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Translational reprogramming drives chromosomal instability during tumorigenesis

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Author contributions

J.V.M. and N.J.C. contributed to conceptualization. J.V.M., K.M.W., E.P.K., E.H.M, S.W., K.P., M.P., M.K.J. and K.G.M. contributed to data curation, validation, investigation. J.V.M, E.P.K., S.B., K.M.F., A.S.T., T.D.J., N.T., Y.F., J.A. and M.W. contributed to formal analysis, methodology and visualization. A.P.B., K.J.S., C.P.D., D.S.L., W.A.P., L.F. and N.J.C. provided resources. A.S.T., W.A.P., L.F. and N.J.C. supervised the study. J.V.M. wrote the original draft. J.V.M. and N.J.C. edited the manuscript. All authors reviewed the manuscript.

Declaration of interests

J.A. and M.W. are employees of Astex Pharmaceuticals.

Ethics statement

All animal experiments were approved by the Peter MacCallum Cancer Centre (PMCC) Animal Ethics Committee and undertaken in accordance with the National Health and Medical Research Council (NHMRC) Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Written informed consent was obtained from patients and the collection and use of tissue for this study as approved by the Peter MacCallum Cancer Centre Human Research Ethics Committee (Project #HREC/44873/PMCC-2018).

Abstract

Chromosomal instability (CIN) is a defining hallmark of cancer that drives genomic evolution, yet the molecular mechanisms triggering CIN during early tumorigenesis remain poorly defined. Here, we identify translation as a critical gatekeeper of chromosomal integrity during tumor initiation. Using models of tumorigenesis driven by SMAD4 loss, we integrate CRISPR-Cas9 screening, polysome profiling, and *in vivo* approaches to demonstrate that this disruption triggers a selective translational reprogramming that impairs mitotic fidelity. Specifically, SMAD4 deficiency alters the global translational landscape and suppresses translation of key mitotic regulator CDK11B. Re-expression of the mitosis-specific isoform CDK11B-p58 rescues these defects and suppresses tumorigenesis, establishing it as a primary effector in this pathway. Importantly, patient tumor analyses reveal a robust correlation between SMAD4 and CIN markers across multiple cancer types. Collectively, our findings uncover a translationally mediated axis of CIN, offering a new mechanism for CIN-driven tumor initiation.

Introduction

Genome instability is a defining and enabling hallmark of cancer, characterized by a diverse landscape of ploidy abnormalities, structural rearrangements, and gene mutations that fuel tumor evolution (1). Chromosomal instability (CIN) is the most prevalent form of genome instability and arises from errors during cell division, such as chromosome mis-segregation, spindle assembly checkpoint defects, and centrosome aberrations (2, 3). The fidelity of these processes relies on a coordinated network of regulatory factors, most notably cyclin-dependent kinases (CDKs) (3, 4). Consequently, even subtle impairments within this complex machinery can compromise mitotic integrity and precipitate the CIN that drives malignant transformation.

Most cancers exhibit some degree of CIN (5, 6), and there are many models of CIN driving tumorigenesis (7-9) and resistance to therapy (10, 11). However, precisely how CIN is initiated at the onset of tumorigenesis remains poorly understood. In particular, cancers of the gastrointestinal (GI) tract are characterized by CIN phenotypes (12-14). A prototypical example is esophageal adenocarcinoma (EAC), which develops from a precursor metaplasia named Barrett's esophagus (15); a progression that correlates with increasing CIN (16, 17). We have previously shown that SMAD4 loss, which coincides with the transition from metaplasia to EAC, induces tumorigenesis and increased copy number alterations (CNA) in Barrett's esophagus cells (18). This CNA increase was especially pronounced in the resultant tumor cells, suggesting that accumulation of mitotic errors occurs in these cells throughout their oncogenic progression.

Loss of SMAD4 is common in many solid tumor types, particularly in pancreatic and other GI cancers (19, 20). Consequences of SMAD4 deficiency are frequently attributed to loss of canonical TGF β and BMP signaling, producing effects such as suppression of growth arrest pathways and altered regulation of epithelial-to-mesenchymal transition (EMT) (21, 22). However, given the pronounced accumulation of CNAs observed upon SMAD4 loss, we hypothesized that SMAD4 may play an unappreciated role in maintaining chromosomal stability by regulating mitotic integrity. We therefore aimed to uncover the mechanisms that trigger CIN in early oncogenesis using a model of transformation driven by SMAD4 loss.

In this study, using unbiased approaches we demonstrate that SMAD4 plays a key role in maintaining chromosomal integrity to suppress tumorigenesis. We show that SMAD4 loss in pre-malignant cells triggers a selective reprogramming of the translome. This shift in the translational landscape disrupts mitotic integrity, resulting in accumulation of CIN. These findings point to a translationally mediated avenue to CIN and a novel role for SMAD4 that is distinct from its canonical functions.

Results

SMAD4 loss models chromosomal instability in GI cancers and uncovers a deregulated mitosis signature

To establish the broad relevance of our hypothesis, we first sought to determine whether the link between SMAD4 deficiency and genomic volatility observed in EAC initiation is conserved across other GI malignancies. We interrogated patient datasets from The Cancer Genome Atlas (TCGA) and Memorial Sloan Kettering (MSK) (13, 23-25). Given its prevalence and known role in CIN (26, 27), we stratified samples by presence of *TP53* alteration (i.e., any mutation or CNA) or *SMAD4* alteration and assessed for fraction of genome alteration (FGA). In EAC samples, we identified a significant change in FGA only as a function of *SMAD4* alteration in *TP53* altered cells, with a trend towards the same in *TP53* unaltered samples (Figure 1A). Remarkably, *TP53* alteration alone was not associated with increased FGA compared to wildtype *TP53* unless combined with *SMAD4* loss. Crucially, confirming this reflects a widespread cancer phenotype, a consistent pattern was detected in gastric, colorectal, and pancreatic adenocarcinomas (Figure 1B–D). Among the rare EAC patients with wildtype p53 and altered SMAD4, three of five exhibited *MDM2* copy number gains, and an additional sample showed p53 protein suppression comparable to MDM2-amplified tumors (Supplementary Table S1). Furthermore, significant associations with FGA were identified for *TGFBR2* or *MYC* alterations (Figure S1A), indicating this CIN phenotype extends to broader TGF β pathway disruption. These data validate *SMAD4* status as a primary determinant of genomic alteration across GI malignancies.

To validate this relationship, we evaluated an independent EAC cohort with matched genomic and transcriptomic data (University of Queensland [UQ] dataset; n = 295) (28, 29). Tumors were classified as SMAD4-deficient (n = 54) or SMAD4-intact (n = 116) based on copy number, mutation, and expression status (Table S2, Figure S1B). Where available, relative mRNA expression data from RNAseq were used to verify SMAD4 expression levels in cases of uncertainty, and ambiguous samples were excluded from analysis. Allele-specific copy number analysis (ASCAT) (30) revealed that SMAD4-

deficient tumors exhibited significantly higher copy number alterations (CNA; as indicated by a lower normal fraction), loss of heterozygosity (LOH), and amplifications without complete gene deletion (Figure 1E), indicating a tendency for single-chromosome allelic loss or gain. These findings were maintained under stricter classification criteria (Figure S1C) and after removing chromosome 18q21.2 to eliminate possible confounding effects of SMAD4 deletion (Figure S1E). Further, CIN signature CX2 was enriched in SMAD4-deficient tumors (81% vs. 36% SMAD4 intact, $p = 9.1 \times 10^{-8}$; Figure 1F) (6) which also displayed elevated homologous recombination deficiency (HRD) metrics (31), including increased telomeric allelic imbalance (TAI), LOH, and large-scale transitions (LST) (Figure S1D). SMAD4-deficient tumors were more than twice as likely to display CIN signature CX4 (55% vs. 25%, $p = 4.1 \times 10^{-4}$), which is associated with PI3K/Akt/mTOR-mediated tolerance of whole genome duplication (6). CIN signature Crucially, all but one strict deficient tumor carried concurrent *TP53* alterations, and the remaining wildtype tumor harboured *MDM2* amplification (Table S1, Figure S1F), reinforcing that SMAD4 deficiency may only occur on a background of defective p53. These data confirm that the observed CIN phenotype is dependent on SMAD4 status and not due to segregation of *TP53* mutations between the two groups.

Next, we employed complementary multi-omics to omics to dissect the origins of this structural instability, utilising non-tumorigenic Barrett's esophagus cells (CP-B) that undergo oncogenic transformation upon SMAD4 knockout (18, 32). We obtained significant differentially expressed gene and protein sets from 3' RNA-sequencing (RNAseq) and label-free quantitative proteomics, respectively, of parental and CRISPR-Cas9-mediated *SMAD4*^{-/-} CP-B cells (Figure 1G). We also conducted a genome-wide CRISPR-Cas9 screen to identify gene knockouts that were selectively lethal in the *SMAD4*^{-/-} cells (i.e., *synthetic lethal* hits) (Figure 1H). Metascape (33) functional enrichment analysis of these datasets identified multiple overlapping gene ontologies (GO) heavily centred on mitosis, chromosome segregation, and the metaphase-to-anaphase transition (Figure 1I, Figure S2A–B). At the transcript level, cells significantly downregulated core components of the spindle assembly checkpoint (SAC) and the anaphase-promoting complex/cyclosome (APC/C), including *TTK/MPS1*, *UBE2S*, *CENPE*, *SPDL1*, *BUB1B*, *UBE2C*, *CDC16/ANAPC6*, *CDC20*, and *CDCA8* (Figure 1J) (34). Proteomic analysis independently confirmed the deregulation of this metaphase-to-anaphase axis, driven by alterations in proteasome subunits essential for APC/C function (Figure 1K) (35, 36). Concurrently, the genome-wide knockout screen revealed that SMAD4-deficient cells were dependent on several APC/C components (*ANAPC11*, *ANAPC1*, *ANAPC5*), SAC regulators (*BUB3*, *CDCA8*), and the checkpoint kinase *CHEK1* (Figure 1L), which we validated using genetic and pharmacological approaches (Figure S2C–D). These data demonstrate that SMAD4 loss deregulates mitotic processes (in particular, the metaphase-to-anaphase transition) and engenders an increased dependency on specific components of these processes for cell proliferation and survival.

