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Qing Zhong

qingzhong@shsmu.edu.cn

Shanghai Jiaotong University School of Medicine <https://orcid.org/0000-0001-6979-955X>

Peng Li

Shanghai Jiaotong University School of Medicine

Qian Yang

Shanghai Jiaotong University School of Medicine

Zaiming Tang

Shanghai Jiaotong University School of Medicine <https://orcid.org/0000-0001-9843-8788>

Junjie Ye

Shanghai Shijiang Biotechnology Biotechnology Co., Lt

Yi Pan

Shanghai Jiaotong University School of Medicine

Hanyan Luo

Shanghai Jiaotong University School of Medicine

Article

Keywords: OFD1, Macropinocytosis, KRAS, pancreatic cancer, SDC4

Posted Date: March 9th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-8939646/v1>

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Additional Declarations: (Not answered)

**Targeting OFD1 restrains *KRAS*-driven pancreatic cancer progression by impairing
macropinocytosis**

Qian Yang¹, Junjie Ye¹, Hanyan Luo¹, Yi Pan¹, Zaiming Tang^{1*}, Qing Zhong^{1*} and Peng Li^{1,*}

¹Institute for Translational Medicine on Cell Fate and Disease, Shanghai Ninth People's Hospital, Key Laboratory of Cell Differentiation and Apoptosis of Chinese Ministry of Education, Department of Pathophysiology, Shanghai Jiao Tong University School of Medicine (SJTU-SM), Shanghai 200025, China.

*To whom correspondence should be addressed.

E-mail:

zaimingtang2017@shsmu.edu.cn (Z.-M.T.), pengli@shsmu.edu.cn (P.L.), qingzhong@shsmu.edu.cn (Z.Q.)

Abstract

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive malignancy driven by oncogenic *KRAS* and characterized by profound metabolic rewiring. To sustain proliferation under nutrient stress, PDAC cells rely on macropinocytosis, an cytoskeleton-dependent pathway for extracellular nutrient uptake. The Oral-Facial-Digital syndrome 1 protein OFD1 is implicated in the progression of PDAC and regulates actin cytoskeletal dynamics. However, its role in macropinocytosis remains unclear. Here, we identify OFD1 as a key downstream effector of *KRAS* that orchestrates macropinocytosis. The oncogenic *KRAS* induces OFD1 expression, whereas OFD1 depletion markedly impairs macropinocytic activity and consequently suppresses tumor growth in *KRAS* mutant PDAC xenograft models. Mechanistically, OFD1 sustains NF- κ B-dependent transcription of Syndecan-4 (SDC4), a core component of the macropinocytic machinery. In turn, restoration of SDC4 expression rescues macropinocytosis and tumor growth. Notably, a drug-repurposing screen identifies pharmacological degradation of OFD1 as an effective strategy to inhibit macropinocytosis and restrain PDAC progression. Consistent with these findings, OFD1 expression positively correlates with SDC4 levels in PDAC patient samples, and elevated SDC4 expression is associated with poor patient survival. Together, these findings establish OFD1 as a

central regulator of macropinocytosis and reveal a potential metabolic vulnerability in PDAC.

Keywords: *OFD1*; Macropinocytosis; *KRAS*; pancreatic cancer; *SDC4*.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) remains one of the most lethal malignancies, with a five-year survival rate less than 10% and limited therapeutic options¹. A defining feature of PDAC is its profoundly desmoplastic and nutrient-deprived tumor microenvironment (TME)². The dense fibrotic stroma compresses blood vessels, restricts oxygen and nutrient delivery, and creates a hypoxic, metabolically hostile milieu³⁻⁵. To survive and proliferate under these harsh conditions, PDAC cells undergo extensive metabolic reprogramming and adopt unconventional nutrient acquisition strategies⁶.

Among these adaptive mechanisms, macropinocytosis has emerged as a crucial survival pathway that enables cancer cells to internalize extracellular proteins and fluid as alternative nutrient sources^{7,8}. This process is frequently activated by oncogenic *KRAS*, which is mutated in the vast majority of PDAC cases, to support protein scavenging and sustain tumor growth under nutrient limitation^{9,10}. Despite its established importance, the molecular interface between *KRAS* signaling and the actin cytoskeleton that orchestrates macropinosome formation remains incompletely understood.

KRAS promotes macropinocytosis through activation of the PI3K pathway and small GTPases such as Rac1 and Cdc42¹¹. Additional regulatory inputs have also been described; for example, vacuolar ATPase (V-ATPase) translocates to the plasma membrane via a soluble adenylylate cyclase-PKA-RAC1 axis to facilitate macropinosome formation¹⁰. Members of the syndecan (SDC) family-particularly SDC1, SDC2, and SDC4, modulate this process by regulating small GTPase activity and cytoskeletal remodeling¹²⁻¹⁵. Collectively, these findings delineate a multilayered regulatory network governing macropinocytosis in PDAC.

Pharmacological studies highlight macropinocytosis as a potentially tractable therapeutic vulnerability. The canonical macropinocytosis inhibitor EIPA suppresses Na⁺/H⁺ exchange and

efficiently blocks nutrient uptake in PDAC models⁷. Similarly, mTOR inhibitors disrupt macropinocytosis and synergize with autophagy blockade to limit tumor growth¹⁶. In addition, drug-repurposing screens have identified compounds such as ivermectin, tyrphostin A9, and pyrvinium pamoate as potent macropinocytosis inhibitors that sensitize *KRAS*-mutant PDAC cells to metabolic stress¹⁷. These findings support macropinocytosis inhibition as a promising complement to metabolic and *KRAS*-targeted therapies. Despite these advances, macropinocytosis remains difficult to modulate selectively¹⁸, highlighting the need for more specific and pharmacologically tractable approaches.

Our previous work identified OFD1 as a centrosomal protein markedly overexpressed in PDAC, and its high expression correlates with poor patient survival and contributes to PDAC progression¹⁹. And we further demonstrated that OFD1 functions as a type II nucleation-promoting factor (NPF), facilitating ARP2/3 complex-mediated actin branching to regulate cytokinesis and primary ciliogenesis²⁰. Given that the formation of macropinocytic cups requires ARP2/3-dependent branched actin networks²¹⁻²³, we hypothesized that OFD1 may drive macropinocytosis through cytoskeletal reorganization, thereby supporting PDAC progression.

Here, we show that oncogenic *KRAS* upregulates OFD1 to drive macropinocytosis and tumor growth. *OFD1* knockdown reducing macropinocytic uptake and *SDC4* expression, whereas restoration of *SDC4* partially rescues these defects. Moreover, a drug-repurposing screen identifies digoxin as an OFD1-degrading agent that suppresses macropinocytosis and PDAC progression. *OFD1* and *SDC4* expression levels are positively correlated in tumors, and their co-overexpression predicts poor patient survival. Together, these finding establishes OFD1 as a critical mediator of *KRAS*-driven macropinocytosis and reveal a clinically actionable metabolic vulnerability in PDAC.

Results

***KRAS*-driven macropinocytosis and PDAC progression rely on OFD1.**

We previously reported that *OFD1* is highly expressed in pancreatic cancer, and that its overexpression is associated with poor patient survival¹⁹. Given that oncogenic *KRAS* mutations occur in more than 90% of PDAC cases²⁴, we hypothesized that *OFD1* upregulation may be driven by *KRAS* activation. To test this possibility, we established an inducible *KRAS* ^{G12D}

expression system in human foreskin fibroblasts (HFFs). Western blot analysis revealed a dose-dependent increase in OFD1 protein levels upon *KRAS*^{G12D} induction (**Fig. 1a-1b**), indicating that oncogenic *KRAS* promotes OFD1 expression. To assess the functional contribution of OFD1 in PDAC progression, we employed a MIA PaCa2 xenograft model harboring mutant *KRAS*. Tumors derived from *OFD1*-depleted cells exhibited significantly reduced growth rates and tumor weights compared with control tumors (**Fig. 1c-1e**), supporting a critical role for OFD1 in *KRAS*-driven tumorigenesis *in vivo*.

To investigate the molecular basis underlying the tumor-suppressive effect of *OFD1* depletion, we performed data-independent acquisition (DIA)-based quantitative proteomics profiling of MIA PaCa2 cells following *OFD1* knockdown. Gene Ontology (GO) Cellular Component enrichment analysis revealed that downregulated proteins were predominantly associated with cell surface components, including actin filaments, cell adhesion, and cytoskeletal organization (**Fig. 1f-1g**). Membrane remodeling and actin dynamics are essential for macropinocytosis—a nutrient-scavenging mechanism that supports tumor cell metabolism under stress^{25,26}. We tested the role of OFD1 in macropinocytosis, OFD1 depletion markedly reduced macropinocytic uptake in MIA PaCa-2 cells, a *KRAS*-mutant PDAC cell line, whereas no significant changes were observed in BxPC-3 cells harboring wild-type *KRAS* (**Fig. 1h-1i**, **Supplementary Fig. 1a**). *In vivo* macropinocytosis assays in xenografts yielded similar results, in which a pronounced reduction in macropinocytic activity was observed in *OFD1* knockdown xenografts (**Fig. 1j-1k**). Moreover, *KRAS* activation enhanced macropinocytosis in fibroblasts, whereas OFD1 silencing substantially attenuated this effect (**Fig. 1l**, **Supplementary Fig. 1b-1c**). Collectively, these data indicate that OFD1 promotes macropinocytosis in a *KRAS*-dependent manner and contributes to tumor growth in pancreatic cancer.

OFD1 regulates macropinocytosis in a Syndecan-4-dependent manner

Having established that *OFD1* contributes to *KRAS*-driven macropinocytosis, we next sought to elucidate the underlying molecular mechanism. Macropinocytosis is initiated by plasma membrane ruffling and macropinocytic cup formation, processes that coordinated by cell surface proteins^{9,10,12,27}. Comparative analysis of our DIA-based proteomic dataset with previously reported *KRAS*-induced surface proteomes¹² revealed a substantial overlap between proteins

upregulated by *KRAS* activation and those downregulated upon *OFD1* depletion. Among these candidates, *SDC4* and *ITGA3* emerged as prominent regulators (**Fig. 2a-2b**).

Given the well-established role of *SDC4* in regulating membrane dynamics and macropinocytosis^{15,28,29}, we focused on *SDC4* for further investigation. Western blot analyses across multiple pancreatic cancer cell lines, including MIA PaCa2, SW1990, and AsPC1, demonstrated that *OFD1* knockdown markedly reduced *SDC4* protein abundance (**Supplementary Fig. 2a-2c**). Functionally, depletion of either *OFD1* or *SDC4* resulted in a comparable suppression of macropinocytic uptake (**Fig. 2c-2e**), supporting a functional link between *OFD1* and *SDC4* in this process.

To determine whether *SDC4* mediates the macropinocytic and tumor-promoting functions of *OFD1*, we reintroduced *SDC4* into *OFD1*-knockdown MIA PaCa2 cells. Restoration of *SDC4* substantially restored the impaired macropinocytic uptake and colony-forming capacity induced by *OFD1* knockdown (**Fig. 2f-2h**). *In vivo*, subcutaneous implantation of control, *OFD1*-depleted, *SDC4*-depleted, and *OFD1*-depleted cells with *SDC4* reconstitution into immunodeficient mice further validated this relationship. Restoration of *SDC4* rescued the tumor growth inhibition caused by *OFD1* depletion, as assessed by tumor volume and tumor weight (**Fig. 2i-2l**). Collectively, these data identify *SDC4* as a pivotal downstream effector of *OFD1* that mediates macropinocytosis and supports pancreatic tumor growth.

