTORC1 [41].

C2-C is a sphingolipid that induces caspases-dependent apoptosis, inhibiting PI3K/AKT pathway and increasing the depolarisation of the mitochondrial membrane [42]–[44]. The over-activation of the PI3K/AKT pathway could explain the apoptotic resistance in the case of the C2-C [45], [46]. This fact would agree with the previous proposed mTORC1 inhibition, since phosphorylation of AKT/PKB at s473 leads to inhibition of mTORC1 mediated by FoxO transcription factors [47], which could be related to F-actin filament disorganisation previously described in Alström fibroblasts [22].

In addition to the role of *ALMS1* in G2/M phase cell cycle arrest and resistance to THAP- and C2-C-mediated apoptosis, this study suggests that *ALMS1* depletion leads to inhibition of the canonical (SMAD dependent) signalling pathway via the TGF- β . This could be affecting downstream processes such as cell migration and EMT.

Our results show how the lack of *ALMS1* alters the expression of several genes related to focal adhesion and cell-substrate adherens junction (Figure 4), two processes required for TGF- β 1-induced EMT [48], [49]. Moreover, we have found interesting candidates that could explain or be related to the mentioned cellular disturbances (Figure 3). *S100A6*, under-expressed, is a positive regulator of proliferation, migration and angiogenesis [50]–[52], and also a regulator of endothelial calcium signaling [53]. *PKM*, which was over-expressed in HeLa cells, is associated with apoptosis resistance [54]. *ITGA11*, another gene under-expressed in this cell line and related to fibrosis generation, is expressed due to activation of the TGF- β pathway [55]. Finally, two known EMT markers, *VIM* and *DSP*, showed a reverse pattern to that expected when this process is activated. This could be a first evidence suggesting inhibition of the TGF- β 1-induced EMT when *ALMS1* is missing.

One of the most relevant detected genes was *SNAI1*, which is inhibited in both cell lines (Hela and BJ-5ta). *SNAI1* and *TWIST1* are transcription factors that repress E-cadherin (*CDH1*) expression and activate the expression of N-cadherins (*CDH2*) [56], [57]. E/N-cadherins switch is involved in the induction of EMT [58], [59] through the TGF- β pathway [60]. Inhibition of *SNAI1* could be affecting the normal EMT in these cells despite the over-expression of *TWIST1*. The decreased expression of *SNAI1*, together with the inhibition of *ACTA2* and *POSTN*, also suggests that TGF- β 1-induced EMT would be down-regulated. *SNAI1* is up-regulated by SMAD3, which is phosphorylated when the TGF- β pathway is activated and forms a complex with SMAD2 [17]. The phosphorylated SMAD2-SMAD3 complex translocates to the nucleus, activating the

expression of several genes related to cell migration and EMT such as *SNAI1* [61]. Because we detected consistent inhibition in both cell models, we sought to determine whether SMAD3 activation was also compromised in the BJ-5ta model. We conclude that *ALMS1* depletion inhibits SMAD3 phosphorylation (Figure 6 C, E), which could lead to downstream inhibition of *SNAI1* and affect migration capacity in this cell line (Figure 6 D,F), which could also affect the EMT.

We have previously described that *ALMS1* depletion by siRNA inhibits SMAD2 phosphorylation in the hTERT RPE-1 cell line [10]. This mechanism is probably also active in the HeLa and BJ-5ta cells and together with the inhibition of SMAD3 could explain the inhibition of *SNAI1* (Figure 5,6 A-B) through the TGF- β /SMAD3/SNAI1 axis [20]. This fact could be the main cause of the TGF- β pathway inhibition on the canonical part of the route, responsible for the expression pattern of the above-mentioned genes.

CONCLUSION:

ALMS1 appears to have a fairly conserved role in the regulation of cell cycle and apoptotic processes but does not affect mitochondrial reduction capacity. *ALMS1* depletion affects signal transduction through the canonical TGF- β pathway by inhibiting it. Inhibition of the TGF- β pathway would be affecting other downstream processes such as cell polarity control, EMT and migration capacity through the axis TGF- β /SMAD3/SNAI1.

MATERIALS AND METHODS:

Cell culture:

HeLa human cervical cancer cell line from American Type Culture Collection (ATCC), hTERT BJ-5ta human dermal fibroblasts (ATCC) and 293T human embryonic kidney cell line from ATCC were used. HeLa and 293T cells were maintained in Dulbecco's minimum essential medium (DMEM, Gibco, Invitrogen, NY, USA), supplemented with 10% fetal bovine serum (FBS) (Gibco, Invitrogen, NY, USA) and 2% penicillin/streptomycin (P/S) (Gibco, Invitrogen, NY, USA). In the case of BJ-5ta line, cells were maintained in DMEM:199, supplemented with 10% of FBS, 2% P/S and Hygromycin B (0.01mg/mL). All cell lines were grown at 37°C with 5% CO₂ for up to one month. After this, the cells were discarded and a new culture was started.

Crispr assay and edition validation:

HeLa cells were edited with a dual episomal system for homology directed repair (HDR) (Santa Cruz Biotechnology, Inc., Dallas, USA). Cells were transfected with Lipofectamine 2000 (Thermo Fisher, Waltham, EE. UU) using 3 gRNAs directed to *ALMS1* exons 1 and 3 (Supplementary Table S1). After plasmid transfection, cells were selected with puromycin (1 µg/mL) for 5 days.

Supplementary Table S1. gRNAs of the constructs used for gene editing. The forward and reverse sequences are shown in the table. The red colour represents the base pairs added for ligation in the plasmid.