Finally, we observed that SMAD4 protein levels in TCGA EAC patient samples positively correlated with the expression of key mitotic regulators, including *BUB1B*, *CDCA8*, *CDC20*, and *KIF2C* (Figure S2E). Highlighting that this mechanism represents a broader pan-cancer phenomenon beyond our specific EAC model, this association was heavily conserved across diverse cell lines in the Cancer

Dependency Map (DepMap) (37), where SMAD4 protein levels consistently correlated with both mRNA and protein levels of *BUB1B*, *CDCA8*, *SGO1*, *NDC80*, and *FBXO8* (Figure S2F–G). Comparing our transcriptional profile to established signatures of CIN (38, 39) and esophageal cancer-specific mitotic defects (40) revealed a highly significant convergence on the "mitotic metaphase and anaphase" GO cluster (Figure S2H). Specifically, the SMAD4-deficiency signature significantly overlapped with both the pan-cancer CIN70 signature (38) (representation factor: 3.4; $p < 1.83 \times 10^{-4}$) and the dysregulated mitotic spindle signature identified in esophageal cancer (40) (representation factor: 4.6; $p < 3.65 \times 10^{-9}$). Collectively, these data indicate that utilising SMAD4 deficiency correlates with a clinically relevant deregulated mitosis signature, making it an ideal tool to study the mechanics governing the onset of CIN.

Loss of SMAD4 increases mitosis defects in Barrett's esophagus cells

To investigate this deregulated mitosis phenotype, we performed live-cell confocal, time-lapse imaging of CP-B parental and *SMAD4*^{-/-} cells, as well as an additional Barrett's esophagus cell line, CP-D, from which we derived further *SMAD4*^{-/-} populations (Figure S3A). Chromosome dynamics were visualized using a DNA stain and images recorded at eight-minute intervals for 16-18 hours (Figure 2A-C). As expected, due to endogenous *TP53* mutations in these cell lines, a fraction of each of the parental cell lines exhibited defects in mitosis such as anaphase bridges, lagging chromosomes, failed cytokinesis or mitotic slippage, whilst many progressed through mitosis without error. Interestingly, the percentage of mitotic defects observed in *SMAD4*^{-/-} CP-B lines was greater than respective parental cells, but no overall difference in total defects was observed in CP-D cells with or without SMAD4 (Figure 2D-E). However, *SMAD4*^{-/-} in both cell lines led to an increased proportion of mitoses with lagging chromosomes, resulting in micronuclei in the daughter cells, and a trend towards more multinuclear cells (Figure 2F-G). Interestingly, there was no difference in mitotic index (Figure S3B), nor in time spent in mitosis overall or from nuclear envelope breakdown to anaphase (Figure S3C-D). This suggests similar rates of cell division and no induction of mitotic checkpoints that might prolong mitotic duration in SMAD4-deficient cells.

We analyzed the size of nuclei in CP-B parental and *SMAD4*^{-/-} cells, as well as a new cell line derived from *SMAD4*^{-/-} tumors that grew from xenografts of these cells (Figure S3A) (18). The cell lines were grown into spheroids, from which cross sections were analyzed for nuclei size (Figure 2H-I). As expected, the size and variability of nuclei size increased across the cell line spectrum, where the parental CP-B cells displayed relatively small and uniform nuclei (mean = 452 pixels², range = 202-912, SD = 197) compared to the *SMAD4*^{-/-} (mean = 490 pixels², range = 200-1099, SD = 234) and tumor-derived cells, the latter of which exhibited very large nuclei (mean = 595 pixels², range = 204-1065, SD = 234). This increase in size and variability of nuclei suggests a concordant increase in mitotic defects leading to progressive aberrant chromosome numbers (41) during cell transformation.

SMAD4 loss and mTOR deregulation co-operate to drive tumorigenesis

Our tumorigenesis model of SMAD4 deficiency is characterized by a period of latency before tumor initiation, whereas there was no latency when cells derived from these tumors were injected

subcutaneously into a subsequent generation of mice (18). We therefore theorized that the cumulative mitotic defects associated with SMAD4 loss may result in specific genomic alterations that would contribute to tumorigenic potential. Thus, we sought to identify potential tumor suppressor genes whose loss may co-operate with that of SMAD4 to accelerate tumor onset. We conducted a pooled genome-wide *in vivo* CRISPR-Cas9 (Brunello^{-/-} library (42)) enrichment screen in CP-B cells, on a background of either prior CRISPR-Cas9 mediated SMAD4 knockout or SMAD4 wildtype (Figure 1D). This screen resulted in tumor initiation four-fold faster than our original model of SMAD4^{-/-}-induced tumorigenesis, with palpable tumors noted after a mean of 21 days versus 160 days with SMAD4^{-/-} alone (Figure 3A). In one case, Cas9-only expressing CP-B cells transduced with the genome-wide library also formed a tumor, however the time to tumor onset (273 days) was substantially longer than the SMAD4^{-/-} population. All tumors of sufficient volume were sequenced to identify the sgRNAs present in the population and their enrichment compared to the representation of sgRNAs in the cells before xenotransplantation. sgRNAs targeting *PTEN*, *TSC2* and *NF2*, notably all genes with downstream negative regulation effects on the PI3K/Akt/mTOR pathway (43-45), were all identified in the SMAD4^{-/-} Brunello^{-/-} tumors (Figure 3B). Additional genes with lesser known or less direct influence on this pathway were also identified in this screen (*GALNT8* (46), *PARVB* (47), *SCN1A* (48), *PPP4R2* (49), *RALGAPB* (50) and *KRT7* (51)), strengthening the co-operative link between SMAD4 deficiency and PI3K/Akt/mTOR pathway deregulation in tumorigenesis.

To validate these findings, *PTEN*^{-/-} and *TSC2*^{-/-} populations were generated in wildtype SMAD4 and SMAD4^{-/-} cells (Figure 3C, Figure S4A), which were then injected into new mice. Both *PTEN*^{-/-} and *TSC2*^{-/-} promoted rapid tumorigenesis of SMAD4^{-/-} cells and were less potently tumorigenic on a wildtype SMAD4 background (Figure 3C). Interestingly, whilst *PTEN*^{-/-} only cells rapidly produced palpable tumors, these neoplasms grew very slowly for about 80 days, remaining relatively dormant until approximately day 150, after which time growth rate drastically increased (Figure 3D). This was in stark contrast to the combined SMAD4^{-/-}*PTEN*^{-/-} cells, which grew quickly immediately after initiation, supporting the notion of a co-operative effect of loss of these two tumor suppressors. We also validated these findings in two patient-derived organoid (PDO) models of Barrett's esophagus. We found that dual SMAD4/*PTEN*^{-/-} PDOs exhibited a dramatically faster growth rate than wildtype PDOs, with development of very large, irregular-shaped organoids (Figure S4B). These data additionally suggest a co-operative effect between loss of SMAD4 and deregulation of the PI3K/Akt/mTOR pathway in promoting tumor formation.

SMAD4-deficient cells and tumors upregulate mTOR signaling and cap-dependent translation initiation

We next aimed to dissect the mechanism underlying this interplay between SMAD4 and the PI3K/Akt/mTOR signaling pathway (Figure 4A). To understand the status of mTOR signaling in SMAD4 deficient cells, reverse phase protein array (RPPA) analysis was performed on a panel of mTOR-related proteins in cells with either SMAD4^{-/-}, *PTEN*^{-/-}, or both SMAD4^{-/-}*PTEN*^{-/-} (Figure 4B). Parental cells displayed generally lower phosphorylation of Akt (S473, T308), mTOR (S2448, S2481), and downstream targets p70 S6 Kinase (p70S6K) and RPS6. *PTEN*^{-/-} cells showed a modest increase in phosphorylation of several mTOR pathway components relative to parental cells, such as p-Akt (S473,

T308), p-p70S6K (T389, P421, S424) and p-RPS6 (S235, S236, S240, S244). In contrast, *SMAD4*^{-/-} cells exhibited broadly elevated expression across multiple nodes of the pathway, including MYC, mTOR phospho-sites and p70S6K, suggesting stronger pathway activation. Analyses of TCGA data revealed strong negative correlations between SMAD4 protein level and levels of PI3K/Akt/mTOR family proteins and phospho-proteins in EAC patients (Figure 4C), supporting our model. The dual *SMAD4*^{-/-}*PTEN*^{-/-} cells showed a mixed pattern, with some targets resembling the high-expression profile of *SMAD4*^{-/-} cells (e.g., RPS6 phosphorylation) and others more closely aligning with the lower expression profile of *PTEN*^{-/-} cells. A similar trend was identified in *TSC2*^{-/-} and *SMAD4*^{-/-}*TSC2*^{-/-} cells (Figure S5A). Together, these data point to augmented mTOR pathway activity in *SMAD4*^{-/-} cells, and a combined effect in *SMAD4*^{-/-}*PTEN*^{-/-} or *SMAD4*^{-/-}*TSC2*^{-/-} dual knockout populations.

Of note was the impact of the gene perturbations on components of the cap-dependent translation initiation machinery. In the scanning model of translation initiation, eIF4E binds to a m⁷GpppX motif at the 5' end of mRNA (5' cap) and associates with eIF4G to form the eIF4F complex (52, 53). The formation of eIF4F is inhibited by 4E-BP1 binding to eIF4E, an association that is inhibited by phosphorylation of 4E-BP1 by mTOR (54). We observed increased phosphorylation of 4E-BP1 (S65, T37, T46) in *SMAD4*^{-/-} cells which was amplified further in dual knockout cells (Figure 4B, Figure S5A). Combined with the increased total eIF4E and eIF4G protein observed in these cells, this may demonstrate greater availability of eIF4E to associate with eIF4G to induce cap-dependent translation. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) revealed a trend towards decreased expression of 4E-BP1 mRNA in *SMAD4*^{-/-} cells and tumors derived from *SMAD4*^{-/-} cells (Figure 4D). It was also found that treating parental CP-B cells with TGFβ induced phosphorylation of 4E-BP1 (Figure 4E), in keeping with previous reports of activation of mTOR by a non-canonical TGFβ cascade (55). Moreover, whilst TGFβ appeared to induce phosphorylation of 4E-BP1 in *SMAD4*^{-/-} cells, overall levels of 4E-BP1 were decreased in these cells (Figure 4E). Given that phosphorylated 4E-BP1 is unable to bind to and thereby inhibit eIF4E, these data allude to an increase in available eIF4E in *SMAD4*^{-/-} cells, particularly in the presence of TGFβ.