OFD1 modulates NF- κ B activity to control *SDC4* transcription

We next examined whether *OFD1* regulates *SDC4* expression at the transcriptional or post-transcriptional level. Quantitative RT-qPCR analyses showed that *OFD1* knockdown markedly reduced *SDC4* mRNA expression in MIA PaCa2 and SW1990 cells (**Fig. 3a-3b**). Treatment with the transcriptional inhibitor actinomycin D revealed comparable *SDC4* mRNA half-lives between control and *OFD1*-depleted cells (**Fig. 3c-3d**), indicating that *OFD1* does not affect *SDC4* mRNA stability. In contrast, luciferase reporter assays demonstrated a significant reduction in *SDC4* promoter activity following *OFD1* depletion (**Fig. 3e**), indicating that *OFD1* primarily regulates *SDC4* expression at the transcriptional level.

To identify transcription factors mediating this regulation, we integrated analyses from two

complementary resources: ChEA3, a database that predicts transcription factors responsible for observed gene expression changes³⁰, and ChIP-Atlas, a comprehensive data-mining platform integrates publicly ChIP-seq datasets³¹. ChEA3 analysis of differentially expressed proteins in *OFD1*-depleted MIA PaCa2 cells identified 57 candidate transcription factors potentially acting downstream of *OFD1*. In parallel, ChIP-Atlas predicted 33 transcription factors with putative binding sites within the *SDC4* promoter region. The intersection of these datasets highlighted p65 (RelA), a principal transactivation subunit of the NF- κ B family, as a candidate mediator of *SDC4* transcriptional regulation (**Fig. 3f**).

Previous studies have shown that *OFD1* depletion attenuates NF- κ B signaling through I κ B accumulation³², whereas NF- κ B directly activates *SDC4* transcription^{33,34}. Consistent with this framework, we observed a marked reduction in phosphorylated p65 at Ser536, a surrogate marker of transcriptionally active NF- κ B³⁵, upon *OFD1* knockdown in pancreatic cancer cells (**Fig. 3g**). Furthermore, chromatin immunoprecipitation (ChIP) assays revealed significantly reduced occupancy of NF- κ B p65 at the *SDC4* promoter in *OFD1*-deficient cells (**Fig. 3h-3i**, **Supplementary Fig. 3a**). Together, these data indicate that *OFD1* sustains NF- κ B activity to promote *SDC4* transcription, thereby linking *OFD1* expression to a macropinocytic regulatory module via *SDC4*.

Small-molecule screening identifies digoxin as a compound reducing *OFD1* protein levels

Given the lack of pharmacological strategies targeting *OFD1* despite its clear oncogenic relevance¹⁹, we sought to identify small molecules capable of reducing *OFD1* protein abundance. To this end, we conducted a drug repurposing screen using an FDA-approved compound library, with *OFD1* protein levels as the primary readout (**Fig. 4a-4b**). Compounds were subsequently classified according to pharmacological categories. Among the top ten classes, DNA-damaging agents and endocrinology/hormone-related drugs predominantly increased *OFD1* levels, whereas inhibitors targeting EGFR, protein tyrosine kinases (PTKs) and cyclin-dependent kinases (CDKs) were highly enriched among those that reduced *OFD1* expression (**Supplementary Fig. 4a**), consistent with a role for *OFD1* within oncogenic signaling networks¹⁹. From this screen, the ten compounds that most effectively reduced *OFD1* expression were selected for further evaluation across a range of decreasing concentrations in two pancreatic cancer cell line (**Supplementary**

Fig. 4b). Notably, digoxin, a clinically approved cardiac glycoside³⁶, exhibited the strongest and most selective suppression of OFD1 protein levels, achieving significant reductions at nanomolar concentrations in both two pancreatic cancer cell lines (**Fig. 4c-4d, Supplementary Fig. 4c-4d**). Western blot analysis further confirmed a dose-dependent decrease in OFD1 protein abundance following digoxin treatment across multiple PDAC cell lines (**Fig. 4e-4f**).

To assess mechanistic concordance between digoxin treatment and OFD1 depletion, we performed quantitative proteomic profiling. This analysis revealed a strong similarity between digoxin-induced and OFD1 knockdown-associated global proteomic changes (**Fig. 4g-4h**). Consistently, Reactome and KEGG pathway demonstrated convergent enrichment signatures, particularly in pathways related to membrane trafficking, cytoskeletal organization, and metabolic signaling (**Fig. 4i, Supplementary Fig. 4e-4h**). Collectively, these results identify digoxin as an unexpected pharmacological modulator of OFD1 that phenocopies key molecular and signaling consequences of OFD1 depletion, thereby highlighting OFD1 as a pharmacologically tractable vulnerability in PDAC.

Digoxin reduces OFD1 protein stability in a SYVN1-dependent manner in PDAC cells

Digoxin exerts its classical pharmacological effects through Na⁺/K⁺ ATPase, with ATP1A1 serving as a principal α -subunit^{37,38}. To determine whether Na⁺/K⁺ ATPase mediates the effect of digoxin on OFD1, we depleted *ATP1A1* and observed no change in OFD1 protein abundance (**Fig. 5a**), indicating that digoxin regulates OFD1 independently of Na⁺/K⁺ ATPase engagement. To assess whether digoxin modulates OFD1 expression at the transcriptional level, we performed qPCR analysis, which revealed no significant changes in OFD1 mRNA abundance following digoxin treatment (**Fig. 5b-5c, Supplementary Fig. 5a**). By contrast, Cycloheximide (CHX) chase assays demonstrated accelerated OFD1 protein turnover in digoxin-treated cells (**Fig. 5d-5e**), implicating a post-translational destabilization mechanism. To determine which E3 ligase mediates Digoxin-induced OFD1 degradation, we first predicted potential OFD1-targeting E3 ligases using the UbiBrowser database. siRNA-mediated knockdown of candidate E3 ligases was performed to examine whether Digoxin-induced OFD1 degradation could be blocked (**Fig. 5f, Supplementary Fig. 5b-5e**). This analysis identified SYVN1 as the critical E3 ligase responsible for OFD1 degradation upon Digoxin treatment (**Fig. 5g**). Moreover, immunofluorescence analysis

demonstrated a progressive reduction in centrosomal OFD1 signal over time following digoxin treatment (**Fig. 5h-5i**), further supporting the conclusion that digoxin promotes OFD1 proteolysis at its primary subcellular localization. Together, these data demonstrate that digoxin reduces OFD1 protein abundance by accelerating its neddylation-dependent turnover, thereby destabilizing a core regulator of macropinocytosis in PDAC cells.

Digoxin mimics OFD1 depletion to suppress macropinocytosis and PDAC growth

To determine whether digoxin functionally recapitulates genetic *OFD1* depletion, we performed integrated *in vitro* and *in vivo* analyses. Consistent with genetic *OFD1* knockdown, digoxin treatment markedly reduced SDC4 expression and impaired macropinocytic uptake in PDAC cells (**Fig. 6a-6b**). In MIA PaCa2 xenograft models, systemic administration of digoxin resulted in pronounced suppression of tumor progression, as evidenced by significantly reduced tumor volume and tumor weight compared with vehicle-treated cohorts (**Fig. 6c-6g**). Notably, digoxin-treated animals exhibited no detectable body weight loss or overt treatment-associated toxicity over the course of the study (**Fig. 6h**), indicating tolerability within the dosing regimen tested. Together, these findings demonstrate that digoxin phenocopies *OFD1* depletion at both molecular and functional levels, suppressing macropinocytosis and constraining PDAC growth *in vivo*, and support pharmacological destabilization of OFD1 as a viable therapeutic vulnerability in pancreatic cancer.

OFD1 expression correlates with SDC4 levels and poor patient survival in PDAC

Having established that *OFD1* regulates macropinocytosis through *SDC4*, we next investigated their relationship between OFD1 and SDC4 *in vivo* and in human PDAC samples. Using the LSL-*Kras*^{G12D}; *Trp53*^{R172H/+}; *Pdx1*-Cre (KPC) mouse model, we generated KPCO mice by selectively deleting *Ofd1* in pancreatic ductal epithelial cells via Cre-mediated recombination. Immunohistochemical (IHC) analysis revealed a marked reduction in SDC4 expression in KPCO tumors compared with KPC controls (**Fig. 7a**), demonstrating that OFD1 is required to sustain SDC4 expression *in vivo*.

To further assess KRAS dependency, inducible *KRAS*^{G12D} activation in HFF fibroblasts led to coordinated upregulation of both OFD1 and SDC4 protein levels (**Fig. 7b**), confirming that their

expression is driven by oncogenic *KRAS* signaling. Consistent with these findings, analysis of TCGA and the PDAC GEO dataset³⁹ revealed a strong positive correlation between *OFD1* and *SDC4* expression in human pancreatic tumors (**Fig. 7c-7d**). Kaplan-Meier survival analysis further demonstrated that high *SDC4* expression was significantly associated with poorer overall survival (**Fig. 7e**). In parallel, IHC staining showed elevated *SDC4* expression in tumor tissues compared with adjacent normal pancreas (**Fig. 7f**). Collectively, these data establish that the *OFD1*-*SDC4* axis is conserved across genetically engineered mouse models and human PDAC, and that its activation is clinically relevant. The tight co-expression of *OFD1* and *SDC4*, together with their association with adverse patient outcomes, highlights this axis as a potential prognostic indicator and a therapeutically actionable vulnerability in PDAC.

Discussion

Macropinocytosis constitutes a critical metabolic adaptation that supports the growth and survival of *KRAS*-driven PDAC cells under nutrient-limiting conditions, rendering this pathway an attractive therapeutic target^{26,40}. Despite its importance, selective pharmacological modulation of macropinocytosis has remained challenging, underscoring the need for more tractable regulatory nodes^{18,26}. In this study, we identify the centrosomal protein *OFD1* as a previously unrecognized regulator of macropinocytosis in *KRAS*-driven PDAC. Oncogenic *KRAS* signaling induces *OFD1* expression, and depletion of *OFD1* markedly attenuates *KRAS*-driven macropinocytosis uptake, supporting a critical role for *OFD1* in sustaining this adaptive metabolic program. Mechanistically, *OFD1* regulates the expression of *SDC4*, a key component of the macropinocytic machinery^{14,28,41,42}, thereby enhancing the capacity of cancer cells to scavenge extracellular proteins and maintain tumor growth under nutrient-limited conditions. Together, these findings position *OFD1* as an important downstream effector of *KRAS*-driven macropinocytosis and reveal a previously underappreciated metabolic vulnerability in PDAC.

The regulation of *SDC4* expression by *OFD1* highlights a broader functional repertoire of centrosomal proteins beyond their canonical roles in microtubule organization and ciliogenesis. While the centrosome is increasingly recognized as a hub for actin organization and signal

integration^{43,44}, our data indicate that OFD1-mediated control of protein homeostasis at the centrosome can propagate to transcriptional programs that govern cytoskeletal remodeling and nutrient scavenging. In line with prior studies implicating OFD1 in centrosome-associated proteasomal regulation³², we observed that OFD1 depletion significantly reduced NF-κB activity in PDAC cells. Given extensive evidence that NF-κB directly regulates SDC4 transcription⁴⁵, these results support a model in which OFD1 modulates SDC4 expression, at least in part, through NF-κB signaling, thereby functionally linking centrosomal protein homeostasis to the regulation of macropinocytic capacity.