ID	Cell line	Exon	Forward primer (gRNA sequence 5' - 3')	Reverse primer (5' - 3')	Strand	PAM	On-target Score	Off-Target Score
gRNA 1 HeLa	HeLa	1	GCCCCGCAGCACCGCTACT	AGTAGCGGTGCTGCGGGGGC	+	CGG	51,4	89,3
gRNA 2 HeLa	HeLa	1	TCCGCTCGCCCTCCGAGTAG	CTACTCGGAGGGCGAGCGGA	-	CGG	56	91
gRNA 3 HeLa	HeLa	3	TTCTCACATTAAACCTAGTC	GACTAGGTTTAATGTGAGAA	-	TGG	29,1	76,7
gRNA 1 BJ	hTERT-BJ-5ta	3	CACCGCCTAGTCTGGGATGTATCCA	aaacTGGATACATCCAGACTAGGCC	-	AGG	56,2	77,1
gRNA 2 BJ	hTERT-BJ-5ta	3	CACCGAACAGAAGAGATCTCTGTT	aaacAACAGAGATCTCTTCTGTTC	-	GGG	63,3	58,1

BJ-5ta cells were edited using a lentivirus system according to the protocol described by Sanjana et al [62], with some modifications. Check supplemental material for more information. After lentiviral transduction, cells were selected with puromycin (1µg/mL) for 5 days.

Surviving cells were expanded and isolated to obtain individual clones. Of the isolation was performed using Fluorescence-Activated Cell Sorting (FACS) with Hoechst (BD Bioscience, San José, USA) in the flow cytometer FACS ARIA III (BD Bioscience, San José, USA). Inhibition of gene expression was validated by qPCR in a StepOnePlus instrument (Thermo Fisher, Waltham, USA).

Mitochondrial activity assay:

Mitochondrial activity was evaluated using Presto Blue reagent (Thermo Fisher, Waltham, USA). Wild type (*ALMS1* +/+) and Hela Knockout cells (*ALMS1* -/-) were seeded in 96-well plates by triplicate in concentrations from 5×10^4 to 1.5×10^3 cells per well. The plates were incubated for 24 hours as previously described. After incubation, 10 μ L of Presto Blue were added to each well and cells were incubated for 2 hours. Next, the plate was centrifuged at 1500 rpm for 5 min and the supernatant was transferred to a fluorescence plate (Corning, NY, EE. UU). Finally, the plates were measured in the Envision plate reader (Perkin-Elmer, Waltham, USA). This experiment was repeated 3 times on different days.

Apoptosis assay:

The apoptosis assay was performed in flat-bottom 96-well plates. HeLa *ALMS1* +/+ and *ALMS1* -/- cells were seeded in triplicate at a concentration of 3×10^4 cells/per well and incubated 24 hours before treatment. Next, apoptotic stimuli were added at the established concentrations, followed by incubation for 24-48 hours depending on the stimuli. After that, cells were washed twice with 100 μ L PBS. 50 μ L trypsin was added per well and the plate was incubated for 5 min at 37°C. After that, trypsin was inactivated with 50 μ L DMEM 10% FBS 2% P/S, and cells were transferred to 96-well plates with U bottom. The plate was centrifuged at 1500 rpm for 5 min, the supernatant was discarded and the cells were resuspended in 200 μ L binding buffer with 2 μ L 7-AAD and 2 μ L annexin V-FITC (Biolegend Inc, San Diego, CA, USA). The plate was incubated for 15 min in darkness and measured in the flow cytometer FC 500 (Beckman-Coulter, Brea, CA, USA). This experiment was repeated 3 times on different days.

Cell cycle assay:

To determine cell cycle alterations, we studied sub-populations in the HeLa *ALMS1* -/- model with Hoechst staining (BD Bioscience, San José, USA). *ALMS1* +/+ and *ALMS1* -/- cells were seeded in a 6-well plate at a concentration of 3×10^5 cells/per well and incubated overnight. The next day, Hoechst staining was added to the medium and the plate was incubated for 1 hour. Then, the medium was removed, cells were washed twice with 1 mL PBS per well, and trypsinised

following the protocol described above. Cells were resuspended in PBS and analysed in the flow cytometer FACS ARIA III.

Protein extraction:

Cells were seeded in 6-well plates by triplicate in DMEM 2% P/S and incubated overnight for serum starvation. One the next day, cells were stimulated with rh-TGF- β 1 (2ng/mL; R&D Systems) during 24 hours. After that, DMEM was removed and wells were washed twice with PBS. Cells were scrapped in PBS. Then, cells were pelleted at 11000 rpm for 5 min, and 100 μ L of RIPA Buffer were added to each sample. Samples were incubated on ice for 20 min in constant agitation. Finally, cell debris was pelleted by centrifugation for 30 min at 12000 rpm and 4 °C. Samples were aliquoted and stored at -80 °C until the next protocol's step. Protein quantification was performed by Bradford microplate assay (Bio-Rad, Hercules, USA).

Protein concentration, reduction and alkylation:

Initially, 20 μ g of each sample was concentrated and stained with Coomassie in a 12% SDS-PAGE gel. Bands were cut and faded. Band distinction was performed by adding 100 μ L of ammonium bicarbonate 25mM/50% acetonitrile and incubated 20 min. This step was repeated 4 times. After distinction, the supernatant was removed and 100 μ L of ammonium bicarbonate 25mM-50% EtOH was added to samples, and incubated for 20 min. Then, the supernatant was removed and 100 μ L EtOH was added and incubated for 15 min. In the next step, 200 μ L 10mM DTT in ammonium bicarbonate were added and samples were incubated for 1 hour at 56°C. Then, DTT solution was removed and 200 μ L iodoacetamide (IAA) 55mM in 50 mM ammonium bicarbonate was added. Samples were incubated again for 30 min at room temperature. Finally, the supernatant was removed, 200 μ L ammonium bicarbonate 50mM were added and samples were incubated for 15 min.

Protein digestion:

After reduction and alkylation, samples were digested. 100 μ L ammonium bicarbonate 25mM-50% acetonitrile (ACN) was added to each sample and incubated for 30 min. The supernatant was removed and 100 μ L ACN 100% was added and incubated for 10 min. The supernatant was removed again and 15 μ L Trypsin in ammonium bicarbonate 25mM were added to each sample. Samples were maintained at 4°C for 30 min. Finally, trypsin was removed, 5 μ L ammonium bicarbonate 25 mM were added and samples were incubated overnight.