Thus, a cap-binding pull-down assay was implemented to verify whether eIF4E activity (as well as expression level) was upregulated in *SMAD4*^{-/-} cells. As expected, parental cells showed an increase in eIF4G binding to the m⁷-GTP cap-bound eIF4E in response to TGFβ (Figure 4F). Furthermore, *SMAD4*^{-/-} cells had higher basal levels of eIF4G binding, which was further increased upon TGFβ stimulation. A SURface SENSing of Translation (SUnSET) puromycin incorporation assay (56) confirmed a decrease in global translation rates in both CP-B and CP-D cells without SMAD4, with preferential translation of transcripts encoding only low-to-medium molecular weight proteins (Figure 4G). In line with this interpretation, our multi-omics analyses identified mRNA processing and translation as key clusters of deregulation in SMAD4-deficient cells (Figure 1E). In addition to mitosis-related genes, our genome-wide CRISPR-Cas9 screen identified multiple genes related to translation, including EIF4E, as synthetic lethal in the context of SMAD4 deficiency (Figure S5B). To interrogate this further, we treated cells with a range of translation inhibitors: Briciclib (eIF4E inhibitor, required for cap-dependent translation), Episilvestrol (EIF4A inhibitor, required for global translation) and Homoharringtonine

(translation elongation inhibitor). We found a change in sensitivity to only Briciclib in *SMAD4*^{-/-} cells (Figure 4H-J, Figure S5C-E), suggesting a specific dependency of SMAD4-deficient cells on cap-dependent translation initiation but not global translation or elongation. These data point to an increase in cap-dependent translation initiation of specific mRNAs in SMAD4-deficient cells, but without a concomitant increase in global translation.

Translational reprogramming in SMAD4-deficient cells results in post-transcriptional repression of CDK11B

As these data suggested possible reprogramming of mRNA translation in our models, we next investigated the translational profiles of SMAD4-deficient cells via polysome analyses (Figure 5A-B). Here, CP-B parental, *SMAD4*^{-/-} and *SMAD4*^{-/-} tumor-derived cells were lysed and fractionated on a sucrose density gradient to isolate polysome-associated mRNAs. Changes in total mRNA abundance and polysome-bound mRNAs were analyzed via RNAseq. This revealed an altered global translational landscape in SMAD4-deficient cells (Figure 5C). SMAD4-deficient cells displayed an increase in free 60S subunits and 80S monosomes, with a concurrent decrease in low-order polysomes (peaks labelled 2-5) and enrichment of high-order polysomes (peaks labelled 7-8). This suggests that, following loss of SMAD4, cells have selectively altered ribosome occupancy on specific subsets of mRNAs, resulting in higher polysome associations on some transcripts at the expense of others. This could represent either increased translation or ribosome stalling on these mRNAs (57). Although the *SMAD4*^{-/-} cell lines displayed enrichment of higher order polysomes, the tumor-derived cell line exhibited a modest reduction in 7-8 ribosome peaks. This pattern is consistent with highly efficient translation (57) and a translome biased towards shorter, growth-related mRNAs (58). To determine if specific transcript characteristics dictated this selective translational sensitivity, we analyzed the mRNA structural features. We found that transcripts exhibiting reduced translational efficiency in *SMAD4*^{-/-} cells were characterized by significantly longer coding sequences (CDS) and increased exon number compared to unchanged or up-regulated mRNAs (Figure S6A-E). Consistent with this structural bias, the *SMAD4*^{-/-} tumor-derived cell line also displayed increased abundance of ribosome and translation-related mRNAs, including EIF4A2, RPL7, RPL8, RPL9, RPL21, RPL23, RPL23A, RPL34, RPL39L, RPS3A, and RPS20 (Figure 5D). Together, these data suggest an association between SMAD4 loss and translational reprogramming with differential ribosome density on a subset of transcripts, potentially defined by their structural complexity, and subsequent further changes during transformation to restore efficient translation.

Next, the polysome-seq was analyzed to detect specific mRNAs that were differentially translated in SMAD4-deficient cells, with a particular focus on mitosis-related genes. We identified that the CDK11B transcript, of which the whole cell mRNA levels remained unchanged across cell lines, was decreased in polysome fractions in *SMAD4*^{-/-} and tumor-derived cells (Figure 5E). Indeed, the translation efficiency quotients for this gene were significantly lower in *SMAD4*^{-/-} and tumor-derived cells (Figure 5F), indicating a reduction in polysome-bound CDK11B mRNA compared to that in the cytoplasmic lysate. Notably, CDK11B serves as a clear example of a structurally regulated transcript, where its unusually long coding sequence and high number of exons place it among the most extreme cases in the group

of transcripts with reduced translation (Figure S6D-E). Interestingly, our genome-wide CRISPR-Cas9 screen also revealed dependency of *SMAD4*^{-/-} cells on CDK11B (Figure 5G). The tight translational control of CDK11B has been well described (59), whereby the CDK11B transcript gives rise to multiple protein isoforms (Figure 5H). For most of the cell cycle, CDK11B mRNA produces its full-length 110 kDa protein by way of cap-dependent translation, whilst an additional isoform (p46) is translated from mRNA alternative splicing. However, a third isoform, p58, is translated only during mitosis using an internal start codon (59), and is essential for progression of correct mitosis (60, 61). These data therefore indicate disrupted translational control of CDK11B in *SMAD4*-deficient cells and tumors.

Re-introduction of p58 isoform of CDK11B rescues mitosis defects and impairs tumorigenesis in *SMAD4* knockout cells

Given its multiple known roles in maintaining mitotic integrity, we finally aimed to determine whether our observed reduction in CDK11B protein synthesis was influencing the defective mitosis phenotype seen in *SMAD4*^{-/-} cells. To do this, we ectopically expressed cDNA encoding the short, p58 isoform of CDK11B in CP-B cells with or without *SMAD4* (Figure 6A) and conducted live-cell imaging to visualize mitoses. Re-expression of CDK11B-p58 significantly reduced the rate of mitotic defects in *SMAD4*^{-/-} cells (Figure 6B-C), especially the occurrence of lagging chromosomes (Figure 6D) and failed cytokinesis (Figure 6E). Cells containing the CDK11B-p58 construct also exhibited a shorter time from nuclear envelope breakdown (NEB) to anaphase (Figure 6F), indicating a less error-prone passage through mitosis, but did not alter the percentage of multinuclear cells (Figure 6G). Importantly, Re-expression of CDK11B-p58 in *SMAD4*^{-/-} cells suppressed tumor formation when xenografted subcutaneously into mice (Figure 6H). Together, these data suggest that lack of CDK11B-p58 protein in *SMAD4*^{-/-} cells may be responsible for the observed mitosis defects and tumorigenicity of these cells.

Discussion

In this study, we delineate a novel role for translational control of mitotic fidelity and CIN during early oncogenesis. While CIN is a defining hallmark of aggressive malignancies, the precise molecular switches that transition pre-malignant tissue from low-level chromosomal alterations to catastrophic, copy-number-driven landscapes remain poorly understood. By investigating these dynamics through the lens of *SMAD4* deficiency – a frequent event in gastrointestinal carcinogenesis – we uncover a co-operative mechanism where the loss of transcriptional tumor suppression converges with translation-initiation stress to unleash CIN. We provide evidence of *SMAD4*-associated CIN in multiple patient cohorts, and a deregulated mitosis signature associated with *SMAD4* deficiency in our models of p53-mutant pre-cancerous Barrett's esophagus.

Our findings resolve a long-standing paradox regarding the role of p53 in suppressing CIN. Early Barrett's metaplasia is marked by low levels of chromosome alterations as compared to dysplastic Barrett's esophagus (16), a phenotype attributed to the frequent loss of wildtype p53 function in the latter (27). Whilst EAC has a high mutational burden, it is primarily a cancer of copy number drivers (26, 62-64) and may in fact be characterized by a deregulated mitotic spindle signature (40). The role of p53 in suppressing CIN has been extensively examined, but mutant p53 is frequently detected in pre-

maligant Barrett's tissue long before progression to invasive cancer (65). Therefore, it appears that loss of p53 is unlikely to be the only factor at play in CIN-driven tumorigenesis and is insufficient to induce oncogenesis on its own in this disease. Our multi-cancer analysis answers this conundrum: *TP53*-altered tumors across multiple GI cancer cohorts exhibit limited increase in the fraction of the genome altered compared to wildtype *TP53* tumors, except in combination with *SMAD4* loss (Figure 1A–D). The co-occurrence of *SMAD4* loss with either *TP53* or *MDM2* alteration (Table S1) suggests that loss of p53 function is a prerequisite for a concurrent *SMAD4* alteration, potentially due to an unstable genome requiring defective error-sensing mechanisms to allow cell survival in this state. A recent study of mitotic spindle dynamics through EAC progression also appears to support this (66). Scott *et al.* demonstrated increasing mitosis defects across a panel of esophageal cell lines, from low-grade dysplastic Barrett's esophagus through to EAC, and determined that these effects are due to loss of wildtype p53 function. However, the authors did not examine the striking negative association between *SMAD4* protein level and observed spindle aberrations in these cell lines that may also explain this phenotype, as shown in our data. This also suggests a protective effect of *SMAD4* wild type protein against CIN in mutant p53 cells.

Our combined 'omics approach supported the notion of *SMAD4* maintaining chromosomal stability in this context. Specifically, our transcriptomic and proteomic data revealed that loss of *SMAD4* resulted in perturbation of critical regulation of the mitotic spindle and chromosome segregation (Figure 1I–K). This expression pattern is similar to that described by Xu *et al.* in their comparative analysis of esophageal cancer versus normal esophageal tissues (40). Whilst Xu *et al.* identified defective mitotic spindle regulation as pertinent in esophageal cancer, here we propose that *SMAD4* loss may be one key cause of this deregulation. Crucially, the significant overlap between our *SMAD4*-deficiency signature and genes known to be involved in mitotic spindle function and CIN (Figure S2H) (38, 39) positively correlates across patient samples (Figure S2E) and pan-cancer cell lines (Figure S2F–G), suggesting this control mechanism is common in EAC and potentially other cancer types.

We detected a significant increase in mitotic errors in *SMAD4*-deficient cells (Figure 2D–E) and propose that accumulation of such errors may result in accrual of tumor-promoting gene alterations. The stochastic nature of these errors may explain the lag time before tumor initiation in *SMAD4*^{-/-} cells (18), as not all errors will result in beneficial gene alterations. Supporting this, our genome-wide tumorigenesis screen identified multiple co-operative drivers that dramatically hasten transformation of *SMAD4*^{-/-} Barrett's cells (Figure 3B–C). Many of these drivers converge on the PI3K/Akt/mTOR pathway, suggesting a strong compound effect of mTOR hyperactivation with *SMAD4* loss. Compellingly, *SMAD4*-deficient EAC tumors are enriched for the CX4 CIN signature (Figure 1F), which has been associated with tolerance of whole genome duplication due to increased PI3K/Akt/mTOR signaling (6). Additionally, we propose that the pool of co-operative drivers in *SMAD4*-deficient tumors may be large, where multiple gene alterations may have effects that co-operate with *SMAD4* loss to promote tumor growth. This may be why there are very few significantly co-occurring gene alterations in *SMAD4*-altered tumors (26), but instead a large panel of potential co-operators, including PI3K/Akt/mTOR.