Given the role of macropinocytosis in nutrient uptake and tumor growth across diverse solid cancers, it has emerged as a promising target for small-molecule inhibition¹⁸. EIPA, the preclinical gold-standard NHE inhibitor, blocks macropinosome formation via submembranous acidification and RAC1 inhibition, but its limited potency, poor specificity, and unfavorable pharmacokinetics have hindered clinical translation, emphasizing the need for next-generation inhibitors⁴⁶. Here, we demonstrate that OFD1 is a critical regulator of *KRAS*-driven macropinocytosis, highlighting it as a potential alternative node for pharmacological intervention. Here, we show that OFD1 is essential for *KRAS*-driven macropinocytosis, and its poor tractability as a conventional drug target prompted us to conduct a phenotypic protein-level repurposing screen, an approach previously successful for challenging oncogenic regulators such as c-Myc and PCSK9⁴⁷⁻⁵¹. This screen identified digoxin as a potent agent that reduces OFD1 protein abundance. digoxin treatment phenocopied the effects of OFD1 knockdown, suppressing macropinocytosis and PDAC progression *in vitro* and *in vivo* without detectable toxicity. These findings establish OFD1 as a pharmacologically tractable node to modulate macropinocytosis, providing a potential strategy to target this pathway in PDAC⁵²⁻⁵⁴.

Beyond its metabolic role, *OFD1* inhibition also compromises DNA damage repair by downregulating *BRCA1*, effectively inducing a 'BRCAness' state that sensitizes cancer cells to PARP inhibition¹⁹. This dual functionality positions OFD1 as a point of convergence between metabolic adaptation and the DNA damage response. We propose that pharmacological reduction of OFD1 levels simultaneously restricts macropinocytosis and enhances sensitivity to PARP

inhibitors, thereby providing a mechanistically rational framework for combining metabolic and genomic stress in OFD1-high PDAC tumors.

Future studies are needed to elucidate the upstream mechanisms governing *KRAS*-driven OFD1 upregulation, particularly considering the potential contribution of *KRAS*-induced centrosome amplification and centriole duplication⁵⁵. Furthermore, assessing whether OFD1-mediated regulation of macropinocytosis extends to other *KRAS*-driven malignancies could broaden the translational relevance of this pathway and guide the development of more selective OFD1-targeted strategies.

Methods

This study complies with all relevant ethical regulations and gets approved by the Ethics Committee of Shanghai Jiao Tong University School of Medicine.

Cell culture

The human pancreatic cancer cell lines PANC1, MIA PaCa 2, and BXPc3 used in this study were purchased from the American Type Culture Collection (ATCC). The murine KPC1199 cell line was derived from a spontaneous pancreatic ductal adenocarcinoma (PDAC) mouse model (KPC; LSL-*Kras*^{G12D/+}; *Trp53*^{R172H/+}; *Pdx1*-Cre) on a C57BL/6 genetic background⁵⁵, and was kindly provided by Dr. Jing Xue (Renji Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China). HFF⁵⁶ was kindly provided by Dr Yin Jing (Shanghai Jiao Tong University School of Medicine, Shanghai, China). All cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin streptomycin.

Detection and quantification of macropinocytosis

The macropinocytosis index was determined according to the method described in reference⁵⁷. Briefly, cells were seeded in 24-well plates at approximately 50% confluency. After allowing

cells to adhere for 24 hours, serum starvation was performed for 12 hours. To label macropinosomes, cells were incubated with high-molecular-weight TMR-dextran (Molecular Probes, Inc.) at a final concentration of 1 mg/ml in serum-free medium at 37°C for 30 minutes. Following incubation, cells were washed five times with ice-cold PBS and immediately fixed with 4% paraformaldehyde. Nuclei were counterstained with DAPI, and coverslips were mounted onto slides using DAKO mounting medium (DAKO). Images were acquired using a laser scanning confocal microscope (Olympus IX83), and the labeled red macropinosomes were quantified using the "Analyze Particles" function in ImageJ software (NIH, USA). For each sample, at least eight randomly selected fields were analyzed to measure the total area of labeled red macropinosomes per cell.

Subcutaneous Xenograft PDAC Models *in vivo*

All animal experiments were conducted in the SPF-grade animal facility of Shanghai Jiao Tong University School of Medicine, with approval from the Institutional Animal Care and Use Committee. For the subcutaneous xenograft model, 6- to 8-week-old male athymic nude (nu/nu) mice were acclimated in the animal facility for one week. A total of 5×10^6 MIA PaCa2-Tet-on-sh*OFDI* cells suspended in 100 μ L of PBS were subcutaneously injected into the right flank of each mouse. Mice were randomly assigned into four groups: control, sh*OFDI*, and sh*OFDI* with doxycycline treatment (500 mg/kg) administered via doxycycline-supplemented chow. Tumor volume was measured twice weekly and maintained below 2000 mm³. After four weeks of treatment, mice were sacrificed, and tumors were collected and fixed in 4% paraformaldehyde for subsequent histological analysis. The number of animals used in each group is specified in the corresponding figure legends. Tumor volume was calculated using the formula: tumor volume (mm³) = $0.52 \times (\text{length} \times \text{width}^2)$.

***In vivo* assessment of macropinocytosis**

In vivo macropinocytosis was measured following previously reported methods⁵⁷. Subcutaneous xenograft tumors (300-500 mm³) were established in 4-week-old female athymic nude mice. After anesthesia with isoflurane, 100 μ L of high molecular weight FITC-dextran was injected intratumorally using a 28G insulin syringe, and successful delivery was confirmed by

visible fluorescence within the tumor. Mice were allowed to recover for 10-15 minutes. At 30-60 minutes post-injection, mice were euthanized using a low-flow CO₂ chamber followed by cervical dislocation. Tumors were immediately harvested, embedded in O.C.T. compound, snap-frozen on dry ice, and sectioned using a cryostat. Tissue sections were counterstained with DAPI and imaged using a laser scanning confocal microscope (Olympus IX83) to visualize dextran-labeled macropinosomes. Quantification was performed using ImageJ software (NIH, USA).

DIA-Based Proteomic Quantification

Data-independent acquisition (DIA) proteomic analysis was performed by the Core Facility of Basic Medical Sciences, Shanghai Jiao Tong University School of Medicine. Briefly, total proteins were extracted, quantified, and digested with trypsin. Peptides were desalted and subjected to DIA mass spectrometry using a high-resolution LC-MS/MS system. A spectral library was generated from pooled samples using data-dependent acquisition (DDA). DIA data were analyzed with Spectronaut software for protein identification and quantification. Normalized protein intensities were used for subsequent statistical analysis.

Western Blotting

Cultured cells were lysed using RIPA buffer (Pierce, Thermo Fisher Scientific, Cat. #87787), and protein concentrations were determined using a BCA Protein Assay Kit (Pierce, Thermo Fisher Scientific, Cat. #23227). Cell lysates were mixed with 1× SDS loading buffer and boiled at 99°C for 10 minutes. Proteins were separated by SDS-PAGE and transferred onto PVDF membranes. Membranes were blocked with 5% non-fat milk in PBST at room temperature for 1 hour, followed by three washes with PBST. Membranes were then incubated overnight at 4°C with primary antibodies diluted in PBST containing 5% BSA: anti-*OFD1* (1:50,000), anti-SDC4 (Cell Signaling Technology, Cat. #12236, 1:1,000), and anti-β-Actin (Proteintech, Cat. #66009-1-Ig, 1:10,000). After three washes with PBST, membranes were incubated with HRP-conjugated anti-rabbit or anti-mouse secondary antibodies (Jackson ImmunoResearch, Cat. #111-035-003 & #115-035-003, 1:10,000) at room temperature for 1 hour. Signal detection was performed using enhanced chemiluminescence (ECL) reagents and visualized using a ChemiDoc Touch Imaging System (Bio-Rad) or an Odyssey Imaging System. Band intensities were quantified using ImageJ

software (NIH).

Quantitative Real-Time PCR analysis

Total RNA was extracted from cells using the EasyPure Fast Cell RNA Kit (TransGen, Cat. #ER111-02) following the manufacturer's instructions. Complementary DNA (cDNA) was synthesized using the ABScript Neo RT Master Mix for qPCR with gDNA Remover (ABclonal, Cat. #RK20433). All experiments were performed in biological triplicates. Quantitative PCR was carried out using 2X Universal SYBR Green Fast qPCR Mix (ABclonal, Cat. #RK21203) on an Applied Biosystems QuantStudio 5 real-time PCR system. Relative gene expression levels were normalized to the internal control gene ACTB (β -actin). Fold changes in gene expression were calculated using the $2^{-\Delta\Delta C_t}$ method. Primer sequences used in this study are listed in the supplementary materials.

Colony formation assay

Pancreatic cancer cells were seeded into 12-well plates at a density of 2×10^3 cells per well, with the seeding density adjusted based on the proliferative capacity of each cell line. Cells were subjected to drug treatment or genetic manipulation as indicated and maintained in culture for 10 to 14 days, with medium replaced three times per week. Following treatment, cells were fixed with methanol and stained with 0.1% crystal violet prepared in methanol. Representative images of the treated groups were then captured for analysis.

Luciferase reporter assay

MIA PaCa2 cells were cultured in DMEM and transfected with a pGL4.22 vector containing the core promoter of the *SDC4* gene, as previously reported⁴⁵. Briefly, approximately 3×10^5 MIA PaCa2 cells per well were seeded in six-well plates. After 48 h of doxycycline-induced *OFDI* knockdown or DMSO control treatment, cells were transfected with 1 μ g of the promoter reporter plasmid or empty pGL4.22 vector together with 1 μ g of a Renilla luciferase plasmid. Forty-eight hours post-transfection, cells were harvested and luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, E1910) according to the manufacturer's instructions. Firefly and Renilla luciferase activities were normalized to total

protein levels.

Chromatin immunoprecipitation (ChIP)

ChIP assays were performed using the ChIP Assay kit (Beyotime, P2078) according to the manufacturer's instructions. MIA PaCa-2 cells were crosslinked with 1% formaldehyde, quenched with 0.125 M glycine, and lysed. Chromatin was sonicated to 300 – 600 bp fragments and diluted prior to immunoprecipitation with antibodies against IgG or NF- κ B p65 (CST, Cat#8242). Immune complexes were captured with Protein G magnetic beads, sequentially washed, and DNA was eluted and reverse-crosslinked by proteinase K digestion. ChIP DNA was purified using the Active Motif ChIP DNA Clean & Concentrator kit (AM58002) and subjected to SDC4 promoter ChIP-qPCR.

Immunoprecipitation and immunoblotting

For immunoprecipitation assays, cells were lysed in RIPA Lysis Buffer (Millipore, 20-188) supplemented with protease inhibitor cocktail (APExBIO, K1007). Immunoprecipitations were performed using the Anti-Myc nanobody agarose beads (ABclonal, AE106) at 4°C. The immunoprecipitants were washed at least three times in Wash lysis buffer (150 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl, pH7.8, 1% NP-40) supplemented with protease inhibitor cocktail, before being resolved by SDS-PAGE and immunoblotted with indicated antibodies.

Immunohistochemistry

Tissue specimens were fixed in 4% neutral-buffered formaldehyde, followed by standard paraffin embedding. Paraffin-embedded tissues were sectioned at a thickness of 5 μ m. For immunohistochemical analysis, antigen retrieval was performed by incubating the sections in 10 mM citrate buffer (pH 6.0) at 98°C for 15 minutes. After cooling, sections were incubated with blocking solution for 1 hour at room temperature to minimize non-specific binding. Primary antibodies were applied and incubated overnight at 4°C. Specifically, a homemade anti-*OFDI* antibody (1:4000 dilution) and a monoclonal anti-SDC4 antibody (Santa Cruz, Cat# sc-12766, 1:100 dilution) were used. Sections were then incubated with biotinylated goat anti-rabbit IgG, followed by streptavidin – horseradish peroxidase (Biocare Medical) for 10 minutes at room

temperature. Signal was developed using diaminobenzidine (DAB), and sections were counterstained with hematoxylin. DAB-positive staining was quantified using ImageJ software with a custom macro to define and compare positively stained areas.