Peptide extraction:

40 µL of trifluoroacetic acid (TFA) 0.5% were added to each sample and incubated for 30 min at room temperature. Then, we added 20 µL ACN 100% in each 1.5 mL tube and incubated for 15 min at 37°C. Finally, the samples were dried in a speed-vac at 45°C for 1 hour and stored at -20°C until the analysis.

Mass Spectrometry and bioinformatic analysis:

Samples were resuspended in 24 µL formic acid 0.1%. Then, 10 µL were injected into the HPLC (LC-ESI-MS/MS workflow using a C18 precolumn). Samples were analysed in a mass spectrophotometry Orbitrap ELITE (Thermo-Fisher, Waltham, USA) in long chromatography mode (4 hours). After that, protein identification was performed in the database UniProtKB/TrEMBL for humans, using the Proteome Discoverer software. Downstream analysis was performed with the R package Differential Enrichment analysis of Proteomics data (DEP package) [63]. Finally, Go Terms analysis was performed with Clusterprofiler package [64], and results were visualized with GOplot package [65]. All the code for the analysis was deposited in the GitHub repository (https://github.com/BreisOne/CRISPR_Hela_KO_Alms1).

The mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium via the PRIDE [66] partner repository with the dataset identifier PXD024964.

RNA extraction and RT-PCR:

Cells were seeded in 6-well plates at a concentration of 2×10^5 by triplicate in DMEM 2%P/S and were incubated overnight for serum starvation. The next day, cells were stimulated with rh-TGF-β1 (2 ng/mL) for 24, 48 and 72 hours. DMEM was removed and wells were washed twice with PBS. Cells were scraped in PBS and collected in a 1.5 mL tube. For the RNA extraction, the NYZ total RNA isolation kit (NYZtech, Lisboa, Portugal) was used following the manufacture's protocol.

After RNA elution, the sample concentrations were measured with Nanodrop (Thermo Fisher, Waltham, USA). Next, 300 ng of RNA were retrotranscribed to cDNA using the NYZ First-Strand cDNA Synthesis kit (NYZtech, Lisboa, Portugal) following the manufacture's protocol.

qPCR expression analysis:

qPCRs was performed in a StepOnePlus instrument using SyBr green (Thermo Fisher, Waltham, EE. UU). Primers were synthesized on demand (IDT) (Supplementary Table S2). Samples were analysed by technical triplicates for each sample and gene. Cts for technical triplicates were

averaged using the geometric mean and $\Delta\Delta CT$ method was applied for the normalization, using 2 housekeeping (HK) genes (*ALAS1* and *YWHAZ*).

Supplementary Table S2. Sequences of the primers used for the evaluation of qPCR expression of epithelial-mesenchymal transition markers and genotyping of the HeLa Knockout line.

Gene symbol	Forward primer	Reverse primer
<i>POSTN</i>	CACTCTTTGCTCCCACTAAT	GCCACTTTGTCTCCCATGAT
<i>CDH2</i>	CCTCCAGAGTTTACTGCCATGAC	GTAGGATCTCCGCCACTGATTC
<i>ACTA2</i>	CTATGCCTCTGGACGCACAAC	CAGATCCAGACGCATGATGGCA
<i>SNAI1</i>	GCTGCAGGACTCTAATCCAGAGTT	GACAGAGTCCCAGATGAGCATTG
<i>TWIST1</i>	GCCAGGTACATCGACTTCCTCT	TCCATCCTCCAGACCGAGAAGG
<i>EDIL3</i>	GCGAATGGAACCTCTTGGCTGTG	GAGCGTTCTGAAGATGCTGGAG
<i>VIM</i>	AGGCAAAGCAGGAGTCCACTGA	ATCTGGCGTTCCAGGGACTCAT
<i>DSP</i>	TGACAGACCGCTGGCAAAGGAT	GGCGTTTAGCATCATAGAGCCAC
<i>ALMS1</i> (Exon1)	GCACTGCGCCTAAGCTG	gttgggggtggaggctg
<i>ALMS1</i> (Exon3)	cagttaatgacttagcatgttttct	ctttccctttttgagttaagga
<i>ALMS1</i> (Exon 1 -3)	GAGCGAGACCAACATGGA	ATTGCGTCAATATGCCCTCCT

Wound healing assay:

BJ-5ta *ALMS1* $+/+$ and *ALMS1* $-/-$ were seeded by triplicate in a 6-well plate at a concentration of 1×10^6 and cultured until a 90-95% of confluence. Cells were serum-starved for 24 hours. After that, TGF- $\beta 1$ (2 ng/mL) was added to all wells and a wound performed with a 200 μ L pipette tip. Cells were incubated at 37°C 5% CO₂ and images were taken at 0, 24 and 48 hours with the microscope inverted Nikon TiU. Finally, images were analysed with the imageJ program [67] and the plugin developed by Suarez-Armendo et al [68].

Western-Blot:

Samples were collected from 6-well plates and lysed in RIPA buffer, containing phosphatase inhibitors (dil 1:200) and protease inhibitors (1:1000), for 30min at 4°C. Then samples were centrifugated during 20min at 12000 rpms and 4°C. The supernatant was collected to 1.5mL low binding tubes and pellet was discarded. Quantification was performed using Bradford assay in microplates. Samples were stored at -80°C until analysis.

A total amount of 20 μ g from each sample were mixed with 6,25 μ L of Laemmli Buffer 4X (Biorad, Hércules, EE.UU), 1,25 μ L β -mercaptoethanol (BME) and a volume of H₂O_d up to a final volume of 25 μ L. Then, the mixes were boiled at 97°C for 5 min and loaded in a Mini-PROTEAN® TGX™ Precast Gel (Biorad, Hércules, EE.UU) at 12% acrylamide/bis-acrylamide. The gel was migrated for 1 hour at 150V. Then, gels were transferred to a 0.2 μ m PVDF membrane using the Trans-

Blot Turbo transfer system (Biorad, Hércules, EE.UU) following the mixed molecular weight (MW) protocol. After transfer, membranes were blocked with in-house tris-buffered saline, 0.1% Tween20 and 5% milk (TBS-T 5%) for 90 min. Incubation with the primary antibody was kept at 4°C overnight. The primary antibodies used in this assay were: anti-SMAD3 (1:1000, Abcam,40854) and anti-p-SMAD3 (1:1000, Abcam, 63403).