We investigated the cross-reactivity between SMAD4 loss and the mTOR pathway, showing that SMAD4-deficient cells upregulate mTOR signaling, and this is increased further when combined with loss of PTEN or TSC2 (Figure 4B, Figure S5A). Known links between SMAD4/TGF β signaling and mTOR converge on 4E-BP1 (55), pointing to an effect on cap-dependent translation. In line with this, we see an increase in cap-dependent translation in *SMAD4*^{-/-} cells (Figure 4F) and a concomitant reprogramming of global translation (Figure 5C). Our SuNSET assay demonstrates a minor decrease in puromycin incorporation in SMAD4-deficient cells (Figure 4G), suggesting delayed elongation or premature termination of peptides in these cells. These data likely represent an initiation-elongation imbalance, where slowed elongation is outpaced by enhanced cap-dependent initiation. Our data suggest that post-transformation, SMAD4-deficient tumor cells may alleviate this initiation-induced elongation stress. It is possible that these cells restore translational capacity through accumulation of an additional mTOR-related gene alteration and subsequent upregulation of mTOR-sensitive mRNAs encoding ribosomal proteins and elongation factors (67). This may explain the faster tumor initiation observed in these cells than untransformed, *SMAD4*^{-/-} cells. In the case of untransformed cells, the observed mTOR-driven increase in cap-dependent initiation may influence ribosome density on specific transcripts at the expense of others. We report that translation of CDK11B, a well-described example of translationally controlled protein expression, is altered in *SMAD4*^{-/-} cells (Figure 5E-F). This selectivity may be governed by transcript architecture; our global analysis indicates that mRNAs with high structural complexity (i.e. those with long CDS and high exon density) are specifically reduced in polysome-bound fractions in *SMAD4*^{-/-} cells (Figure S6). Whilst whole cell mRNA levels remain the same, ribosome-bound CDK11B transcripts, which sit at the extreme end of this structural distribution, are decreased in SMAD4-deficient tumors. The mitotic index of parental versus *SMAD4*^{-/-} cells is similar (Figure S3B), suggesting that a difference in CDK11B protein levels is unlikely due to an imbalance in cell cycle stage. Given our model depicts upregulation of cap-dependent translation initiation in *SMAD*^{-/-} cells, we propose that alternative translation mechanisms are suppressed in these cells resulting in lower expression of CDK11B-p58.

Finally, we show that reintroduction of CDK11B-p58 is sufficient to rescue multiple mitotic defects in *SMAD4*^{-/-} cells, including lagging chromosomes and failed cytokinesis, and restrain tumor formation. This is somewhat unsurprising, given that CDK11-p58 has been shown to be essential for maintaining cohesion of sister chromatids, as well as recruitment of ESCRT-III complex and abscission during cytokinesis (60, 61). Lagging chromosomes and errors in chromosome segregation can result in formation of micronuclei (68, 69), which are abundant in our *SMAD4*^{-/-} cells. Interestingly, re-expression of CDK11B-p58 did not significantly reduce the percentage of cells with multiple nuclei in our models. A recent study found that TGFBR2-deficient cells became enriched in cell populations with micronuclei (70), suggesting a protective effect of loss of TGF β signaling in cell survival with micronuclei. We propose that such complex interplay between multiple signaling pathways may result in perpetuation of multinuclear cells despite re-introduction of CDK11B-p58 alleviating the original cause of these abnormalities. In this way, CDK11B-p58 re-expression may mitigate accumulation of additional mitotic errors that lead to tumorigenesis.

In sum, our data present a model for SMAD4 as a gatekeeper for CIN through regulation of mitosis via translational control. We demonstrate that SMAD4 loss triggers a cascade of PI3K/Akt/mTOR upregulation, translational reprogramming, mitosis defects, and CIN. A product of this altered system is decreased translation of CDK11B, which is essential for progression of correct mitosis. Importantly, we show that re-introduction of the CDK11B-p58 isoform is sufficient to reduce mitotic errors in SMAD4-deficient cells, plausibly diminishing their tumorigenic potential. However, there remains a delicate balance between tumor-promoting CIN and lethal volatility of the genome, as excessive genome disruption can result in mitotic catastrophe and cell death (3, 71). Further, cells displaying high levels of CIN are acutely sensitive to inhibitors of mitosis and the DNA damage response (DDR) (72, 73). Mounting evidence suggests that tumor progression can be driven by CIN and ploidy abnormalities that promote evolution but may also predispose cells to sensitivity to further mitotic insults. Through our CRISPR-Cas9 dependency screen, we reveal that *SMAD4*^{-/-} cells are indeed more susceptible to loss of secondary mitosis regulatory genes. Taken together, these data reveal a new mechanistic basis of tumorigenesis and unveil promising therapeutic avenues for translationally reprogrammed, CIN-driven cancers.

Materials and methods

Experimental models

Cell lines

The human telomerase reverse transcriptase (hTERT) immortalized Barrett's epithelial cell lines, CP-B (CP-52731 RRID: CVCL_C452) and CP-D (CP-18821, RRID: CVCL_C454), were obtained from Professor Peter Rabinovitch (University of Washington, Seattle, WA) and cultured in MCDB-153 medium supplemented with 400 ng/mL hydrocortisone, 20 ng/mL epidermal growth factor (EGF), 20 mg/mL adenine, 84 mg/mL bovine pituitary extract (BPE), ITS Liquid Media Supplement (100X) for final concentrations of 2.5 mg/mL insulin, 1.4 mg/mL transferrin, 1.3 ng/mL selenium (all from Sigma-Aldrich, Sydney, Australia), 10 nM cholera toxin, 5% v/v fetal bovine serum (FBS), 375 ng/mL fluconazole (Sigma-Aldrich), and 4 mM L-glutamine (GlutaMAX; Thermo Fisher Scientific, Carlsbad, CA), adjusted to pH 7.2. All cells were maintained at 37°C with 5% CO₂. Authentication of cell lines was performed via short tandem repeat (STR) analysis with the PowerPlex16 genotyping system (Promega, Madison, WI) and cultures were confirmed to be free from mycoplasma by polymerase chain reaction (PCR) (Cerberus Sciences, Scoresby, VIC). Cells were thawed from early passage stocks and passaged no more than 10 times.

Mouse models

NOD-SCID IL-2R γ ^{KO} (NSG) mice were obtained from in-house breeding facility. All animal experiments were approved by the Peter MacCallum Cancer Centre (PMCC) Animal Ethics Committee and undertaken in accordance with the National Health and Medical Research Council (NHMRC) Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Patient-derived Barrett's esophagus organoids

Patients were recruited during hospital visits for either routine surveillance endoscopy or esophagectomy procedure at Peter MacCallum Cancer Centre (PMCC). Written informed consent was obtained from patients and the collection and use of tissue for this study as approved by the Peter MacCallum Cancer Centre Human Research Ethics Committee (Project #HREC/44873/PMCC-2018). Biopsy specimens were collected from regions of Barrett's metaplasia in the distal esophagus using 2.8 mm biopsy forceps. Biopsies were transported on ice in organoid wash media, made with base medium Advanced Dulbecco's Modified Eagle Medium/F12 (AdMEM/F12; Thermo Fisher Scientific, Waltham, MA) supplemented with 1X GlutaMAX (Thermo Fisher Scientific), 10 mM HEPES (Thermo Fisher Scientific), 2.5 µg/mL Amphotericin B and 0.3 mg/mL Imipenem/Cilastatin. Specimens were processed using a modified protocol, combining previously established methods (74, 75) for optimal conditions. Biopsies were minced using a size 23 scalpel blade and digested with Dulbecco's Modified Eagle Medium (DMEM; Thermo Fisher Scientific) supplemented with 0.3 mg/mL Imipenem/Cilastatin, 1.5 mg/mL Collagenase II (Thermo Fisher Scientific), 800 U hyaluronidase (Sigma-Aldrich, St. Louis, MO) and 10 µM Y-27632 (ROCK inhibitor; Tocris Bioscience, Bristol, UK). Tissues were incubated in a 37°C water bath for 2-3 h, with regular inversion. The digested suspension was triturated passed through a

70 µm filter and centrifuged for 5 min at 350 x *g* at 4°C. Red blood cells were lysed by adding 2 mL ACK solution (150 mM ammonium chloride (NH₄Cl; Sigma-Aldrich), 10 mM potassium bicarbonate (KHCO₃; Sigma-Aldrich) and 100 µM EDTA in water, adjusted to pH 7.2-7.4). After 2 min, 13 mL DMEM with 10% FBS was added and the tube centrifuged again for 5 min at 4°C, the supernatant carefully aspirated and the tube kept on ice. The pellet was resuspended in R&D Systems Cultrex Basement Membrane Extract Type II (BME; In Vitro Technologies, Noble Park, VIC), at approximately 100 µL Cultrex BME for every 1 mm of pellet. Cells were then seeded into 15 µL domes onto pre-warmed (37°C) 48-well flat-bottomed plates, with one dome per well. Plates were inverted to promote dispersal of cells throughout the dome while setting and incubated at 37°C for 30 min before re-inversion and addition of growth medium (detailed below), including 10 µM Y-27632. All cultures were grown in Intesticult™ Organoid Growth Medium (StemCell Technologies, Vancouver, Canada), supplemented with 1 µg/mL gastrin, 100 ng/mL FGF-10 and 0.25 mg/mL imipenim/cilastatin. During derivation and passage, media was supplemented with 10 µM Y-27632.

Patient data analysis

For fraction of genome alteration analyses, relevant mRNA and protein expression data as well as clinical attributes were downloaded from publicly available databases. EAC patient samples sourced from The Cancer Genome Atlas (TCGA) Firehose Legacy and Memorial Sloan Kettering (MSK) studies using cBioPortal [<https://cbioportal.org>] (23, 24). These two studies were selected due to their comprehensive profiling of CNA and mutations. Putative CNAs were determined using the GISTIC 2.0 algorithm. Data were last accessed on 9 August 2025.