Compound Screening and Data Analysis:

For chemical screening, bioactive compounds were from FDA-approved Drug Library (Selleck), that comprised about 1430 compounds were used. Potential compounds were screened by western blot assay. PDAC cells were seeded into 12-well plates, and when cell density reached 70%, the compounds were added at the concentration of 10 μ M in the preliminary screening, and through 24 hours of incubation, the cells were collected and lysed. After western blot, the gray values of the internal reference β -Actin and OFD1 bands were counted and converted. Mock-treated (DMSO) cells and *OFD1* shRNA transfected cells with DMSO were included in each gel as standard controls. To exclude compounds possibly acting as cytotoxic drugs at the concentration of 10 μ M, the concentration of compounds was reduced by one order of magnitude, and repeat the screen treatment. All experimental data was analyzed using Image Studio and GraphPad Prism. Normalized Values was then calculated with the gray values using Eq. Normalized Values = [Compound X *OFD1* / Compound X β -Actin] / [DMSO *OFD1*/DMSO β -Actin]. Robust z score = [Log₂ Normalized Values - Average]/Standard deviation.

Bioinformatic analysis

SDC4 expression and its association with patient survival in pancreatic cancer, as well as the correlation between *OFD1* and SDC4 expression, were analyzed using TCGA data via GEPIA (<http://gepia2.cancer-pku.cn/>). In addition, the correlation between SDC4 and *OFD1* expression was further evaluated using the GEO dataset GSE62452 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE62452>).

Acknowledgments

We thank Dr. Zhigang Zhang for providing the LSL-KrasG12D/+; p53R172H/+; Pdx1-Cre (KPC) genetically modified mice. This work was supported, in part, by the National Key Research and Development Program of China (2023YFA0914900) to Q.Z., National Natural

Science Foundation of China (W2511018 , 32530055 , 92254307) to Q.Z., National Natural Science Foundation of China (82473225, 32070741) to Z.T., the National Natural Science Foundation of China (82504055) to P.L., Shanghai Municipal Health Commission Seed Program for Research and Translation of Medical Technologies (2024ZZ1031) to Q.Z., the Shanghai Frontiers Science Center of Cellular Homeostasis and Human Diseases; the Fundamental Research Funds for the Central Universities, and Shanghai Science and Technology Commission (20JC1410100).

Author contributions

Q.Y. and P. L. started the project and performed most of the experiments; H.L., J.Y. and W.N. contributed with animal experiments and PDAC tumor sample preparation; P.L., and Q.Y., Z.T. and Q.Z. edited the manuscript; P. L., Z.T. and Q.Z conceived the project, designed the experiments, analyzed the data and wrote the manuscript with the help of all authors. All authors discussed the results and commented on the manuscript.

Competing Interests Statement

The authors declare no competing interests.

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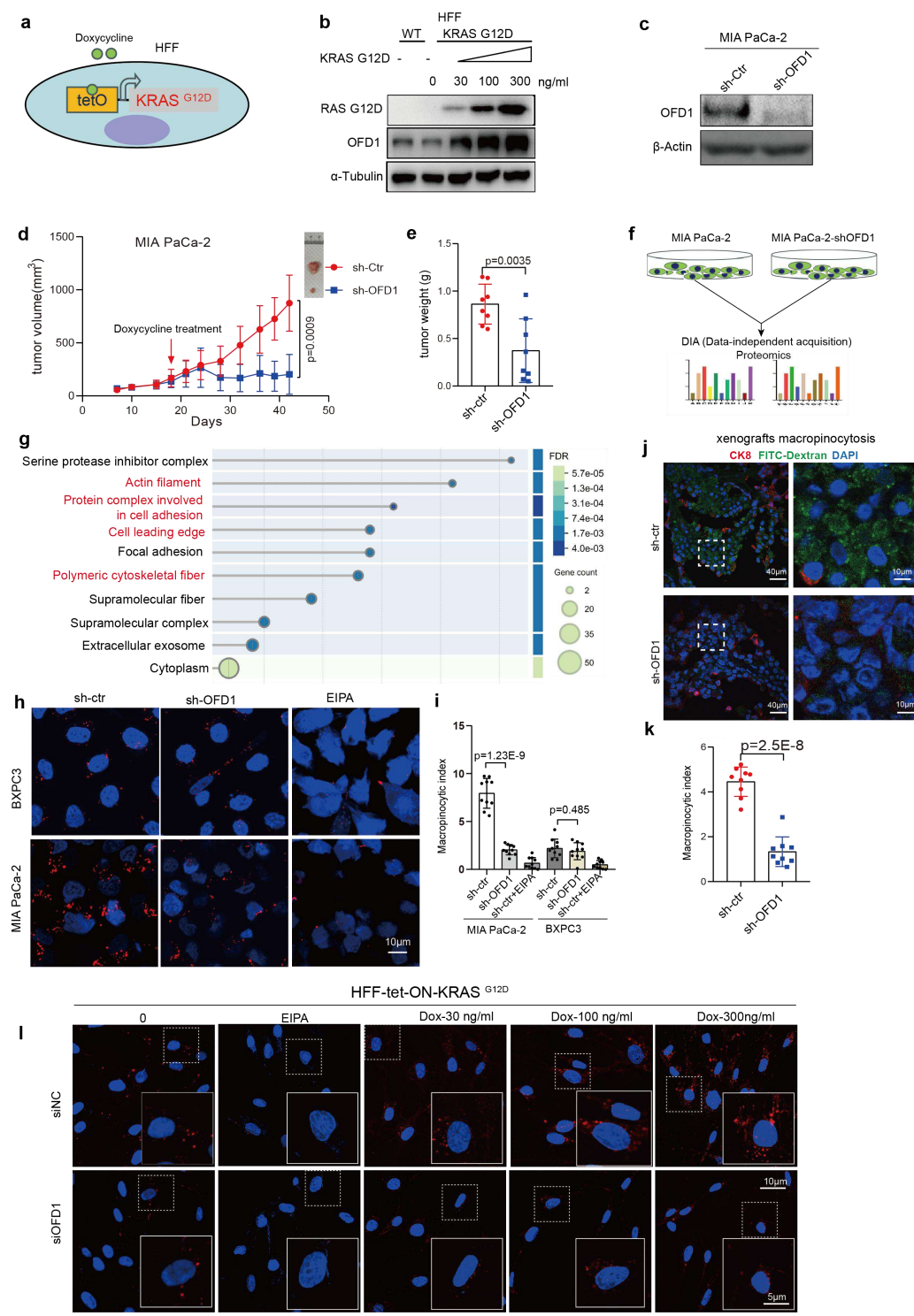


Fig. 1| OFD1 regulates KRAS-driven macropinocytosis and PDAC progression.

a. Schematic of the engineered HFF (human foreskin fibroblast) line stably expressing a doxycycline-inducible *KRAS*^{G12D} on/off system.

b. Western blot analysis of OFD1 and *RAS*^{G12D} expression in HFF cells treated with increasing concentrations of doxycycline (0, 30, 100, 300 ng/ml).

682 **c.** Western blot validation of shRNA-mediated *OFD1* knockdown in MIA PaCa-2 cells.

683 **d-e.** Effect of *OFD1* knockdown on tumor growth in MIA PaCa-2 xenografts. Doxycycline was
684 administered when tumors reached approximately 100 mm³. Tumor volumes were monitored twice
685 weekly (**d**). and tumor weights were measured at the experimental endpoint (**e**).

686 **f.** Workflow schematic of data-independent acquisition (DIA)-based quantitative proteomic analysis
687 performed in MIA PaCa-2 cells following *OFD1* knockdown.

688 **g.** Gene Ontology (GO) cellular component enrichment analysis of downregulated proteins upon
689 *OFD1* knockdown, determined using STRING functional protein association networks.

690 **h-i.** Effect of *OFD1* knockdown on macropinocytosis in BXPC-3 and MIA PaCa-2 cells. **i.**
691 Representative images of macropinocytosis visualized by TMR-dextran uptake; EIPA was included
692 as a negative control. **j.** Quantification of the macropinocytosis index across treatment groups (10
693 randomly selected fields per group, n = 10). Statistical significance was evaluated using a two-tailed
694 Student's t-test.

695 **j-k.** *In vivo* macropinocytosis assay in MIA PaCa-2 xenografts following *OFD1* knockdown. **j.**
696 Representative confocal images showing FITC-dextran uptake in tumor sections, with CK8 staining
697 marking tumor cells. Insets show magnified regions (scale bars, 40 μm for main images; 10 μm for
698 insets). **k.** Quantification of macropinocytosis index across groups (n > 10 fields). Statistical
699 significance was assessed using a two-tailed Student's t-test.

700 **l.** Representative fluorescence images of macropinocytosis in HFF cells cultured with doxycycline (0,
701 30, 100, or 300 ng ml⁻¹) for 48 h to induce *KRAS*^{G12D}, before and after siRNA-mediated *OFD1*
702 knockdown (scale bars, 10 μm; magnified view, 5 μm).

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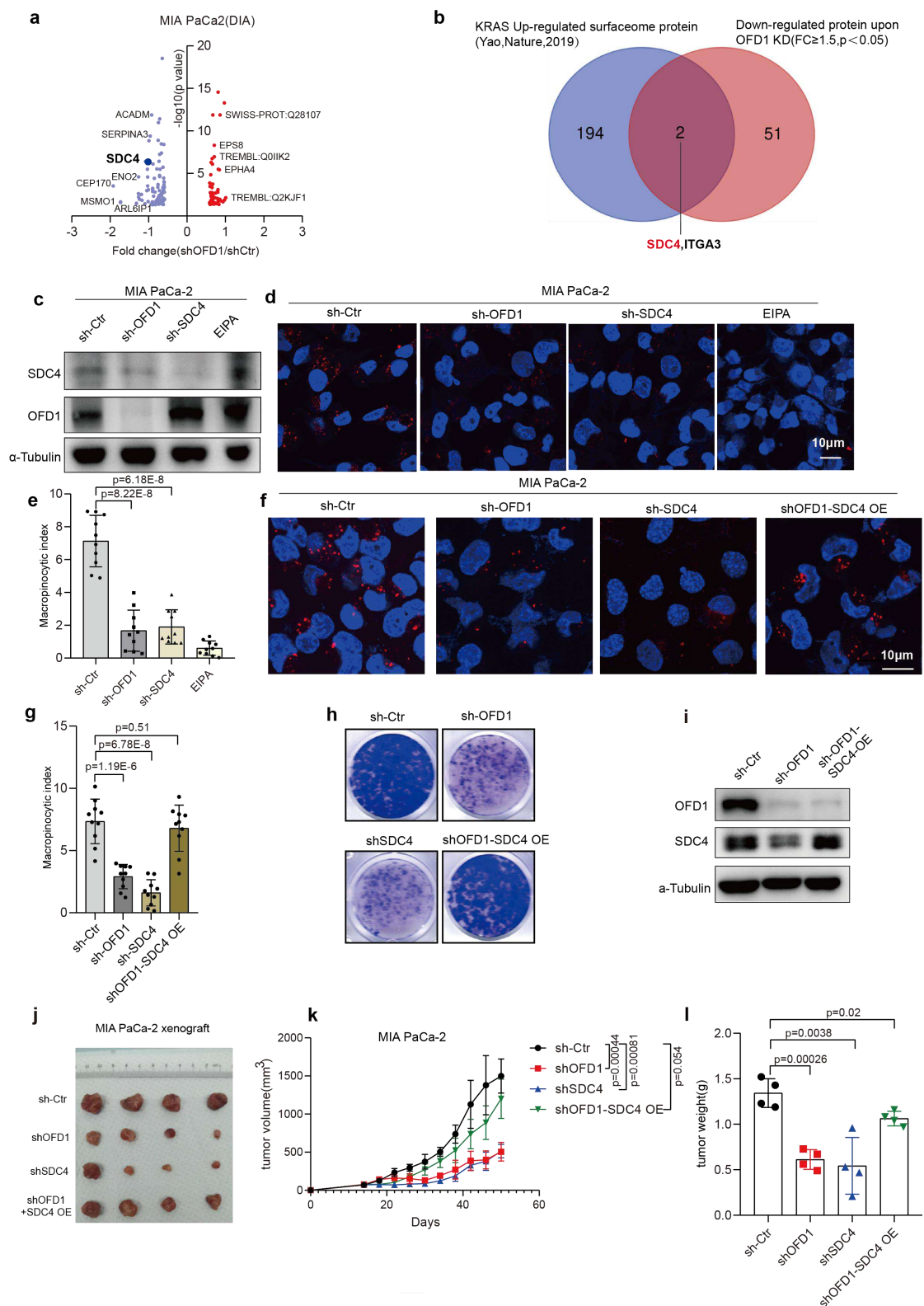
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714 **Fig. 2| OFD1 regulates macropinocytosis in a Syndecan-4-dependent manner.**