Next day, incubation with secondary antibody was performed by 45min in TBST 5%, after washed the membrane 3 times in TBST 5%. The secondary antibody used in this assay was goat anti-rabbit IgG H&L (HRP) (Abcam, 205718) at 1:5000 for p-SMAD3 and 1:15000 for SMAD3. Finally, for protein visualization Clarity western ECL substrate (Biorad, Hércules, EE.UU) was used and membranes were exposed in the ChemiDoc system (Biorad, Hércules, EE.UU). Quantification of protein bands was carried on Image Lab software (Biorad, Hércules, EE.UU) using the total protein standardisation method.

Statistical analysis:

On one hand, mitochondrial activity, apoptosis and cell cycle data visualization and statistical analysis were performed with the software GraphPad Prism 7.00.

Mitochondrial activity was analysed using a linear regression model. Comparison of fluorescence increase with cell concentration did not show significant differences between both slopes ($\alpha = 0.05$). The apoptosis experiment results were analysed with 2ways-Anova to compare each different population between genotypes. A Sidak's multiple comparisons test was applied to correct the analysis. Finally, cell cycle results were analysed with a two-tailed unpaired t-test for each population (G2/M, S and G0/G1).

On the other hand, proteomic data analysis included different statistical analyses and data transformations along the pipeline. Initially, the data were normalised by variance stabilizing transformation (VSN). The data imputation for missing values was performed like missing not at random (MNAR) using the Minprob method with FDR < 0.01. Finally, differential expression analysis was performed using protein-wise linear models combined with empirical Bayes statistics implemented in the limma package, FDR < 0.05.

A third analysis was performed for the qPCR data. After normalization, fold changes (FC) were calculated concerning the geometric mean of the expression of each gene in the biological triplicates for *ALMS1* +/+. Significant differences were calculates using a multiple t-test with a significant threshold of FDR < 0.05.

Finally western-blot data were normalized using total protein method. After normalization FCs were calculated using the geometric mean of the expression/activation of the protein in each clone. Significant differences were calculated using a multiple t-test with a significant threshold of FDR < 0.05. For wound healing assay, an unpaired t-test was applied and a significant threshold of p-value <0.05 was set.

Author Contributions: BB-M and DV designed the study. BB-M performed the experiments and analysed the data. EN-G assisted in performing apoptosis, cell cycle and mitochondrial activity assays. AI-R assisted in the quantification of the expression of EMT markers in the BJ-5ta model and in the cell migration assay. BB-M and DV wrote the manuscript. All authors have read the draft and provided approval for publication.

Data Availability Statement: Proteomic data are available via ProteomeXchange with identifier PXD024964.

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BIBLIOGRAPHY:

- [1] J. D. Marshall, P. Maffei, G. B. Collin, and J. K. Naggert, "Alström syndrome: genetics and clinical overview," *Curr. Genomics*, vol. 12, no. 3, pp. 225–235, May 2011, doi: 10.2174/138920211795677912.
- [2] A. B. Jaykumar, P. Caceres, E. L. Henson, W. H. Beierwaltes, and P. A. Ortiz, "Deletion of ALMS1 (Alstrom Syndrome 1) enhances salt-sensitive hypertension, and induces insulin resistance and obesity in rats," *FASEB J.*, vol. 31, no. 1_supplement, pp. 857.26-857.26, Apr. 2017, doi: 10.1096/fasebj.31.1_supplement.857.26.
- [3] G. B. Collin *et al.*, "Mutations in ALMS1 cause obesity, type 2 diabetes and neurosensory degeneration in Alström syndrome," *Nat. Genet.*, vol. 31, p. 74, Apr. 2002, [Online]. Available: <https://doi.org/10.1038/ng867>.
- [4] J. D. Marshall *et al.*, "Spectrum of ALMS1 variants and evaluation of genotype-phenotype correlations in Alström syndrome," *Hum. Mutat.*, vol. 28, no. 11, pp. 1114–1123, Nov. 2007, doi: 10.1002/humu.20577.
- [5] T. Hearn *et al.*, "Subcellular Localization of ALMS1 Supports Involvement of Centrosome and Basal Body Dysfunction in the Pathogenesis of Obesity, Insulin Resistance, and Type 2 Diabetes," *Diabetes*, vol. 54, no. 5, pp. 1581 LP – 1587, May 2005, doi: 10.2337/diabetes.54.5.1581.
- [6] D. Jagger *et al.*, "Alström Syndrome protein ALMS1 localizes to basal bodies of cochlear hair cells and regulates cilium-dependent planar cell polarity," *Hum. Mol. Genet.*, vol. 20, no. 3, pp. 466–481, Feb. 2011, doi: 10.1093/hmg/ddq493.
- [7] G. Li *et al.*, "A role for Alström syndrome protein, alms1, in kidney ciliogenesis and cellular quiescence," *PLoS Genet.*, vol. 3, no. 1, pp. e8–e8, Jan. 2007, doi: 10.1371/journal.pgen.0030008.
- [8] E. Zulato *et al.*, "ALMS1-deficient fibroblasts over-express extra-cellular matrix components, display cell cycle delay and are resistant to apoptosis," *PLoS One*, vol. 6, no. 4, pp. e19081–e19081, Apr. 2011, doi: 10.1371/journal.pone.0019081.
- [9] C. C. Leitch, S. Lodh, V. Prieto-Echagüe, J. L. Badano, and N. A. Zaghloul, "Basal body proteins regulate Notch signaling through endosomal trafficking," *J. Cell Sci.*, vol. 127, no. Pt 11, pp. 2407–2419, Jun. 2014, doi: 10.1242/jcs.130344.