UQ EAC samples were collected from patients that gave written informed consent (HREC/2020/QMS/62117, UQ/2020/HE001913). DNA and RNA were extracted using the Qiagen AllPrep DNA/RNA mini kit according to standard protocol (Qiagen, Germany). DNA was used for Illumina SNP arrays and whole-genome or whole-exome sequencing as previously described (150 bp paired-end; Illumina HiSeq (28, 76). RNA sequencing was performed and processed as previously described (29). These data are accessible through the EGA (<https://ega-archive.org/studies/EGAS00001002864>). The sequence data are generated from patient samples and therefore are available under restricted access. Data access can be granted via the EGA with completion of an institute data transfer agreement, and data will be available for a defined period once access has been granted. SNP array data was analyzed using ASCAT (version 3.2.0), and HRD scores calculated using published formula (31).

Generation of knockout cell lines

Top and bottom-strand oligonucleotides were designed with the following overhangs to allow for cloning into the pLentiGuide-Puro vector (Addgene #52963): top strand 5'-CACCG... and bottom strand 5'-AAACNNNNNNNNNNNNNNNNNNNNNC. Oligos were annealed and amplified and cloned into the vector backbone using Esp3I restriction sites as described previously (77). Cloned plasmids were amplified using NucleoSpin Plasmid Miniprep Kit (Macherey-Nagel, Düren, Germany) as per the manufacturer's specifications.

For production of lentivirus, 5.5×10^5 HEK293T cells/well were seeded in a 6-well plate and left to adhere overnight at 37°C. Third generation lentivirus packaging mix was created by combining 0.5 µg/µL pMDI, 0.25 µg/µL RSV-REV, 0.3 µg/µL VSV and 1 µg/µL plasmid DNA in neat DMEM (Thermo Fisher Scientific). This mix was first vortexed before adding 9.2 µL polyethylenimine (PEI; Thermo Fisher Scientific) at 1 mg/mL, vortexing again and incubating for 15 min at RT. This mix was then added dropwise to HEK293T cells, which were then incubated at 37°C overnight. After 24 h, the media was removed and replaced with fresh media relative to the target cells (i.e. complete MCDB media for CP-B cells). At 48 h post-transfection, the viral supernatant was collected and passed through a 0.45 µm filter and stored at -80°C.

For lentiviral transduction, 5×10^5 cells/well were seeded in a 12-well plate in 400 µL complete media. One third of the harvested viral stock (approx. 600 µL) was added to each well to a total volume of 1 mL/well plus 8 µg/mL sequabrene. Cells were then centrifuged for 30 min at 1200 x *g* at 30°C and incubated at 37°C for 24 h. Cells were then lifted and transferred to flasks supplemented with 2 µg/mL puromycin (selection marker for pLentiGuide-Puro) and incubated at 37°C for three days to select for successfully transduced cells. sgRNA sequences: *SMAD4* (ATAACAGCTATAACTACAAA), *PTEN* (CCAATTCAGGACCCACACGA), *TSC2* (TCCTTGCGATGTACTCGTCG).

RNA-sequencing

Total cell RNA was isolated from cells using Nucleospin RNA extraction kit (Macherey-Nagel, Germany), in biological triplicate. RNA concentration and purity were measured with a NanoDrop1000 spectrophotometer (Thermo Fisher Scientific). RNA-sequencing library was prepared with QuantSeq Lexogen 3'mRNA-FW on the Illumina NextSeq 500 system using Single End 75 base reads with a read depth of 3-4 million reads per sample. Sequencing reads were aligned using HISAT2 (78), and gene expression was quantified using HTSeq software (79). Normalized expression was measured in counts-per-million (CPM) on a log₂ scale, with library size adjustment conducted using the Trimmed Mean of M-values method with edgeR (80) R package. Differential expression analysis was performed using the LIMMA-Voom workflow (81). Gene set enrichment analysis was performed using GSEA. MSigDB gene set collections were used as reference gene sets. Dataset can be found in Table S3.

Quantitative proteomics

CP-B cells (5.0×10^6) were plated in 15 cm plates and allowed to adhere for 24 h. Cells were washed with ice-cold PBS and lysed at 4°C in RIPA buffer (1 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM sodium fluoride, 1 mM sodium pyrophosphate in PBS) supplemented with protease and phosphatase inhibitors (Roche, Basel, Switzerland). Protein purification, tryptic digest and peptide elution were performed, and liquid chromatography tandem mass spectrometry (LC-MS/MS) was conducted, all as previously described (82). Data analysis was performed using MaxQuant (83) and Perseus (84) platforms. Dataset can be found in Table S4.

CRISPR screening

CP-B cells were engineered to express the Cas9 endonuclease by transduction with the FUCas9Cherry vector (Addgene, Watertown, MA; #70182) and subsequent selection for mCherry-positive cells. Cells were then transduced with a genome-wide sgRNA Brunello (76,441 sgRNAs, Addgene; #73178 (42)), at a MOI of 0.3, aiming for 500-fold starting representation of each guide. Puromycin selection (2 µg/mL) was applied for 3 days and surviving cells were passaged every three days in T175 flasks with 8×10^6 cells per flask for a total of 6 days. 3.2×10^7 cells were used for xenograft transplants. Genomic DNA of cells and xenograft tumors was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) and sequencing libraries were generated by PCR as previously detailed (85). The libraries were sequenced on a NextSeq 500 (Illumina, San Diego, CA) to generate 75 base pair single-end reads, which were demultiplexed with Bcl2fastq (v2.17.1.14). The sequencing reads were subsequently trimmed with Cutadapt (v1.7) to remove sgRNA vector derived sequences, then sgRNA reads were counted and the distribution analyzed using MAGeCK (v0.5.8) using read depth (--norm-method total) normalization. MAGeCK score was determined by $-\log_{10}(\text{neg } P\text{-value}) + \log_{10}(\text{pos } P\text{-value})$ and datasets were visualized on GraphPad Prism (v9.0). Dataset can be found in Table S5.

Metascape analysis

Gene lists including top *SMAD4*^{-/-} synthetic lethal hits (MAGeCK score > 3), RNA-sequencing differentially expressed genes (q-value < 0.05) and proteomics differentially abundant proteins (q-value < 0.05) were entered into the Metascape online resource [<https://Metascape.org>] (33). Gene lists were analyzed for exact gene matches and overlapping enrichment of the following gene ontology sources: KEGG Pathway, GO Biological Processes, Reactome Gene Sets, Canonical Pathways, Cell Type Signatures, CORUM, TRRUST, DisGeNET, PaGenBase, Transcription Factor Targets, WikiPathways, PANTHER Pathway and COVID. Using all genes in the human genome as the background for enrichment, “enrichment factors” were calculated as the ratio of observed counts to the number of counts expected by chance. Clusters were designated as > 3 similar terms with enrichment factors > 1.5 and p-values < 0.01 (calculated using accumulative hypergeometric distribution).

Competitive growth assays

For confluency-based assays, Cas9-expressing CP-B cells and two *SMAD4*^{-/-} CP-B clonal populations were transduced with sgRNA targeting *ANAPC11*, and treated with 2 µg/mL puromycin for 72 h. The three cell populations were then pooled and re-seeded at 1.0×10^5 cells/well in 6-well plates and T0 confluency determined utilizing an IncuCyte FLR (Essen BioScience, Ann Arbor, MI) imaging phase and green channels. Media was changed after 3 days and an endpoint reading was taken at T6 (i.e. 6 days post-selection). Percentage of *SMAD4*^{-/-} populations was determined as GFP confluency/total cell confluency. Empty vector control wells were set up and confluency quantified to account for differences in transduction efficiency.

Drug dosing

For dose response assays, serial dilutions of pharmacological compounds (Prexasertib, SelleckChem S7178; Bricilib, SelleckChem S6533; Episilvestrol, MedChemExpress HY-15359; Homoharringtonine,

Sigma SML1091) were added to 96-well plates containing 2×10^3 cells/well. After 120 h incubation, cell viability was determined using AlamarBlue reagent (Life Technologies) and fluorescence was read at 550 nm/590 nm on a Cytation 3 Imaging Reader (BioTek).

DepMap Analysis

Cell line dependency, mRNA expression and proteomics data were obtained from the cancer Dependency Map (DepMap) portal [<https://depmap.org>], last accessed on 9 August 2025. The relevant data were extracted from DepMap Public 22Q2 release of the Cancer Cell Line Encyclopedia (CCLE) project RNA-sequencing and quantitative proteomics (37). The DepMap platform calculates RNA expression values as transcripts per million (TPM) using the RNA-Seq by Expectation-Maximisation (RSEM) tool and transformed using the formula $[\log_2(\text{TPM}+1)]$. Proteomics on the DepMap portal are obtained from quantitative mass spectrometry of 375 cancer cell lines by the Gygi lab (37).

Live cell imaging

Cells were seeded at 4×10^3 cells/well in black 96-well glass bottom μ -Plates (Ibidi, Fitchburg, WI) in relevant growth medium and incubated at 37°C with 5% CO₂ for 72 h to allow for adherence and for cells to reach exponential growth rate. One hour prior to recording, media was replaced to contain 0.75X solution SPY650-DNA dye (Spirochrome, Switzerland). Cells were imaged on Olympus FV3000 (Olympus, Japan) in a controlled chamber at 37°C with 5% CO₂ for 16-18 h using 60x silicone objective (UPLSAPO-S 60x, NA 1.35, Olympus, Japan). Live cell images were captured in z stacks every 8 min. Transmitted channel was acquired and SPY650-DNA dye was excited by 635nm laser. Image processing was performed using Fiji software (86).

Nucleus size quantification

For immunofluorescence of cell spheroids, cells were grown on low-attachment plates (Corning, Corning, NY) and allowed to form three-dimensional spheroid structures before suspension in agarose and paraffin embedding. Cross sections of the spheroids were stained with DAPI and coverslipped as above. Slides were imaged with fluorescent microscopy, and the size of nuclei analyzed and quantified using Fiji software. Nucleus size was determined by converting images to 8-bit greyscale, applying default colour threshold adjustment uniformly to all images, and analyzing particles with a lower limit of 200 pixels² to exclude debris and an upper limit of 1100 pixels² to exclude overlapping nuclei.

Animal experiments

For cell line xenografts, 5×10^6 cells (8×10^6 for the CRISPR screen) suspended in 100 μ L of 1:1 PBS and Matrigel (BD Bioscience, Franklin Lakes, NJ) were subcutaneously injected into the right flank of ~6 week-old female NSG mice. Tumor volume was assessed with caliper measurements every ~7 days and calculated using the formula $(\text{length} \times \text{width}^2)/2$. At experimental endpoint (tumor volume > 1000 mm³), mice were culled, tumors and organs were harvested.