715 **a.** Volcano plot depicting differentially expressed proteins identified by data-independent
716 acquisition (DIA)-based quantitative proteomic analysis of MIA PaCa-2 cells following *OFD1*

knockdown.

b. Venn diagram showing the overlap between *KRAS*-upregulated surfaceome proteins (Yao et al., Nature 2019) and proteins downregulated upon *OFD1* knockdown (fold change ≥ 1.5 , $P < 0.05$), highlighting two overlapping hits: SDC4 and ITGA3.

c-e. Western blot analysis and macropinocytosis assay in MIA PaCa-2 cells following shRNA-mediated *OFD1* knockdown, SDC4 knockdown, or EIPA treatment (**c**). Representative images of TMR-dextran uptake are shown (**d**), and quantification of the macropinocytosis index is presented (**e**). Macropinocytosis was quantified from 10 randomly selected fields per group ($n = 10$). Statistical significance was determined using a two-tailed Student's t-test.

f-g. Restoration of SDC4 rescues the reduced macropinocytosis by *OFD1* knockdown in MIA PaCa-2 cells. Representative fluorescence images showing TMR-dextran uptake in the indicated treatment groups (**f**; scale bars: 10 μm) and corresponding quantification of macropinocytosis index (**g**; $n = 10$ random areas; data are mean $\pm$ SD). Data are representative of three independent experiments.

h-l. Restoration of SDC4 rescues the reduced tumorigenicity induced by *OFD1* knockdown in MIA PaCa-2 cells. **h.** Colony formation assay of MIA PaCa-2 cells under the following conditions: sh-Ctr control, *OFD1* knockdown, SDC4 knockdown, and *OFD1* knockdown rescued with SDC4 overexpression. **i.** Immunoblot analysis of *OFD1* and SDC4 protein levels in MIA PaCa-2 cells infected with scrambled shRNA (sh-Ctr), sh-*OFD1*, or sh-*OFD1* in combination with SDC4 overexpression. **j.** Representative tumors image at the experimental endpoint are shown. Subcutaneous xenograft experiments in nude mice implanted with MIA PaCa-2 cells corresponding to the treatment groups in panel. **k.** Tumor volumes were monitored over time in each treatment group. **l.** tumor weights were measured at the experimental endpoint. Statistical significance was assessed using a two-tailed Student's t-test.

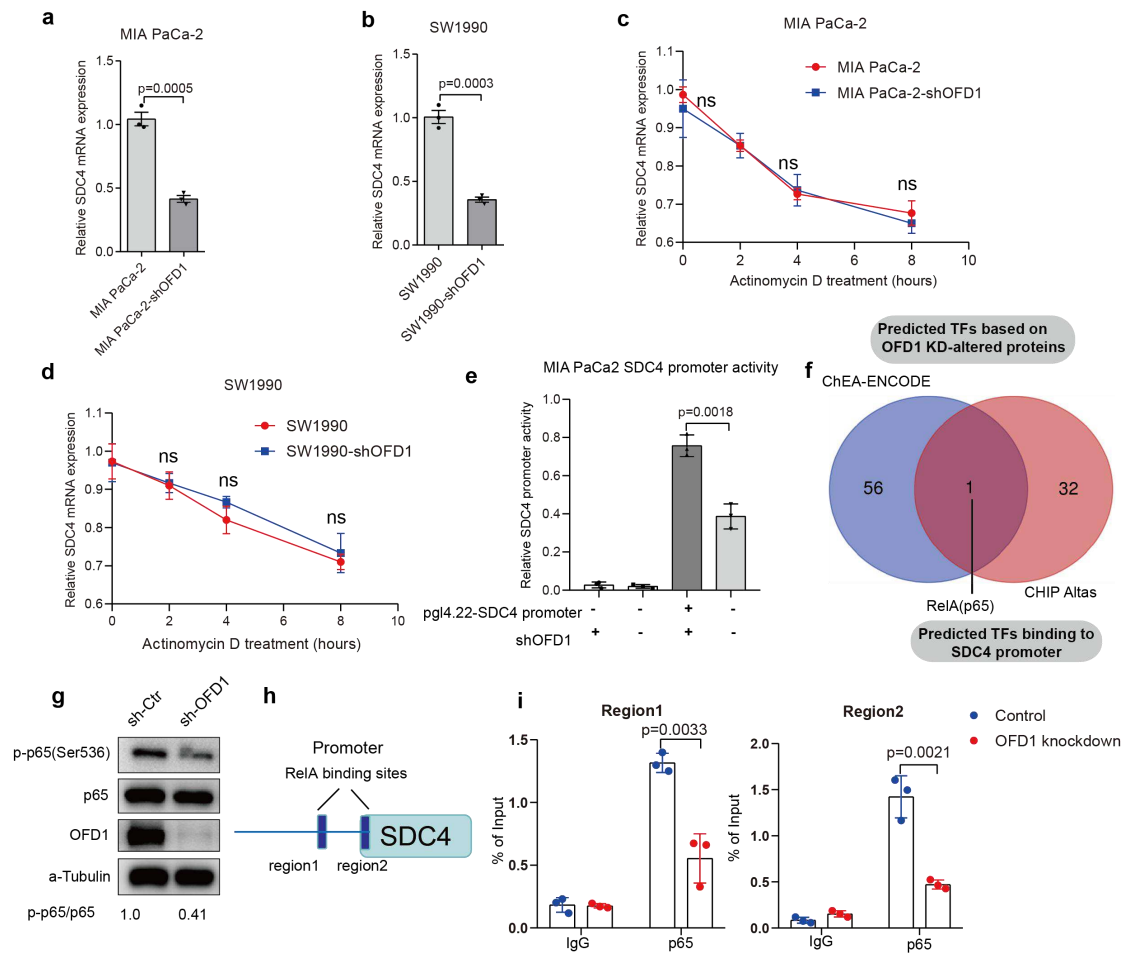


Fig. 3| OFD1 modulates NF- κ B activity to control SDC4 transcription.

a-b. Quantitative PCR analysis of *SDC4* mRNA levels in MIA PaCa-2 (**a**) and SW1990 (**b**) cells following *OFD1* knockdown. Data are presented as mean $\pm$ SD from three independent experiments. Statistical significance was assessed using a two-tailed Student's t-test.

c-d. Assessment of *SDC4* mRNA stability in MIA PaCa-2 (**c**) and SW1990 (**d**) cells with or without *OFD1* knockdown after treatment with the transcription inhibitor actinomycin D (5 μ g mL⁻¹). $P < 0.05$; ns, not significant.

e. Luciferase reporter assay of *SDC4* promoter activity in MIA PaCa-2 cells. Cells were transfected with the pGL4.22-*SDC4* promoter-luciferase reporter or empty vector and subjected to doxycycline-induced *OFD1* knockdown for 48 h. Luciferase activity was measured using a dual-luciferase system, with Renilla luciferase as the reference control. Data are presented as mean $\pm$ SD ($n = 3$), and statistical significance was determined by two-tailed Student's t-test.

f. Venn diagram summarizing the integrative bioinformatic analysis to identify transcription factors (TFs) regulating *SDC4*. Proteins significantly downregulated upon *OFDI* knockdown in MIA PaCa-2 cells were overlapped with TFs predicted by ChEA3 (adjusted $P \leq 0.01$) and ChIP-Atlas (adjusted $P \leq 0.01$) to bind the *SDC4* promoter. NF- κ B subunit p65 was identified as a candidate regulator.

g. Immunoblot analysis of total p65 and phosphorylated p65 (p-p65) protein levels in MIA PaCa-2 cells following *OFDI* knockdown.

h. Schematic of two predicted p65 binding sites within the *SDC4* promoter region. Region1(-209 ~-137) ,Region2(72 ~171)

i. Chromatin immunoprecipitation (ChIP) assay showing p65 binding to the two promoter sites in MIA PaCa-2 cells upon *OFDI* knockdown.

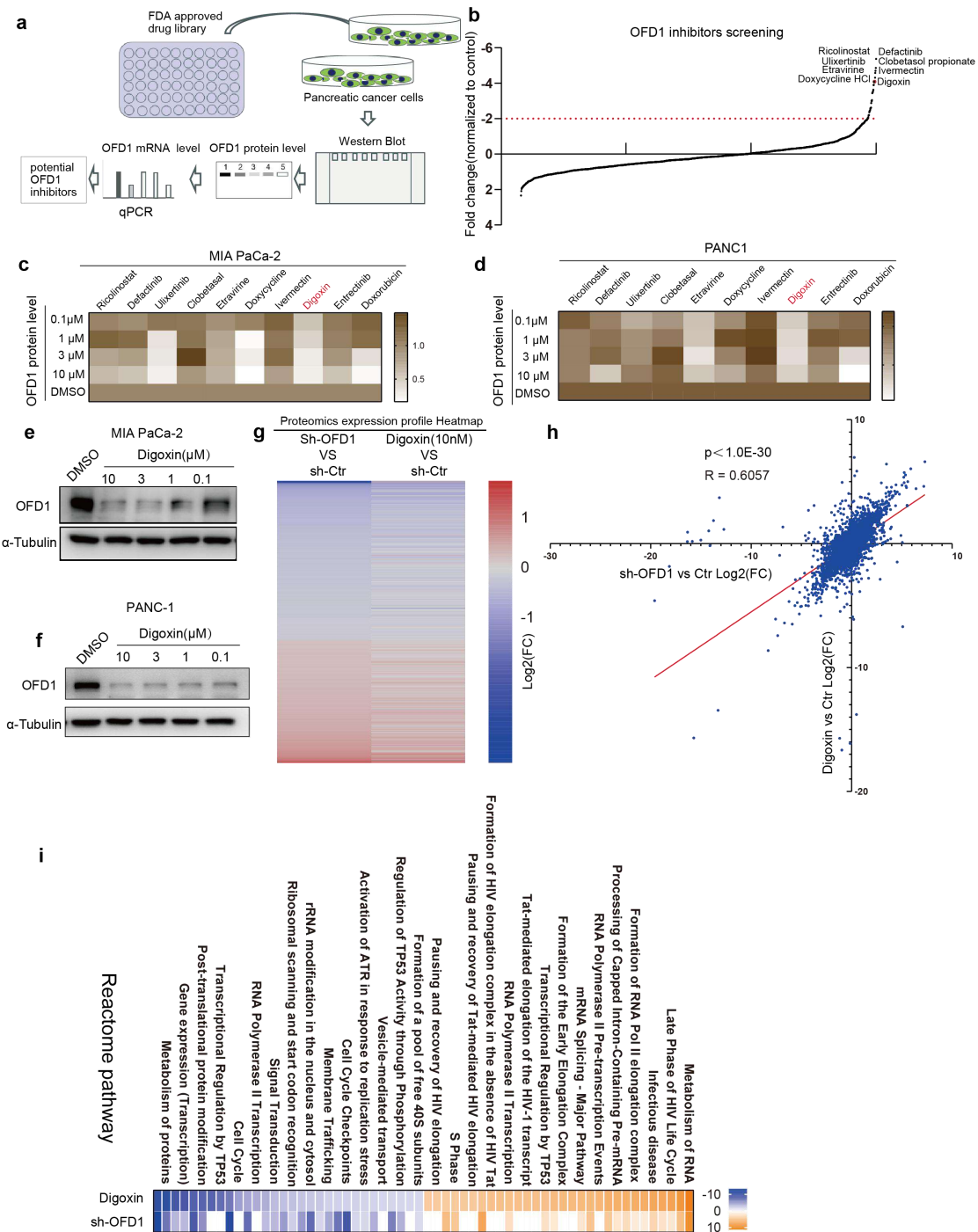


Fig. 4| Small-molecule screening identifies digoxin as a compound reducing OFD1 protein

levels.