- [10] M. Álvarez-Satta *et al.*, "ALMS1 Regulates TGF- β Signaling and Morphology of Primary Cilia," *Frontiers in Cell and Developmental Biology*, vol. 9, p. 112, 2021.
- [11] A. B. Roberts *et al.*, "Smad3 is key to TGF- β -mediated epithelial-to-mesenchymal transition, fibrosis, tumor suppression and metastasis," *Cytokine Growth Factor Rev.*, vol. 17, no. 1–2, pp. 19–27, Feb. 2006, doi: 10.1016/J.CYTOGFR.2005.09.008.
- [12] W. KL, J. KE, and H. CA, "Targeting TGF- β Mediated SMAD Signaling for the Prevention of Fibrosis," *Front. Pharmacol.*, vol. 8, no. JUL, Jul. 2017, doi: 10.3389/FPHAR.2017.00461.
- [13] C. A. Clement *et al.*, "TGF- β Signaling Is Associated with Endocytosis at the Pocket Region of the Primary Cilium," *Cell Rep.*, vol. 3, no. 6, pp. 1806–1814, 2013, doi: <https://doi.org/10.1016/j.celrep.2013.05.020>.
- [14] Y. Zhang, P. B. Alexander, and X. F. Wang, "TGF- β family signaling in the control of cell proliferation and survival," *Cold Spring Harb. Perspect. Biol.*, vol. 9, no. 4, Apr. 2017, doi: 10.1101/cshperspect.a022145.
- [15] L. K, "Signaling Cross Talk between TGF- β /Smad and Other Signaling Pathways," *Cold Spring Harb. Perspect. Biol.*, vol. 9, no. 1, Jan. 2017, doi: 10.1101/CSHPERSPECT.A022137.
- [16] J. Massagué, "TGF β signalling in context," *Nature Reviews Molecular Cell Biology*, vol. 13, no. 10. NIH Public Access, pp. 616–630, Oct. 2012, doi: 10.1038/nrm3434.
- [17] A. Nakao *et al.*, "TGF- β receptor-mediated signalling through Smad2, Smad3 and Smad4," *EMBO J.*, vol. 16, no. 17, pp. 5353–5362, Sep. 1997, doi: 10.1093/EMBOJ/16.17.5353.
- [18] S. Thuault, E.-J. Tan, H. Peinado, A. Cano, C.-H. Heldin, and A. Moustakas, "HMGA2 and Smads Co-regulate SNAIL1 Expression during Induction of Epithelial-to-Mesenchymal Transition," *J. Biol. Chem.*, vol. 283, no. 48, p. 33437, Nov. 2008, doi: 10.1074/JBC.M802016200.
- [19] Y. Wang, J. Shi, K. Chai, X. Ying, and B. P. Zhou, "The Role of Snail in EMT and Tumorigenesis."
- [20] T. Vincent *et al.*, "A SNAIL1-SMAD3/4 transcriptional repressor complex promotes TGF- β mediated epithelial-mesenchymal transition," *Nat. Cell Biol.*, vol. 11, no. 8, pp. 943–950, 2009, doi: 10.1038/ncb1905.
- [21] X. Yan *et al.*, "Smad7 Protein Interacts with Receptor-regulated Smads (R-Smads) to Inhibit Transforming Growth Factor- β (TGF- β)/Smad Signaling *," *J. Biol. Chem.*, vol. 291,

no. 1, pp. 382–392, Jan. 2016, doi: 10.1074/JBC.M115.694281.

[22] G. B. Collin *et al.*, “The Alström syndrome protein, ALMS1, interacts with α -actinin and components of the endosome recycling pathway,” *PLoS One*, vol. 7, no. 5, pp. e37925–e37925, 2012, doi: 10.1371/journal.pone.0037925.

[23] L. T. Shenje *et al.*, “Mutations in Alström protein impair terminal differentiation of cardiomyocytes,” *Nat. Commun.*, vol. 5, p. 3416, Mar. 2014, doi: 10.1038/ncomms4416.

[24] R. Horbay and R. Bilyy, “Mitochondrial dynamics during cell cycling,” *Apoptosis*, vol. 21, no. 12. Springer New York LLC, pp. 1327–1335, Dec. 01, 2016, doi: 10.1007/s10495-016-1295-5.

[25] M. H. Fox, “A model for the computer analysis of synchronous DNA distributions obtained by flow cytometry,” *Cytometry*, vol. 1, no. 1, pp. 71–77, Jul. 1980, doi: 10.1002/cyto.990010114.

[26] L. M. Westrate, A. D. Sayfie, D. M. Burgenske, and J. P. MacKeigan, “Persistent mitochondrial hyperfusion promotes G2/M accumulation and caspase-dependent cell death,” *PLoS One*, vol. 9, no. 3, p. 91911, Mar. 2014, doi: 10.1371/journal.pone.0091911.

[27] D. Arita, M. Kambe, C. Ishioka, and R. Kanamaru, “Induction of p53-independent apoptosis associated with G2M arrest following DNA damage in human colon cancer cell lines,” *Japanese J. Cancer Res.*, vol. 88, no. 1, pp. 39–43, Jan. 1997, doi: 10.1111/j.1349-7006.1997.tb00299.x.

[28] I. Yoshida, A. Monji, K. Tashiro, K. Nakamura, R. Inoue, and S. Kanba, “Depletion of intracellular Ca²⁺ store itself may be a major factor in thapsigargin-induced ER stress and apoptosis in PC12 cells,” *Neurochem. Int.*, vol. 48, no. 8, pp. 696–702, 2006, doi: <https://doi.org/10.1016/j.neuint.2005.12.012>.

[29] S. T. Christensen, S. K. Morthorst, J. B. Mogensen, and L. B. Pedersen, “Primary cilia and coordination of receptor tyrosine kinase (RTK) and transforming growth factor β (TGF- β) signaling,” *Cold Spring Harbor Perspectives in Biology*, vol. 9, no. 6. Cold Spring Harbor Laboratory Press, p. a028167, Jun. 01, 2017, doi: 10.1101/cshperspect.a028167.

[30] F. Cheng *et al.*, “Vimentin coordinates fibroblast proliferation and keratinocyte differentiation in wound healing via TGF- β -Slug signaling,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 113, no. 30, pp. E4320–E4327, Jul. 2016, doi: 10.1073/pnas.1519197113.