Immunoblotting

As per established protocols (87), cells were lysed at 4°C in RIPA buffer (1 mM EDTA, 1% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 50 mM sodium fluoride, 1 mM sodium pyrophosphate in PBS) supplemented with cOmplete Mini protease inhibitor and PhosSTOP phosphatase inhibitor cocktails (Roche, Basel, Switzerland). Equal amounts of protein were boiled, resolved by SDS-PAGE and transferred to PVDF membranes. Membranes were incubated in blocking buffer (5% w/v bovine serum albumin (BSA; Bovogen Biologicals, Keilor East, VIC) in Tris-buffered saline with 0.1% v/v Tween-20 (0.1% TBS-T) for 1 h at RT and probed overnight in primary antibody at 4°C. Blots were washed thrice in 0.1% TBS-T, followed by incubation with peroxidase-conjugated secondary antibody (Dako, Denmark) for 1 h at RT. Protein levels were detected using Clarity Western ECL Substrate (BioRad, Hercules, CA) or ECL Plus Western blotting substrate kit (Thermo Fisher Scientific). Antibodies are detailed in Table S6.

Reverse phase protein array (RPPA)

Cells were seeded at 1×10^6 cells/well in 6-well plates and allowed to adhere overnight before lysis in CeLyA Lysis Buffer (CLB1; Zeptosens, Bayer, Witterswil, Switzerland), and protein quantified using a Pierce™ Coomassie Plus (Bradford) Protein Assay Kit. Using a Sciclone/Caliper ALH3000 liquid handling robot (Perkin Elmer, Waltham, MA), samples were prepared in 4 dilutions (100%, 63%, 40% and 25%) in 10% CLB1:90% CeLyA Spotting Buffer (CSBL1; Zeptosens) and spotted onto ZeptoChips (Zeptosens) in 3-4 technical replicates using a Nano-plotter-NP2.1 non-contact microarray system (GeSiM, Radeburg, Germany). Chips were blocked for 1 h with blocking buffer (BB1; Zeptosens), incubated with pre-validated primary antibodies (1:500, 20 h), and Alexa Fluor® 647 anti-rabbit secondary antibody (1:1000, 4 h) (Thermo Fisher Scientific). Chips were read on a Zeptosens instrument and software version 3.1 used to calculate the Relative Fluorescence Intensity (RFI). All samples were normalized to the background values reported in the secondary antibody-only negative control. Antibodies are detailed in Table S7.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Cells were seeded 4×10^5 cells/well in 6-well plates and allowed to adhere overnight. Extraction and purification of RNA was conducted using Nucleospin RNA kit (Macherey–Nagel) before reverse transcription to cDNA using Transcriptor First Strand cDNA Synthesis Kit (Roche) as per manufacturer's instructions, including the optional denaturing step. Quantitative PCR (qPCR) was conducted using LightCycler 480 SYBR-Green qPCR (Roche) as per manufacturer's protocol, with gene expression normalized within each sample to GAPDH and analyzed using the $\Delta\Delta C_t$ method. Primer sequences were obtained using the NCBI Primer Blast application and are detailed in Table S8.

m7-GTP cap-binding assay

Cells were seeded at 1.0×10^6 cells/well in 6-well plates and allowed to adhere overnight. Cells were treated with 10 nM TGFβ or vehicle (citric acid) for 4 h (or cycloheximide for 6 h) before washing with warm PBS. Cells were then lifted with warm 0.05% trypsin-EDTA and centrifuged at $6000 \times g$ for 90 s. The trypsin-EDTA was removed and the pellet washed with warm PBS before centrifuging again at $6000 \times g$ for 90 s. The supernatant was removed and cells were lysed in non-denaturing lysis buffer (7

mL dH₂O, 160 µL 5 M NaCl, 160 µL 1 M Tris-HCl pH 7.4, 40 µL 200 mM NaF, 40 µL 1 M MgCl₂, 40 µL 1 mM sodium orthovanadate, and 40 µL Igepal) with gentle agitation on ice for 2 h. Cells were centrifuged at 12000 x *g* for 15 min at 4°C to remove cellular debris and the supernatant was retained. Five percent of this supernatant was reserved to be used as the whole cell lysate control and stored at -20°C. The remaining cell lysate was added to a microfuge tube containing pre-washed (with TBS) blank agarose control beads (Thermo Fisher Scientific) in a pre-clearing step to remove proteins that interact with the agarose beads, with gentle agitation on ice for 10 min. The blank beads were pelleted by centrifuging at 500 x *g* for 30 s and the lysate transferred to a microfuge tube containing pre-washed (with TBS) γ-aminophenyl-m⁷GTP agarose C10-linked beads (Jena Bioscience, Jena, Germany). Lysis buffer was then added to wash the blank agarose beads, which were pelleted by centrifugation at 500 x *g* for 30 s and this wash process repeated 5 times before resuspension in lysis buffer and stored at -20°C for use as the non-specific agarose bead binding control. The tube containing cell lysate and γ-aminophenyl-m⁷GTP agarose C10-linked beads was incubated with gentle agitation on ice for 1 h to capture cap-binding proteins. The beads were pelleted by centrifuging at 500 x *g* for 30 s and the supernatant removed and stored at -20°C. Lysis buffer was then added to wash the beads, which were pelleted by centrifugation at 500 x *g* for 30 s and this wash process repeated 5 times before resuspension in lysis buffer and GTP added to a final concentration of 1 mM. The beads + 1 mM GTP was incubated on ice with gentle agitation for 1 h to disassociate proteins that non-specifically interact with m⁷GTP. The beads were then pelleted by centrifuging at 500 x *g* for 30 s and the supernatant removed and stored at -20°C for use as the non-specific GTP-binding control. Lysis buffer was added to wash the beads, which were pelleted by centrifugation at 500 x *g* for 30 s and this wash process repeated 5 times before resuspension in a small volume of lysis buffer and storage at -20°C. This final solution contains the m⁷GTP-bound proteins. All samples and controls were boiled to dissolve the agarose beads prior to immunoblotting as mentioned previously, using 4-20% gradient gels (BioRad).

SuNSET assay

Cells were seeded 4 x 10⁵ cells/well in 6-well plates and allowed to adhere overnight. Cells were then treated with 100 µg/mL cycloheximide for 2 h and/or 1 µM puromycin for 30 min (56) before harvesting and immunoblotting as mentioned previously.

Polysome profiling

For polysome profiling, cells were treated with 100 µg/mL cycloheximide for 5 min at 37°C, washed with hypotonic wash buffer (5 mM Tris-HCl (pH 7.5), 2.5 mM MgCl₂, 1.5 mM KCl, 100 µg/mL cycloheximide) and lysed in a hypotonic lysis buffer (5 mM Tris-HCl (pH 7.5), 2.5 mM MgCl₂, 1.5 mM KCl, 100 µg/mL cycloheximide, 2 mM DTT, 0.5% Triton X-100, 10 U Promega RNasin and 0.5% sodium deoxycholate). Lysates were centrifuged at 16,000 x *g* for 7 min at 4°C. 10% of supernatant volume was collected to control for input and mixed with TRIzol reagent (Invitrogen). The remaining sample was loaded onto a 10–50% linear sucrose density gradient and centrifuged at 41,000 RPM using a SW41 Ti rotor (Beckman Coulter) for 1.5 h at 4 °C. Gradients were fractionated (16 fractions per sample) and optical density was continuously recorded at 260 nm using a Triax Flow Cell FC1 (BioComp). Fractions were

collected and mixed with an equal volume of TRIzol reagent (Invitrogen). Cytoplasmic and polysome-associated RNA were extracted according to the manufacturer's instructions and purified using the Qiagen RNeasy Mini Kit (Qiagen 74106). For library preparation, rRNA was depleted using NEBNext® Ultra™ II Directional RNA Library Prep Kit. Libraries were sequenced on the Illumina NextSeq2000 platform.

Coding genes were selected using biomaRt. Anota2seq was used for normalization using the "TMM-log2" option, perform QC analyses, residual outlier tests, building the contrast matrix, select significant genes in the total mRNA and translated mRNA fractions, as well as the identification of translation and buffering. To select genes that were significant for translation, a maximum slope of 2 and a minimum slope of -1 was used, and for genes that were significant for buffering we used a minimum slope of -2 and a maximum slope of 1. An adjusted p-value of 0.05 was used as a cutoff.

Transcriptome Architecture and Feature Analysis

To characterize the structural properties of translationally regulated mRNAs, genomic features were extracted from the GRCh38 GTF annotation. To avoid isoform-specific bias, analysis was restricted to the canonical transcript for each gene, defined as the isoform with the longest total width. For each canonical transcript, the lengths of the whole transcript, 5' untranslated region (UTR), 3' UTR, and coding sequence (CDS), as well as total exon count, were calculated.

Gene expression from polysome-seq was quantified using Salmon, and genes with low abundance (BaseMean < 50, normalized via DESeq2, using only whole-cell input samples) were excluded from downstream analysis to ensure robust feature comparison. Translationally regulated genes were identified based on translation efficiency (TE) changes ($\log_2FC > 0.5$, $p_{adj} < 0.05$). High-translated (n=24) and Low-translated (n=25) groups were compared against a count-matched background of "Unchanged" genes ($\log_2FC < 0.1$, $p_{adj} > 0.5$, n=24). Statistical significance for feature length differences (CDS, UTR, Exons) was determined using two-sided Wilcoxon rank-sum tests, comparing regulated groups to the unchanged background.

Statistical analyses

All statistical analyses were carried out using GraphPad Prism, Version 9.2 (GraphPad Software Inc, San Diego, CA) or Microsoft Excel, Version 16.56, unless otherwise specified. GI50 dose of single agents was determined by fitting the Hill equation. The difference between data groups was analyzed using two-tailed Student's *t*-test or ANOVA with Tukey's multiple comparisons post hoc test as indicated in the manuscript. Correlations were determined using Pearson's R. Significant overlap between large gene sets was determined by calculating representation factor [representation factor = # genes in common / (# in group one x # in group 2) / 20471 genes in genome] and using normal approximation of hypergeometric probability <<http://nemates.org/MA/progs/representation.stats.html>> . Total number of genes in human genome obtained from <Permalink: http://Apr2022.archive.ensembl.org/Homo_sapiens/Info/Annotation>. Data are represented as mean ± standard error of the mean (SEM) unless otherwise specified. *P*-values < 0.05 were considered as statistically significant.

Data availability

The data generated in this study are available upon request from the corresponding author.