a-b. Screening of FDA-approved small-molecule compounds for their ability to reduce OFD1 protein levels in MIA PaCa-2 cells. **a.** Representative immunoblots showing OFD1 protein levels. **b.** Quantification of OFD1 fold-change under each drug treatment, normalized to untreated controls. Data are presented as mean $\pm$ SD from three independent experiments.

c. Heat map summarizing Western blot-based quantification of OFD1 protein reduction following 24 h treatment with the top 10 hit compounds at the indicated concentrations (10 μ M, 3 μ M, 1 μ M, and 0.1 μ M) in MIA PaCa2 cells.

d. Heat map summarizing Western blot-based quantification of OFD1 protein reduction following 24 h treatment with the top 10 hit compounds at the indicated concentrations (10 μ M, 3 μ M, 1 μ M, and 0.1 μ M) in PANC1 cells.

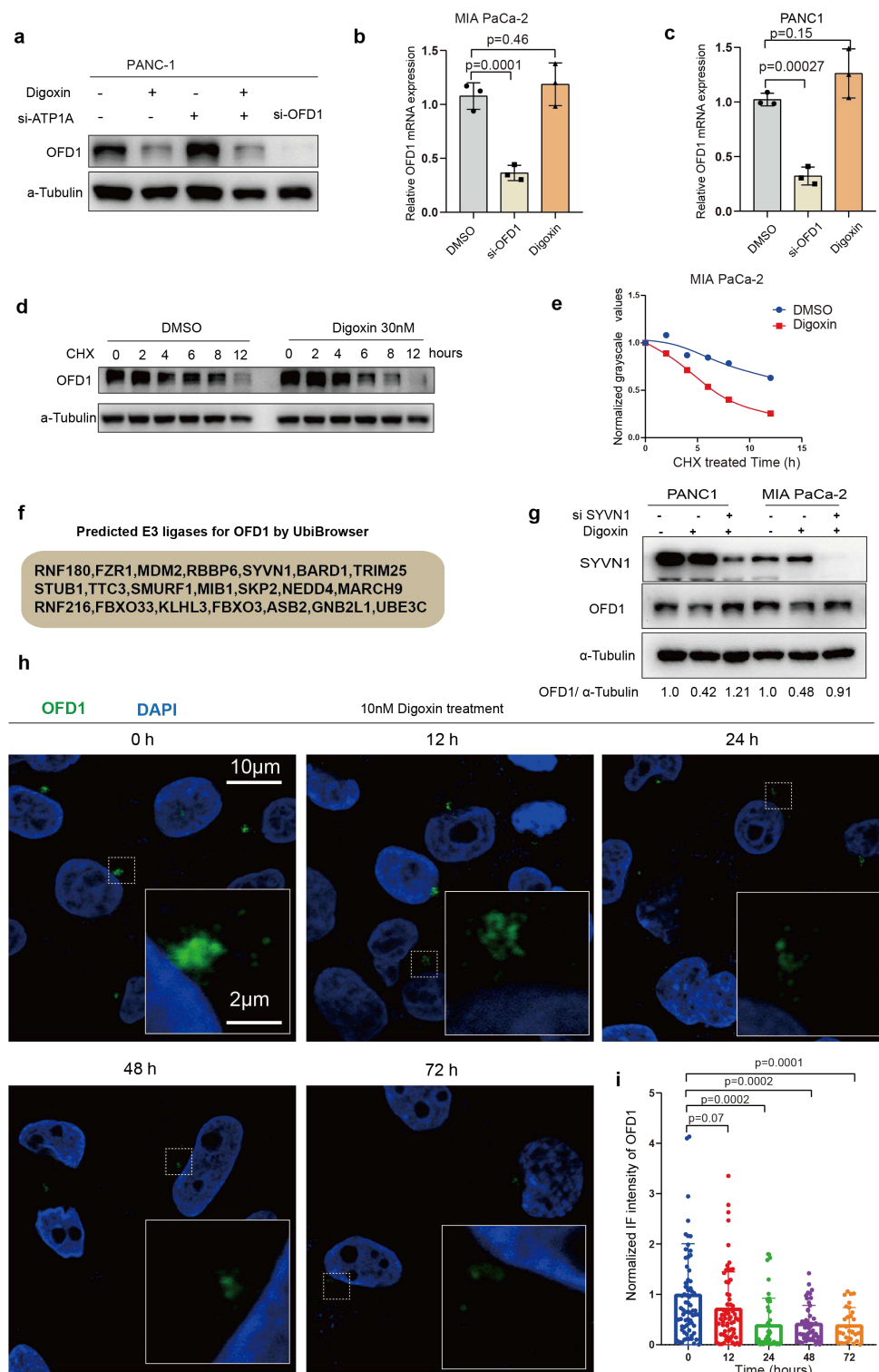
e. Immunoblot analysis of OFD1 protein levels in MIA PaCa-2 cells treated with increasing concentrations of digoxin (0.1, 1, 3, or 10 μ M) for 24 h. α -Tubulin served as a loading control.

f. Immunoblot analysis of OFD1 protein levels in PANC-1 cells following shRNA-mediated *OFDI* knockdown or treatment with 0.1 μ M Digoxin for 24 h. α -Tubulin served as a loading control.

g. Heatmap showing proteomic expression profiles in PANC-1 cells treated with Digoxin(10 nM for 72 h) versus vehicle, alongside the profile observed in shRNA-mediated *OFDI* knockdown versus control.

h. Pearson correlation analysis of protein expression changes between Digoxin-treated and vehicle-treated cells and between shRNA-mediated *OFDI* knockdown and control. Correlation coefficient (R) and *P*-value are indicated.

i. Heatmap of Reactome pathway analysis highlighting pathways altered in digoxin-treated versus vehicle-treated PANC-1 cells and in shRNA-mediated OFD1 knockdown versus control cells.



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832 **Fig. 5| Digoxin reduces OFD1 protein stability in a SYVN1-dependent manner in PDAC**
833 **cells.**

834 **a.** Immunoblot analysis of OFD1 protein levels in PANC1 cells treated with 0.1 μ M Digoxin

following siRNA-mediated knockdown of ATP1A. α -Tubulin was used as a loading control.

b-c. Quantitative PCR analysis of *OFD1* mRNA levels in MIA PaCa-2 (**b**) and PANC-1 (**c**) cells after treatment with 0.1 μ M Digoxin for 24 h. siOFD1 was included as a positive control. Data are presented as mean $\pm$ SD from three independent experiments.

d-e. Analysis of OFD1 protein stability in MIA PaCa-2 cells. Cells were pretreated with 30 nM Digoxin or DMSO for 12 h, followed by cycloheximide (CHX, 20 μ g mL⁻¹) treatment for 0, 2, 4, 6, 8, and 12 h. (**d**) Representative immunoblots showing OFD1 protein levels. (**e**) Quantification of OFD1 levels normalized to α -Tubulin and plotted as a degradation curve, demonstrating accelerated protein turnover upon digoxin treatment.

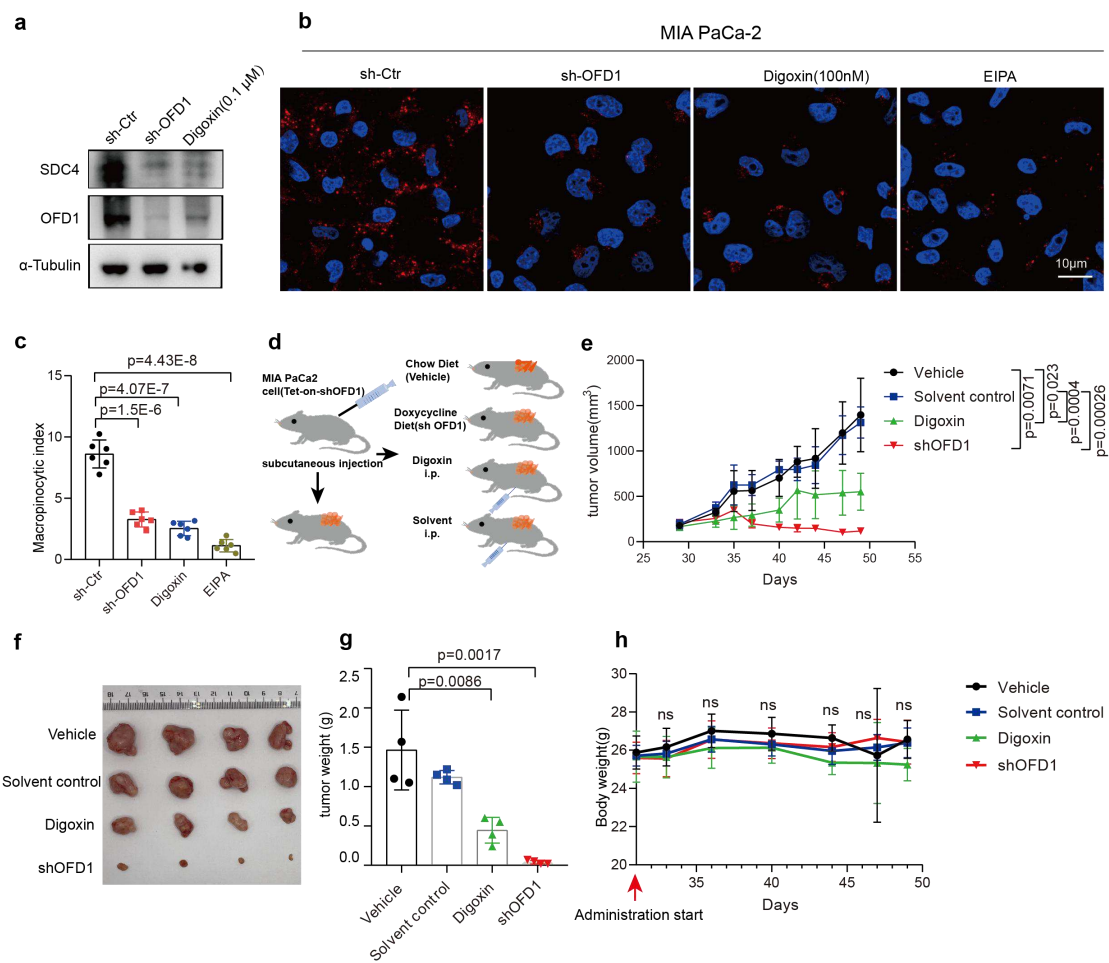
f. Predicted E3 ligase gene list for OFD1 based on the UbiBrowser database.

g. Western blot analysis showing the protein levels of OFD1 and SYVN1 in PANC1 and MIA PaCa-2 cells following digoxin treatment combined with SYVN1 knockdown by siRNA.

h. Immunofluorescence analysis of OFD1 in MIA PaCa-2 cells treated with 10 nM Digoxin for 0, 12, 24, 48, and 72 h. Representative images show a progressive decrease in OFD1 signal intensity with prolonged Digoxin exposure (scale bar, 10 μ m).

i. Quantification of OFD1 fluorescence intensity at the indicated time points. Data represent mean $\pm$ SD from at least 30 cells per condition. Statistical significance was determined using an unpaired two-tailed Student's t-test.