[31] S. J. Serrano-Gomez, M. Maziveyi, and S. K. Alahari, “Regulation of epithelial-

mesenchymal transition through epigenetic and post-translational modifications," *Mol. Cancer*, vol. 15, p. 18, Feb. 2016, doi: 10.1186/s12943-016-0502-x.

[32] L. Yang, T. Y. Besschetnova, C. R. Brooks, J. V Shah, and J. V Bonventre, "Epithelial cell cycle arrest in G2/M mediates kidney fibrosis after injury," *Nat. Med.*, vol. 16, no. 5, pp. 535–543, 2010, doi: 10.1038/nm.2144.

[33] T. L. White, N. Deshpande, V. Kumar, A. G. Gauthier, and U. V Jurkunas, "Cell cycle re-entry and arrest in G2/M phase induces senescence and fibrosis in Fuchs Endothelial Corneal Dystrophy," *Free Radic. Biol. Med.*, vol. 164, pp. 34–43, Feb. 2021, doi: 10.1016/j.freeradbiomed.2020.12.445.

[34] H. Li *et al.*, "Atg5-mediated autophagy deficiency in proximal tubules promotes cell cycle G2/M arrest and renal fibrosis," *Autophagy*, vol. 12, no. 9, pp. 1472–1486, Sep. 2016, doi: 10.1080/15548627.2016.1190071.

[35] J. JAKUBIKOVA, Y. BAO, and J. SEDLAK, "Isothiocyanates Induce Cell Cycle Arrest, Apoptosis and Mitochondrial Potential Depolarization in HL-60 and Multidrug-resistant Cell Lines," *Anticancer Res.*, vol. 25, no. 5, 2005.

[36] N. B. Madungwe *et al.*, "Mitochondrial inner membrane protein (Mitofilin) knockdown induces cell death by apoptosis via an AIF-PARP-dependent mechanism and cell cycle arrest," *Am. J. Physiol. - Cell Physiol.*, vol. 315, no. 1, pp. C28–C43, Jul. 2018, doi: 10.1152/ajpcell.00230.2017.

[37] Y. Liu *et al.*, "Calycosin induces mitochondrial-dependent apoptosis and cell cycle arrest, and inhibits cell migration through a ROS-mediated signaling pathway in HepG2 hepatocellular carcinoma cells," *Toxicol. Vitro.*, vol. 70, p. 105052, Feb. 2021, doi: 10.1016/j.tiv.2020.105052.

[38] P. Lindner, S. B. Christensen, P. Nissen, J. V. Møller, and N. Engedal, "Cell death induced by the ER stressor thapsigargin involves death receptor 5, a non-autophagic function of MAP1LC3B, and distinct contributions from unfolded protein response components," *Cell Commun. Signal.*, vol. 18, no. 1, p. 12, 2020, doi: 10.1186/s12964-019-0499-z.

[39] C. M. Osowski and F. Urano, "Measuring ER stress and the unfolded protein response using mammalian tissue culture system," *Methods Enzymol.*, vol. 490, pp. 71–92, 2011, doi: 10.1016/B978-0-12-385114-7.00004-0.

[40] F. Wang *et al.*, "Thapsigargin Induces Apoptosis by Impairing Cytoskeleton Dynamics in

Human Lung Adenocarcinoma Cells,” *Sci. World J.*, vol. 2014, p. 619050, 2014, doi: 10.1155/2014/619050.

[41] Z. Mu, L. Wang, W. Deng, J. Wang, and G. Wu, “Structural insight into the Ragulator complex which anchors mTORC1 to the lysosomal membrane,” *Cell Discov.*, vol. 3, no. 1, p. 17049, 2017, doi: 10.1038/celldisc.2017.49.

[42] M. J. Zinda, C. J. Vlahos, and M. T. Lai, “Ceramide Induces the Dephosphorylation and Inhibition of Constitutively Activated Akt in PTEN Negative U87MG Cells,” *Biochem. Biophys. Res. Commun.*, vol. 280, no. 4, pp. 1107–1115, 2001, doi: <https://doi.org/10.1006/bbrc.2000.4248>.

[43] R. M. Sánchez-Mora, H. Arboleda, and G. Arboleda, “PINK1 Overexpression Protects Against C2-ceramide-Induced CAD Cell Death Through the PI3K/AKT Pathway,” *J. Mol. Neurosci.*, vol. 47, no. 3, pp. 582–594, 2012, doi: 10.1007/s12031-011-9687-z.

[44] A. S. Arora, B. J. Jones, T. C. Patel, S. F. Bronk, and G. J. Gores, “Ceramide induces hepatocyte cell death through disruption of mitochondrial function in the rat,” *Hepatology*, vol. 25, no. 4, pp. 958–963, Apr. 1997, doi: <https://doi.org/10.1002/hep.510250428>.

[45] S.-J. Jeong *et al.*, “PI3K/AKT inhibition induces caspase-dependent apoptosis in HTLV-1-transformed cells,” *Virology*, vol. 370, no. 2, pp. 264–272, 2008, doi: <https://doi.org/10.1016/j.virol.2007.09.003>.

[46] B. N. Fahy, M. Schlieman, S. Virudachalam, and R. J. Bold, “AKT inhibition is associated with chemosensitisation in the pancreatic cancer cell line MIA-PaCa-2,” *Br. J. Cancer*, vol. 89, no. 2, pp. 391–397, 2003, doi: 10.1038/sj.bjc.6601037.

[47] C.-C. Chen *et al.*, “FoxOs Inhibit mTORC1 and Activate Akt by Inducing the Expression of Sestrin3 and Rictor,” *Dev. Cell*, vol. 18, no. 4, pp. 592–604, 2010, doi: <https://doi.org/10.1016/j.devcel.2010.03.008>.

[48] C. Cicchini *et al.*, “TGFβ-induced EMT requires focal adhesion kinase (FAK) signaling,” *Exp. Cell Res.*, vol. 314, no. 1, pp. 143–152, 2008, doi: <https://doi.org/10.1016/j.yexcr.2007.09.005>.

[49] T. Baronsky *et al.*, “Cell-Substrate Dynamics of the Epithelial-to-Mesenchymal Transition,” *Nano Lett.*, vol. 17, no. 5, pp. 3320–3326, May 2017, doi: 10.1021/acs.nanolett.7b01558.