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Figure 1

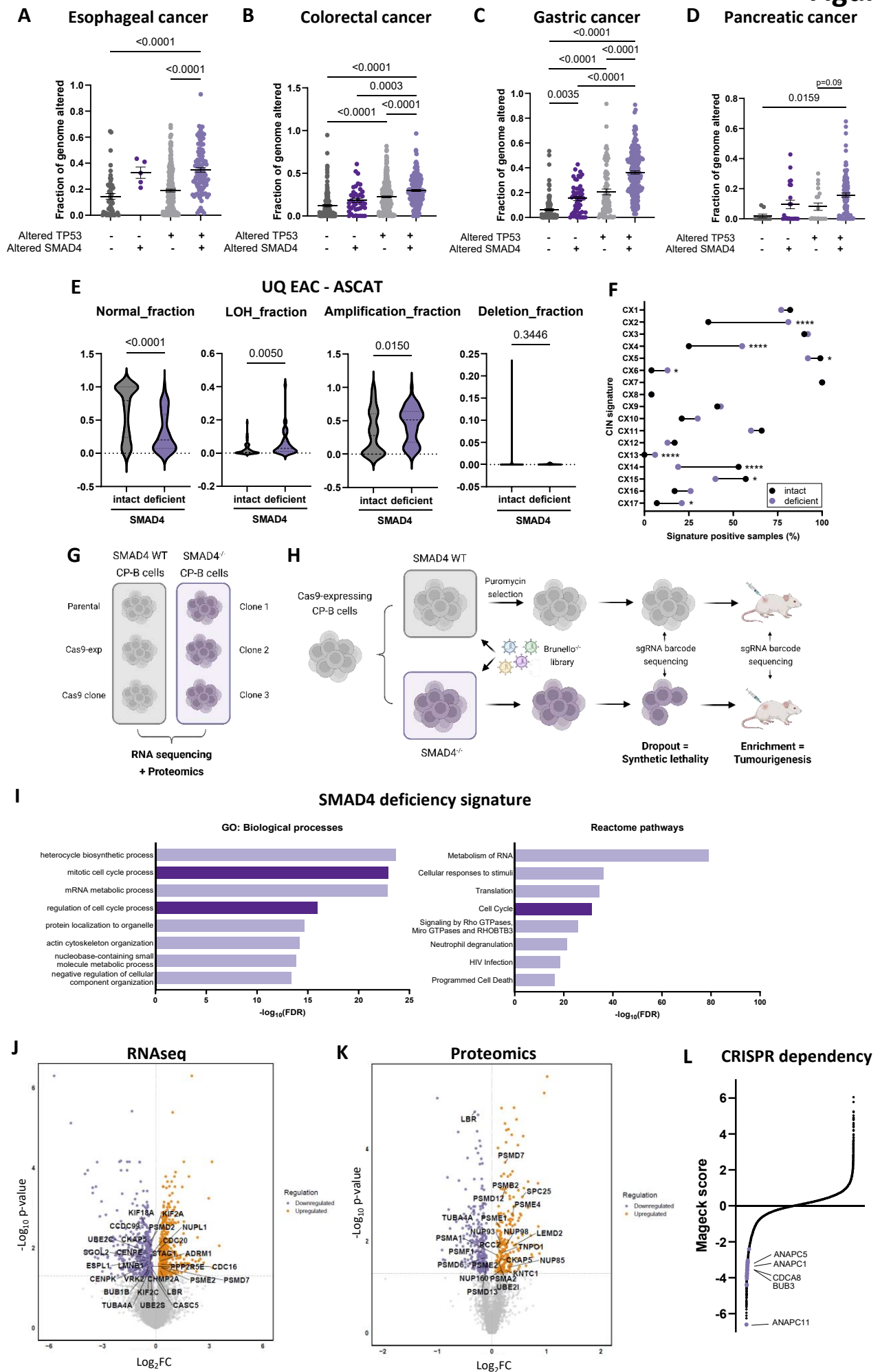


Figure 1 SMAD4 loss models chromosomal instability in GI cancers and uncovers a deregulated mitosis signature.

Dot plots showing fraction of the genome altered in TCGA and MSK (A) esophageal adenocarcinoma, (B) colorectal, (C) gastric or (D) pancreatic cancer patient samples as a function of alteration in *SMAD4* or *TP53* genes. Error bars represent SEM. (E) Violin plots indicating median and quartiles of chromosomal aberrations in UQ EAC patient samples, stratified by *SMAD4* status. (F) Dumbbell plot depicting presence of CIN signatures in UQ EAC patient samples. Schematics showing the workflow for the (G) RNA-sequencing and proteomics experiments and (H) *in vitro* CRISPR-Cas9 knockout screen using Brunello genome-wide knockout library (Brunello^{-/-}). (I) Bar charts depicting top significant Biological processes and Reactome pathways in *SMAD4* deficient cells. Volcano plots depicting differentially expressed (J) genes and (K) proteins between CP-B parental and CP-B *SMAD4*^{-/-} cells as determined by RNA sequencing and LC-MS/MS, respectively. (L) Scatter plot depicting ranked MaGeCK scores, where <0 = dropout and >0 = enrichment of knockout. (A-D) Ordinary one-way ANOVA with Tukey's multiple comparisons test, (E) unpaired Student's t-test, (F) multiple t-tests.

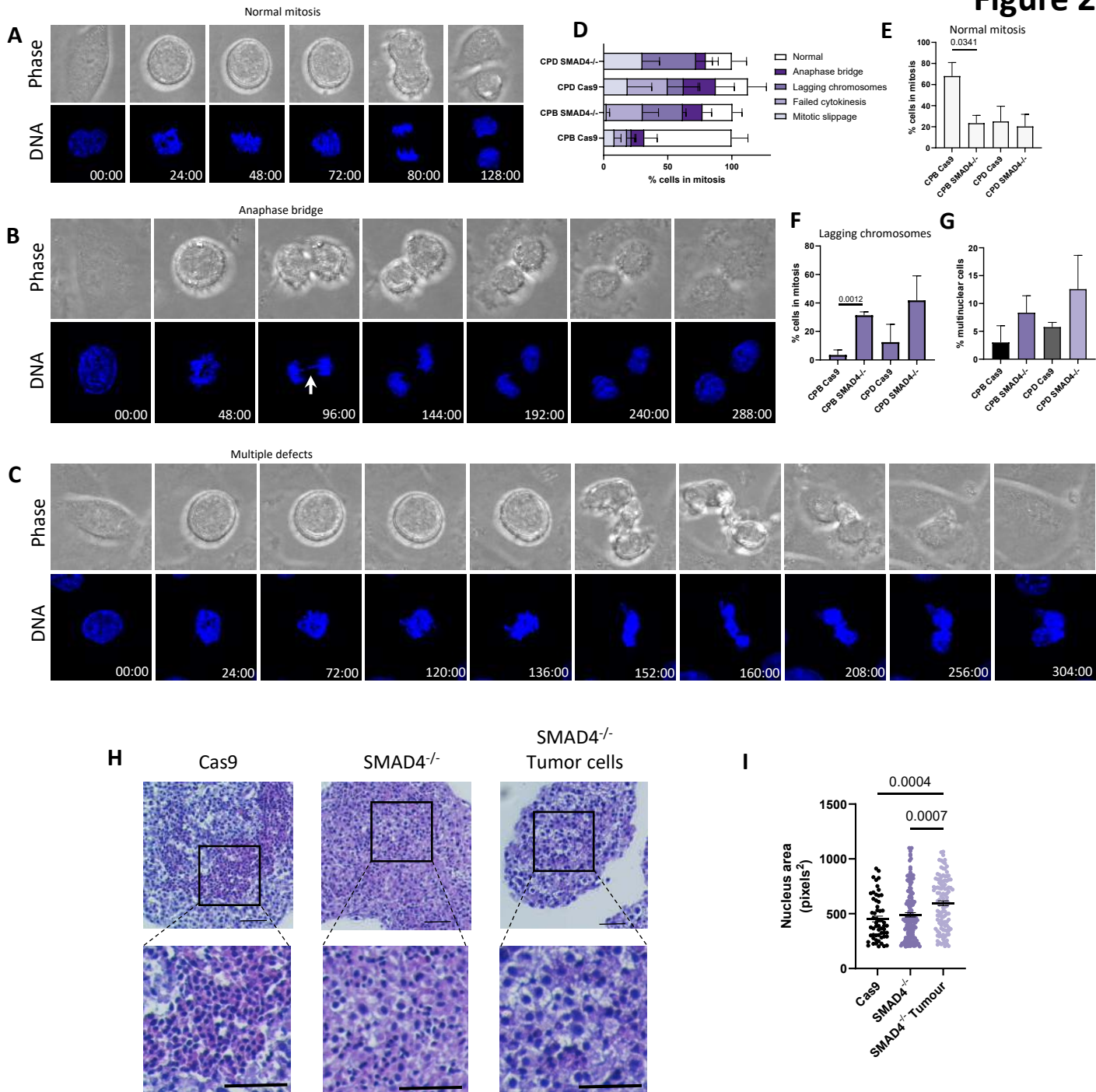


Figure 2 Loss of SMAD4 increases mitosis defects in Barrett's esophagus cells. Time lapse images of CP-B SMAD4^{-/-} cells stained with SPY650-DNA showing examples of (A) normal mitosis, (B) lagging chromosomes and (C) failed cytokinesis. Bar charts showing percentage of (D) mitotic cells with mitosis defects (range 9-38 cells in mitosis visualised per cell line), percentage of cells with (E) normal mitoses, (F) lagging chromosomes, and (G) multiple nuclei (87-170 cells visualised per cell line). (H) Haematoxylin and eosin staining of CP-B cells grown in 3-dimensional spheroids. Scale bars represent 50 μ m. (I) Quantitation of nuclei area in CP-B cell spheroids. Solid bar represents mean, error bars represent SEM. Ordinary one-way ANOVA with Tukey's multiple comparisons test.