Fig. 6



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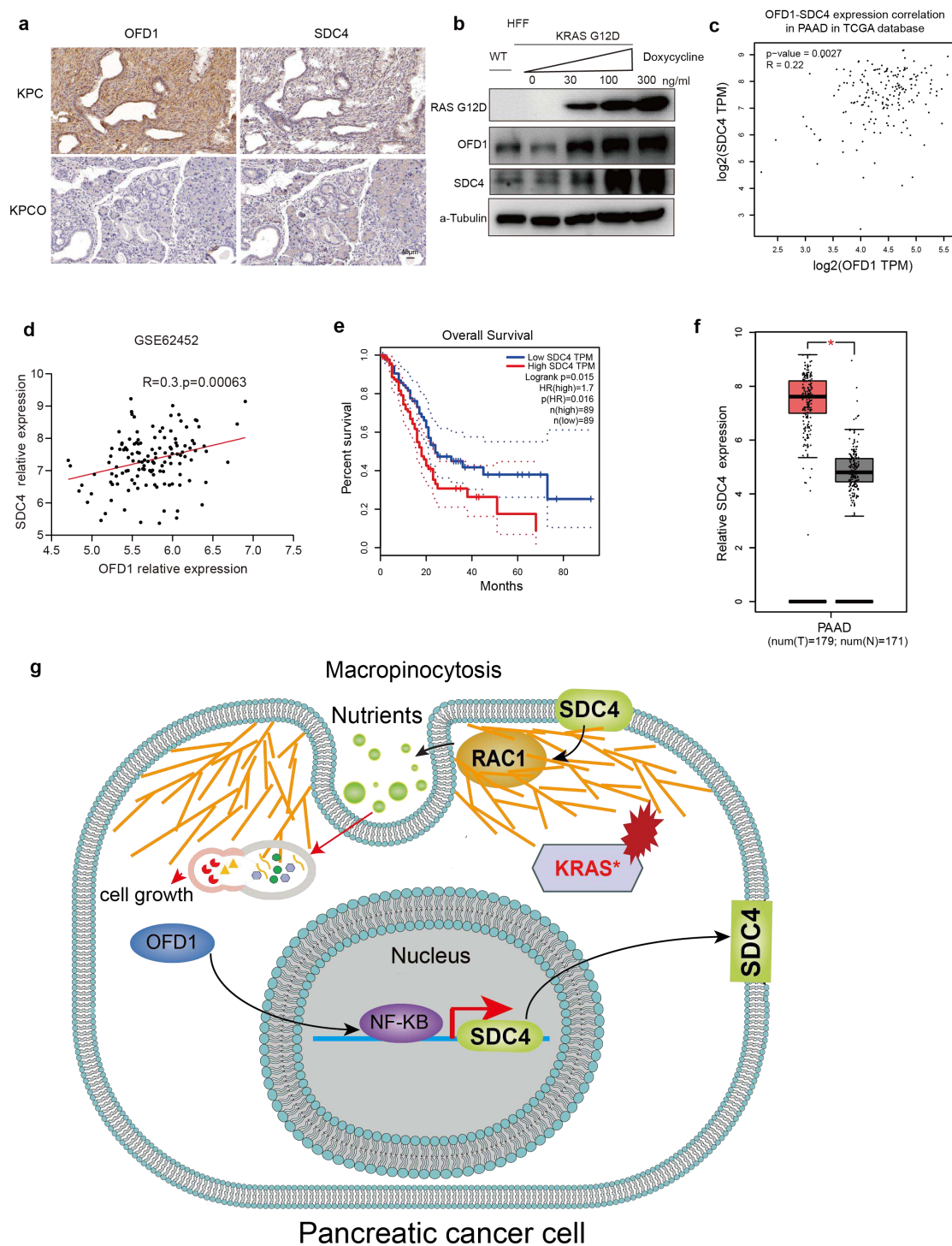
Fig. 6| Digoxin mimics OFD1 depletion to suppress macropinocytosis and PDAC growth.

a. Immunoblot analysis of OFD1 and SDC4 protein levels in MIA PaCa-2 cells subjected to shRNA-mediated *OFD1* knockdown or treatment with 0.1 μM Digoxin for 24 h. α-Tubulin was used as a loading control.

b-c. Macropinocytosis assay in MIA PaCa-2 cells following shRNA-mediated *OFD1* knockdown or treated with 0.1 μM digoxin for 24 h.(b) Representative fluorescence images showing TMR-dextran uptake. (c) Quantification of the macropinocytosis index from 10 randomly selected fields per group (n = 10). Statistical significance was assessed using an unpaired two-tailed Student's t-test.

d-h. *In vivo* evaluation of the effects of OFD1 knockdown and digoxin treatment in MIA PaCa-2 xenograft models. **d.** Schematic overview of the experimental design. Tet-on shOFD1 MIA PaCa-2 cells were subcutaneously implanted into nude mice, followed by treatment with control chow, doxycycline-containing chow to induce shOFD1 expression, oral gavage of digoxin, or

solvent control, as indicated. **e.** Tumor growth curves over time (mean $\pm$ SD). Statistical significance was determined by two-way ANOVA. **f.** Representative images of excised tumors from each treatment group at the experimental endpoint. **g.** Tumor weights measured at endpoint; statistical significance was assessed using one-way ANOVA. **h.** Body weight changes over time (mean $\pm$ SD). No significant differences were observed among groups (ns), as determined by two-way ANOVA. Red arrows indicate the initiation of treatments.



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913 **Fig. 7| OFD1 expression correlates with SDC4 levels and poor patient survival in PDAC.**

914 **a.** Representative IHC staining for OFD1 (anti-OFD1 antibody) and SDC4 in pancreatic tumors

915 from KPC (LSL-*Kras*^{G12D/+}; *Trp53*^{R172H/+}; *Pdx-1*-Cre) and KPCO (LSL-*KRAS*^{G12D/+}; *Trp53*^{R172H/+};

916 *Pdx-1*-Cre; *Ofd1*-null allele) mice, Scale bar ,20 μ m.

917 **b.** Immunoblot analysis of OFD1 and SDC4 protein levels in HFF human foreskin fibroblasts

918 harboring a doxycycline-inducible *KRAS*^{G12D} on/off system, treated with increasing concentrations
919 of doxycycline (0, 30, 100, 300 ng/ml).

920 **c-d.** Pearson correlation analysis between OFD1 and SDC4 expression in pancreatic cancer tissues
921 from the TCGA dataset (**c**) and GEO dataset GSE62452 (**d**), demonstrating a positive association

922 **e.** Kaplan-Meier survival analysis showing the association between SDC4 expression (high versus
923 low) and overall survival in patients with pancreatic cancer based on TCGA data.

924 **f.** Comparison of SDC4 expression levels in tumor (n = 179) versus normal (n = 171) pancreatic
925 samples from TCGA dataset. Statistical significance was assessed by a two-tailed Student's t-test.

926 **g.** Schematic model illustrating that targeting *OFD1* suppresses *KRAS*-driven pancreatic cancer
927 progression by attenuating macropinocytosis through downregulation of SDC4.

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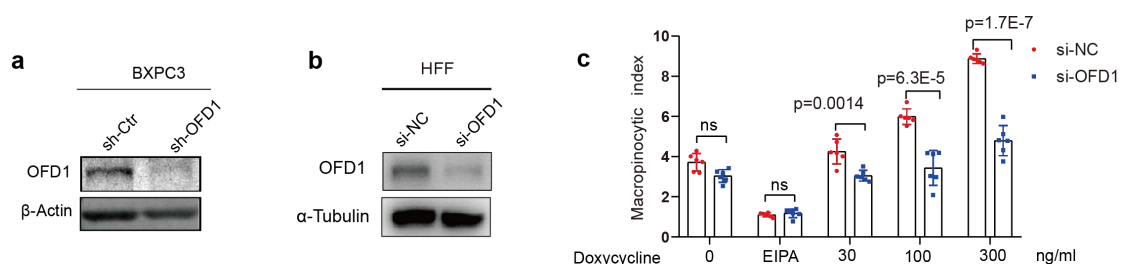
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Supplementary Fig. 1



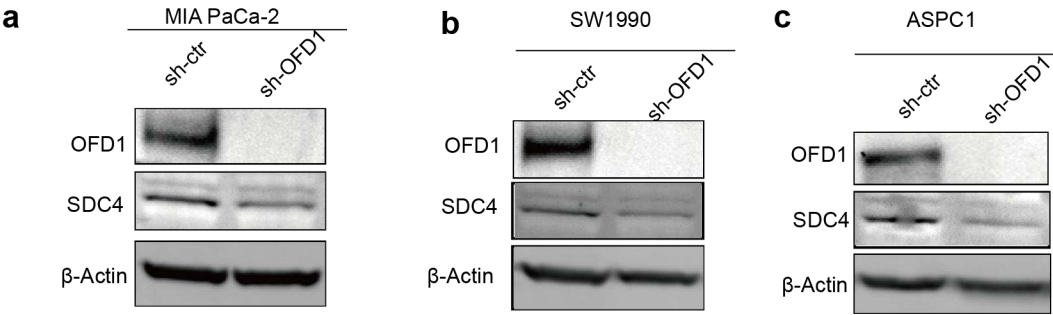
Supplementary Fig. 1| OFD1 facilitates *KRAS*^{G12D} induced macropinocytosis.

a. Western blot validation of shRNA-mediated OFD1 knockdown in BXPC3 cells.

b. Immunoblot analysis of OFD1 protein levels following siRNA-mediated *OFD1* knockdown in HFF cells.

c. Quantification of macropinocytosis in HFF cells cultured with doxycycline (0, 10, 100, or 300 ng/ml) for 24 h to induce *KRAS*^{G12D}. Statistical significance was determined using a two-tailed Student's t-test.

Supplementary Fig. 2

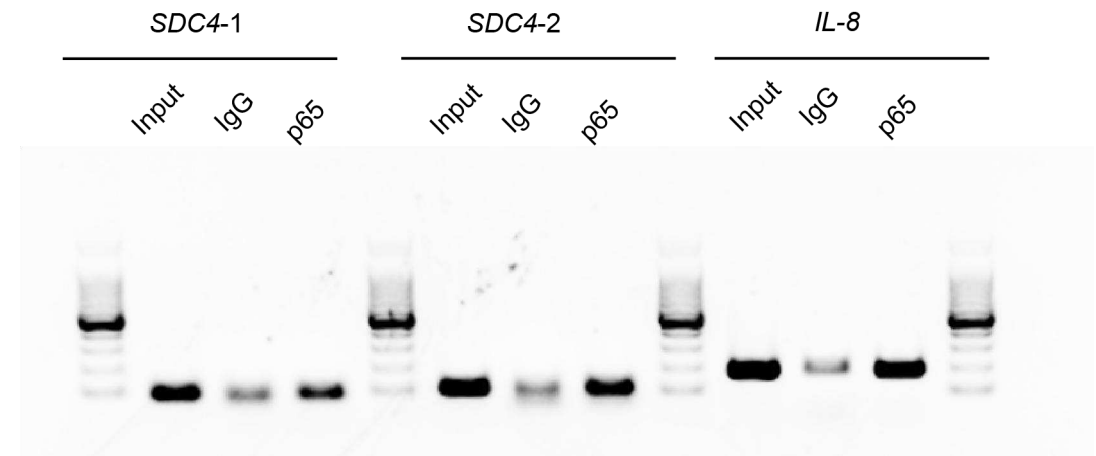


Supplementary Fig. 2| OFD1 regulates SDC4 expression in MIA PaCa-2 cells.

a-c. Immunoblot analysis of OFD1 and SDC4 protein levels in MIA PaCa-2(**a**) ,SW1990(**b**) and ASPC1(**c**)cells infected with scrambled shRNA (sh-Ctr), sh-*OFD1*.