- [50] A. Li *et al.*, "S100A6 promotes the proliferation and migration of cervical cancer cells via the PI3K/Akt signaling pathway.," *Oncol. Lett.*, vol. 15, no. 4, pp. 5685–5693, Apr. 2018, doi: 10.3892/ol.2018.8018.
- [51] A. Li *et al.*, "S100A6 promotes cell proliferation in human nasopharyngeal carcinoma via the p38/MAPK signaling pathway.," *Mol. Carcinog.*, vol. 56, no. 3, pp. 972–984, Mar. 2017, doi: 10.1002/mc.22563.
- [52] P. Li, X. Lv, Z. Zhang, and S. Xie, "S100A6/miR193a regulates the proliferation, invasion, migration and angiogenesis of lung cancer cells through the P53 acetylation.," *Am. J. Transl. Res.*, vol. 11, no. 8, pp. 4634–4649, 2019.
- [53] B. Haldar *et al.*, "S100A6 is a positive regulator of PPP5C-FKBP51-dependent regulation of endothelial calcium signaling.," *FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol.*, vol. 34, no. 2, pp. 3179–3196, Feb. 2020, doi: 10.1096/fj.201901777R.
- [54] S. Calabretta *et al.*, "Modulation of PKM alternative splicing by PTBP1 promotes gemcitabine resistance in pancreatic cancer cells.," *Oncogene*, vol. 35, no. 16, pp. 2031–2039, Apr. 2016, doi: 10.1038/onc.2015.270.
- [55] N. Lu, S. Carracedo, J. Ranta, R. Heuchel, R. Soininen, and D. Gullberg, "The human α 11 integrin promoter drives fibroblast-restricted expression in vivo and is regulated by TGF- β 1 in a Smad- and Sp1-dependent manner," *Matrix Biol.*, vol. 29, no. 3, pp. 166–176, Apr. 2010, doi: 10.1016/j.matbio.2009.11.003.
- [56] W. Jiao, K. Miyazaki, and Y. Kitajima, "Inverse correlation between E-cadherin and Snail expression in hepatocellular carcinoma cell lines in vitro and in vivo," *Br. J. Cancer*, vol. 86, no. 1, pp. 98–101, 2002, doi: 10.1038/sj.bjc.6600017.
- [57] I. Poser, D. Domínguez, A. G. de Herreros, A. Varnai, R. Buettner, and A. K. Bosserhoff, "Loss of E-cadherin Expression in Melanoma Cells Involves Up-regulation of the Transcriptional Repressor Snail*," *J. Biol. Chem.*, vol. 276, no. 27, pp. 24661–24666, 2001, doi: <https://doi.org/10.1074/jbc.M011224200>.
- [58] L. Hao, J. R. Ha, P. Kuzel, E. Garcia, and S. Persad, "Cadherin switch from E- to N-cadherin in melanoma progression is regulated by the PI3K/PTEN pathway through Twist and Snail," *Br. J. Dermatol.*, vol. 166, no. 6, pp. 1184–1197, Jun. 2012, doi: <https://doi.org/10.1111/j.1365-2133.2012.10824.x>.
- [59] K. Gravdal, O. J. Halvorsen, S. A. Haukaas, and L. A. Akslen, "A Switch from E-Cadherin to

N-Cadherin Expression Indicates Epithelial to Mesenchymal Transition and Is of Strong and Independent Importance for the Progress of Prostate Cancer,” *Clin. Cancer Res.*, vol. 13, no. 23, pp. 7003 LP – 7011, Dec. 2007, doi: 10.1158/1078-0432.CCR-07-1263.

[60] K. Araki *et al.*, “E/N-cadherin switch mediates cancer progression via TGF- β -induced epithelial-to-mesenchymal transition in extrahepatic cholangiocarcinoma,” *Br. J. Cancer*, vol. 105, no. 12, pp. 1885–1893, 2011, doi: 10.1038/bjc.2011.452.

[61] A. B. Roberts *et al.*, “Smad3 is key to TGF- β -mediated epithelial-to-mesenchymal transition, fibrosis, tumor suppression and metastasis,” *Cytokine Growth Factor Rev.*, vol. 17, no. 1–2, pp. 19–27, 2006, doi: 10.1016/j.cytogfr.2005.09.008.

[62] N. E. Sanjana, O. Shalem, and F. Zhang, “Improved vectors and genome-wide libraries for CRISPR screening,” *Nat. Methods*, vol. 11, no. 8, pp. 783–784, 2014, doi: 10.1038/nmeth.3047.

[63] X. Zhang, A. H. Smits, G. B. van Tilburg, H. Ovaa, W. Huber, and M. Vermeulen, “Proteome-wide identification of ubiquitin interactions using UbIA-MS,” *Nat. Protoc.*, vol. 13, no. 3, pp. 530–550, Mar. 2018, doi: 10.1038/nprot.2017.147.

[64] G. Yu, L.-G. Wang, Y. Han, and Q.-Y. He, “clusterProfiler: an R package for comparing biological themes among gene clusters,” *OMICS*, vol. 16, no. 5, pp. 284–287, May 2012, doi: 10.1089/omi.2011.0118.

[65] W. Walter, F. Sánchez-Cabo, and M. Ricote, “GOplot: an R package for visually combining expression data with functional analysis,” *Bioinformatics*, vol. 31, no. 17, pp. 2912–2914, Sep. 2015, doi: 10.1093/bioinformatics/btv300.

[66] Y. Perez-Riverol *et al.*, “The PRIDE database and related tools and resources in 2019: improving support for quantification data,” *Nucleic Acids Res.*, vol. 47, no. D1, pp. D442–D450, Jan. 2019, doi: 10.1093/nar/gky1106.

[67] C. A. Schneider, W. S. Rasband, and K. W. Eliceiri, “NIH Image to ImageJ: 25 years of image analysis,” *Nat. Methods* 2012 97, vol. 9, no. 7, pp. 671–675, Jun. 2012, doi: 10.1038/nmeth.2089.

[68] A. Suarez-Arnedo, F. T. Figueroa, C. Clavijo, P. Arbeláez, J. C. Cruz, and C. Muñoz-Camargo, “An image J plugin for the high throughput image analysis of in vitro scratch wound healing assays,” *PLoS One*, vol. 15, no. 7, Jul. 2020, doi: 10.1371/JOURNAL.PONE.0232565.

Supplementary Material and Methods:

CRISPR plasmids construction:

Single guides RNAs (sgRNAs) for BJ-5ta model were designing using benchling to generate a deletion of 113pb in the exon 3 of *ALMS1* (ENSG00000116127). The 2 sgRNA (5'- CCTAGTCTGGGATGTATCCA – 3' and 5'- GAACAGAAGAGATCTCTGTT – 3') result from benchling were cloned into the plasmid lentiCRISPR v2 [1] following a modified protocol from that described by Shalem et al [2]. LentiCRISPR v2 was a gift from Feng Zhang (Addgene plasmid #52961). Initially, 12.5µL (5µg) of LentiCRISPR V2 were digested with 3µL Esp3I (BsmBI), 3µL FastAP and 6µL 10X FastDigest Buffer from Thermo Fisher. Final volume was set at 60µL. Plasmid digestion was checked by migration in an agarose gel at 1%.

sgRNAs were phosphorylated and annealed with its correspondent oligo mixing as follow: 1µL sgRNA (100µM), 1µL of its complement oligo, 2µL of 5X T4 Buffer (Canvax, Cordoba, Spain), 0.5 T4 PNK (NEB, Ipswich, EE.UU) and 5.5 µL distilled H₂O (H₂Od). Mix reaction was incubated at 37°C for 30min and 95°C for 5min. The samples were then left to stand at room temperature for 2 hours and diluted at 1:50, 1:100 and 1:200 ratios.

The unpurified digested plasmid was ligated with the diluted sgRNAs as follows: 0.5µL digested LentiCRISPR v2, 2µL diluted sgRNA, 1µL T4 DNA Ligase (Canvax, Cordoba, Spain), 4.5µL H₂Od. The mix was incubated at 37°C by 30min.

Finally, Stable Competent E.coli (NEB, Ipswich, EE.UU) were transformed by thermal shock according to the manufactured protocol and incubated at 30°C overnight. After that, construct validation was carried out using forward primer for U6 promotor (5'- GACTATCATATGCTTACCGT -3') and reverse sgRNA.

Plasmids Transfection and Lentivirus production:

293T were seeded in a concentration of 3×10^5 into a 6-well plate and incubated overnight. Then, 293T cells were co-transfected with each CRISPR construct and the plasmids psPAX2 and pCMV-VSV-G [3] using lipofectamine 3000 (Thermo Fisher, Waltham, EE. UU). pCMV-VSV-G was a gift from Bob Weinberg (Addgene plasmid #8454) and psPAX2 was a gift from Didier Trono (Addgene plasmid #12260)

Initially, 7.5µL of lipofectamine 3000 were diluted in 125µL of Opti-MEM medium (Thermo Fisher, Waltham, EE. UU) by duplicate. Then a total amount of 5µg of ADN (1.6µL for each

plasmid) were diluted at 250µL Opti-MEM. After that, diluted DNA and diluted lipofectamine were mixed in a 1:1 ratio, centrifugated at 1200rpm and incubated for 20min at room temperature. Finally, the 250µL of mix were applied to the wells containing the cells.

The cells were incubated with the mixture for 24 hours. Then, the mixture was removed and the cell was cultured for a further 72 hours for lentivirus production.

Lentiviral transduction:

After 72 hours of culturing 293T cells transfected for lentivirus production, the culture medium was collected in 15 mL tubes and filtered through a 45µm filter (Sartorius,). In addition, a solution of complete DMEM medium without P/S with polybrene was prepared at a dilution of 1:1000 (8µg/ml).

Then, 300µL lentiviral preparation were mixed with 200µL complete DMEM médium with polybrene for each well. An additional 1 ml of DMEM with polybrene was added and the plates were centrifuged at 800g for 30 minutes. This step was repeated one more time. After the second centrifugation the mix with lentivirus was added again and the plates were incubated for 24 hours. Finally, mix was removed and cells were allowed to settle for 48 hours in DMEM médium. They were then selected with puromycin (1ng/mL) for 5 days.

Edition validation:

After puromycin selection, clonal isolation and expansion we checked the lack of *ALMS1* gene by qPCR in StepOnePlus (Thermo Fisher, Waltham, EE. UU). Finally, we found that these cells had the expected exon 3 deletion by PCR with resolution of samples on a 2% agarose gel and followed by sanger sequencing.

761 Supplementary figures:

Supplementary Figure S1. Characterisation of *ALMS1* $-/-$ cell line HeLa. **(A)** Design of the model. **(B)** Relative gene expression of *ALMS1* $+/+$ and polyclonal cell line for *ALMS1* $-/-$ genotype. **(C)** Relative gene expression of *ALMS1* $+/+$ and a first batch of clones for *ALMS1* $-/-$ genotype. **(D)** Relative gene expression of *ALMS1* $+/+$ and second batch of clones for *ALMS1* $-/-$ genotype. **(E)** Strategy for amplification of exons 1 and 3 from genomic DNA in two clones of the *ALMS1* $-/-$ (KO) line and the *ALMS1* $+/+$ control (WT). **(F)** Amplification strategy of the resulting sequence between exon 1 and 3 after removal of the sequencing between the 2 outermost gRNAs.

Supplementary Figure S2. Genetic characterisation of *ALMS1* $-/-$ cell line BJ-5ta. **(A)** Clone screening. **(B)** Relative gene expression of *ALMS1* $+/+$ and a batch of clones for *ALMS1* $-/-$ genotype. **(C)** Agarose gel (2%) showing the exon 3 amplicon of the control line (*ALMS1* $+/+$) and the F4 clone (*ALMS1* $-/-$) which showed the lowest gene expression. **(D)** Characterisation of the F4 clone mutation by sanger sequencing.