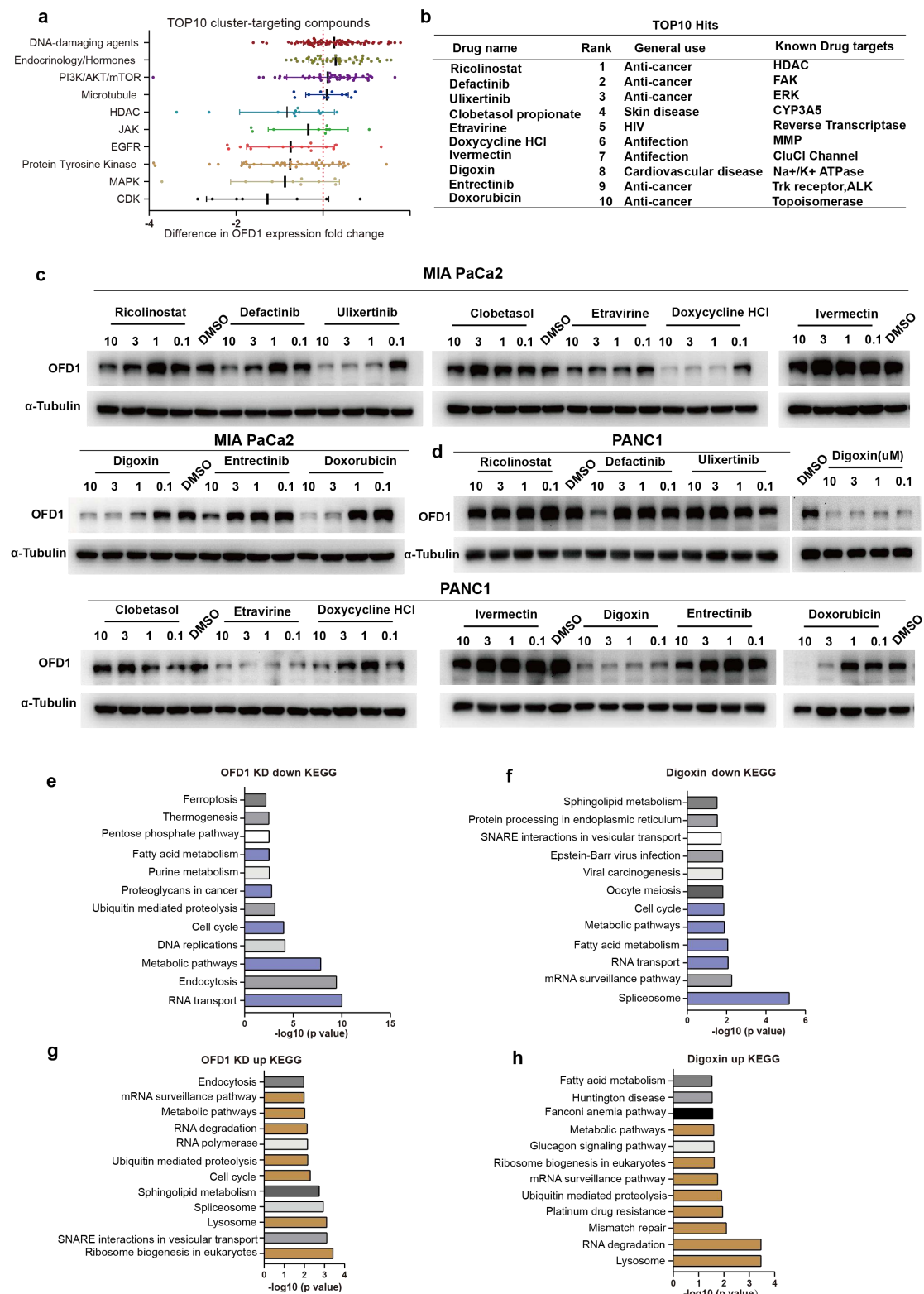
Supplementary Fig. 3

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Supplementary Fig. 3| OFD1 regulates SDC4 expression through NF- κ B-dependent promoter binding in PDAC.

a. Representative agarose gel showing chromatin immunoprecipitation (ChIP) assay results for NF- κ B p65 binding to two predicted NF- κ B binding sites (sites 1 and 2) within the SDC4 promoter in MIAPaCa-2 cells. Input DNA, IgG immunoprecipitation, and p65 immunoprecipitation are shown. The IL-8 promoter was used as a positive control for p65 binding.



Supplementary Fig. 4 | Compound screening prioritized digoxin as a functional mimic of OFD1 depletion.

a. Distribution of OFD1 expression fold changes across ten classes of pathway-targeting

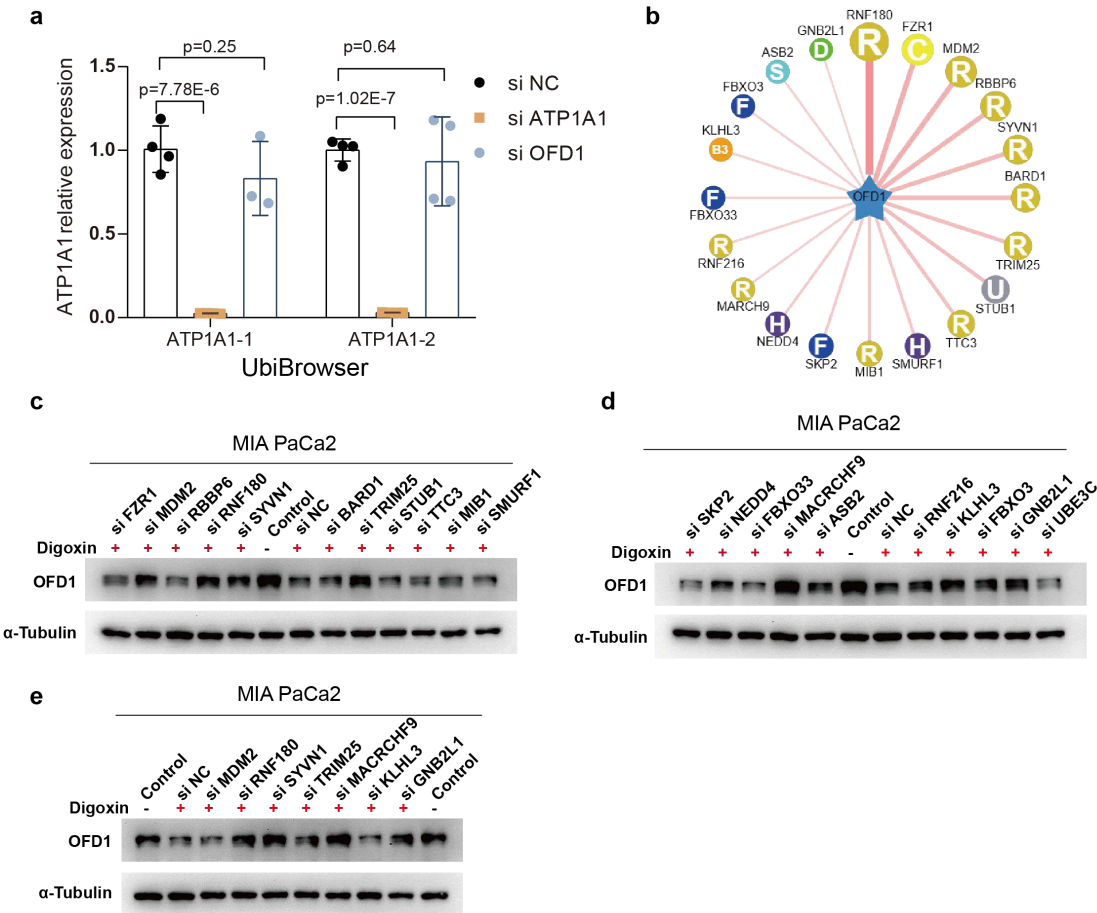
compounds. Each dot represents an individual compound; error bars indicate mean $\pm$ SD. The red dashed line denotes no change in OFD1 expression.

b. Top 10 hits from the drug-repurposing screen targeting OFD1, including compound name, common clinical use, and known molecular targets.

c-d. Immunoblot analysis of OFD1 protein levels in MIA PaCa-2 (**c**) and PANC1 (**d**) cells following 24 h treatment with the indicated compounds at concentrations of 10 μ M, 3 μ M, 1 μ M, or 0.1 μ M: Ricolinostat, Defactinib, Ulixertinib, Clobetasol propionate, Etravirine, Doxycycline HCl, Ivermectin, Digoxin, Entrectinib, and Doxorubicin.

e-h. Bar plots showing KEGG pathways significantly downregulated in PANC-1 cells upon OFD1 knockdown (**e**) or digoxin treatment (10 nM for 72 h) (**f**). Pathways highlighted in the same color indicate those commonly downregulated under both conditions; Bar plots showing KEGG pathways significantly upregulated in PANC-1 cells upon OFD1 knockdown (**g**) or digoxin treatment (0.1 μ M for 24 h) (**h**). Pathways highlighted in the same color indicate those commonly upregulated under both conditions.

Supplementary Fig. 5



Supplementary Fig. 5 | Digoxin induces SYVN1-dependent OFD1 degradation via a Na^+/K^+ ATPase - independent mechanism.

a. Quantitative PCR analysis of *ATP1A1* mRNA levels in MIA PaCa2 cells transfected with siATP1A1 or siOFD1. Data represent mean $\pm$ SD from three independent experiments. Statistical significance was assessed using a two-tailed Student's t-test.

b. Predicted E3 ligase gene list for OFD1 based on the UbiBrowser database.

c-e. Western blot analysis showing the protein levels of OFD1 and predicted E3 ligases in MIA PaCa-2 cells after digoxin treatment in combination with siRNA-mediated knockdown of the indicated E3 ligases.

1028 **Supplementary Table 1. Primers used in this study**

qPCR-SDC4-F	5'-GACGATGAGGATGTAGTGGGG-3'
qPCR-SDC4-R	5'-ACAACTTCAGGGCCGATCAT-3'
qPCR- <i>OFD1</i> -F	5'-AACCTGCGTCTCCTAAACCG-3'
qPCR- <i>OFD1</i> -R	5'-AGCCTTTTCGGCTTTCCGTA-3'
qPCR-ACTB-F	5'-ACTGGCATCGTGATGGACTC-3'
qPCR-ACTB-R	5'-AGGTAGTCAGTCAGGTCCCG-3'
ChIP-SDC4-promoter-F1	5'-AGTTGGAAACCCCAAGGGAC-3'
ChIP-SDC4-promoter-R1	5'-CGGAGGGTAGGACTAGGGG-3'
ChIP-SDC4-promoter-F2	5'-TCCGACGAACAAAGGAGGC-3'
ChIP-SDC4-promoter-R2	5'-GTTCTTCGTAGGCGGAGTCG-3'
Luciferase-SDC4-promoter-F	5'-TTCACCTCTCTGGCTCAAGCAGT-3'
Luciferase -SDC4-promoter-R	5'-TGGCACCGCGGACTGGAGAAG-3'
ATP1A-siRNA-sense	5'-UGAUGAACUUCAUCGUAAATT-3'
ATP1A-siRNA-anti-sense	5'-UUUACGAUGAAGUUCAUCAAG-3'
Sh-OFD1-sense	5'-GAACGAAGAGAACTAGAAA-3'
Sh-SDC4-sense	5'-GCCAGGTTCTTCTTGAGC-3'