Figure 3

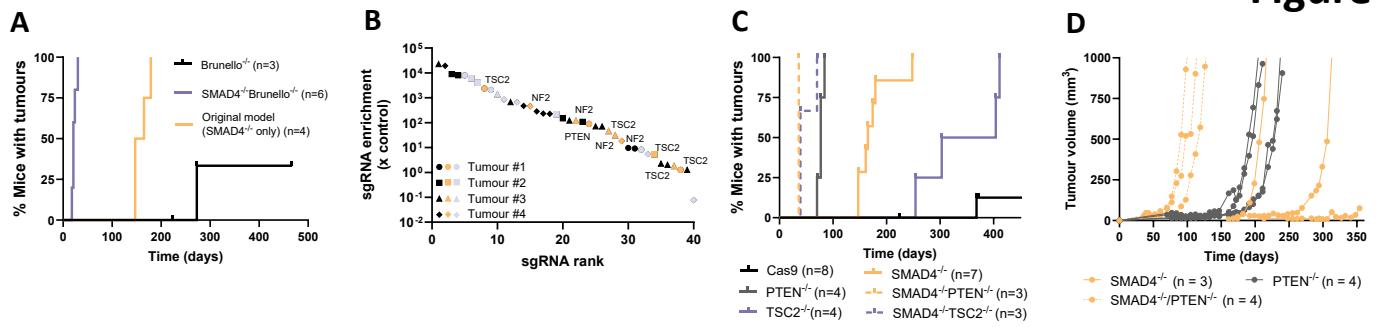


Figure 3 SMAD4 loss and mTOR deregulation co-operate to drive tumorigenesis. (A) Kaplan-Meier plot depicting percentage of mice with tumors over time in CRISPR-Cas9 knockout screen. (B) Scatter plot showing enrichment of individual sgRNAs as identified in tumors from tumorigenesis screen. Orange symbols represent canonical regulators of PI3K/Akt/mTOR signaling and grey symbols represent secondary regulators. (C) Kaplan-Meier plot depicting percentage of mice with tumors over time in single sgRNA validation experiments. (D) Plot showing growth rate of tumors in single sgRNA validation experiments.

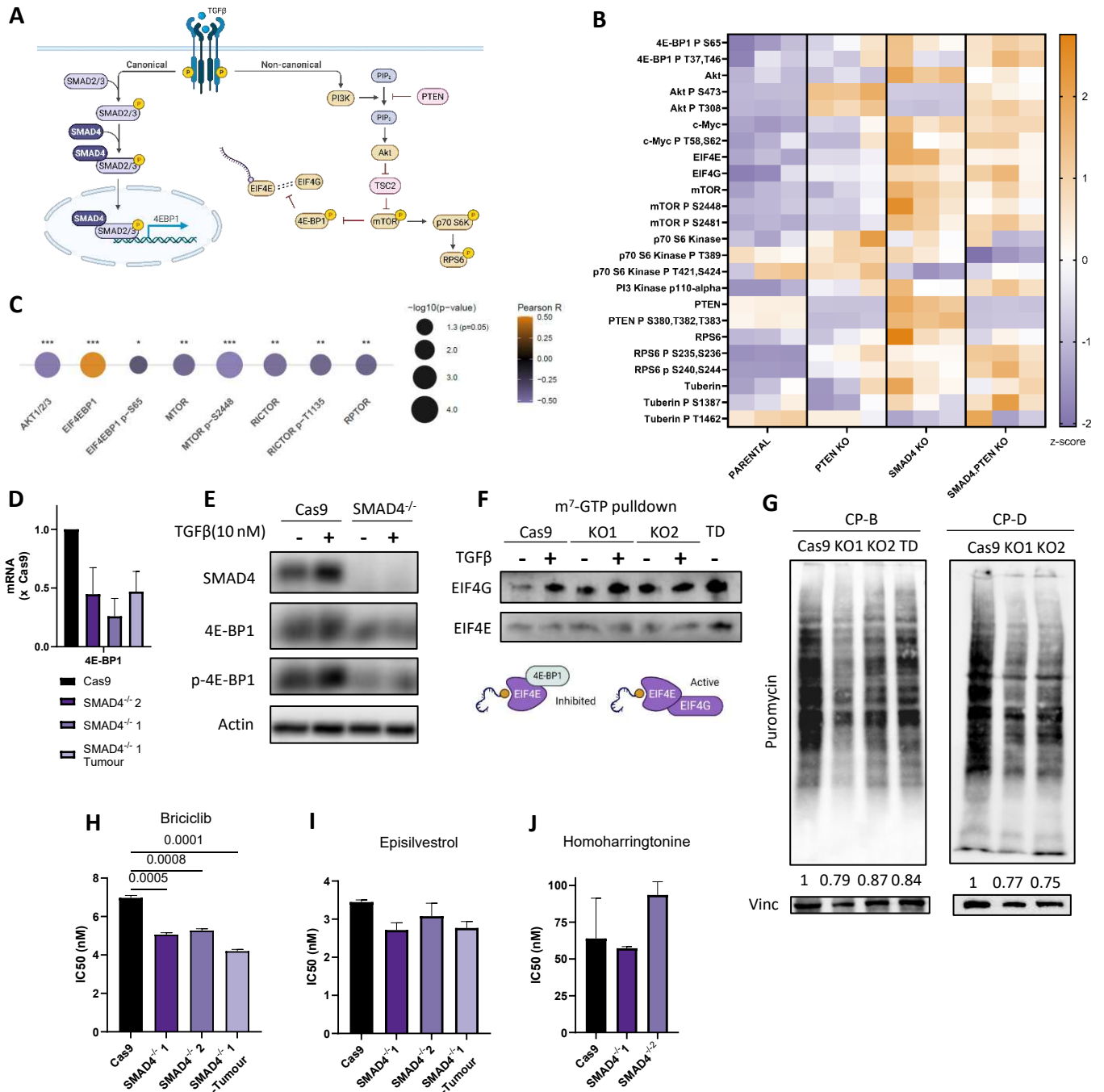


Figure 4 SMAD4-deficient cells and tumors upregulate mTOR signaling and cap-dependent translation initiation. (A) Schematic depicting intersection of TGF β and PI3K/Akt/mTOR pathways. (B) Heat map depicting protein and phospho-protein expression in CP-B cells as determined by RPPA, depicted as z-scores. (C) Bubble plot showing correlations between SMAD4 protein and PI3K/Akt/mTOR pathway proteins and phospho-proteins in TCGA EAC patient samples. (D) Histogram depicting 4E-BP1 mRNA levels in CP-B cells ($n = 3$). Error bars represent SEM. (E) Immunoblot showing SMAD4, 4E-BP1 and p-4E-BP1 protein levels in CP-B cells in response to 6 h exposure to TGF β (10 nM). Actin was used as a loading control. (F) (Top) Immunoblot of m7-GTP cap-binding assay showing eIF4G and eIF4E proteins bound to m7-GTP resin. KO1/2 = SMAD4 $^{-/-}$ 1/2, TD = SMAD4 $^{-/-}$ tumor-derived and (bottom) simplified schematic of eIF4E activated and inhibited states. (G) Immunoblot showing puromycin incorporation into cell lines in SuNSET assay. Vinculin (Vinc) was used as a loading control. Histograms depicting IC50 of (H) Briciclib, (I) Episilvestrol and (J) Homoharringtonine in cell lines ($n=3$).

Figure 5

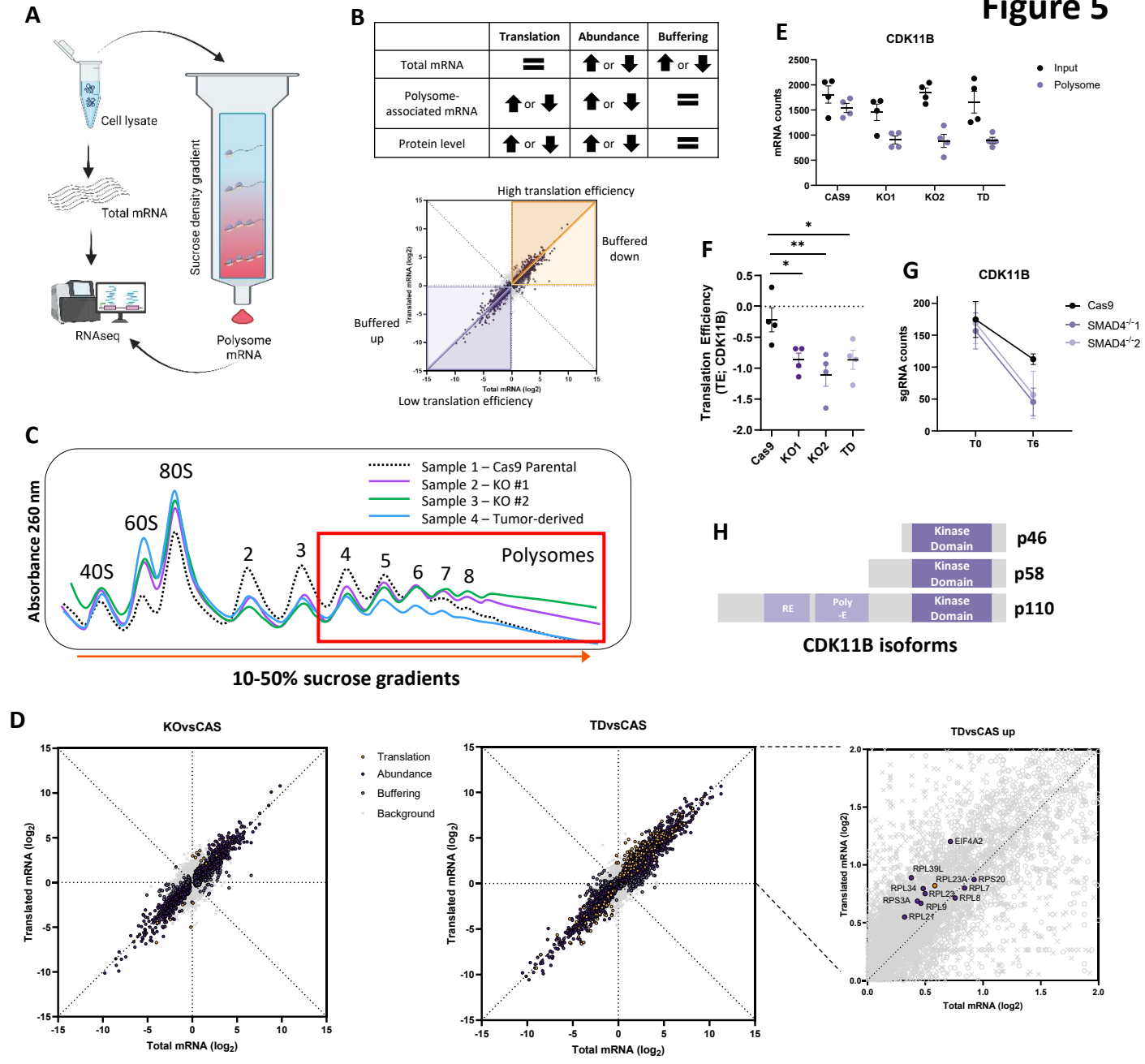


Figure 5 Translational reprogramming in SMAD4-deficient cells results in post-transcriptional repression of CDK11B.

Schematics depicting (A) workflow of polysome profiling and (B) interpretation of polysome-sequencing. (C) Polysome traces CP-B cells treated on a 10–40% sucrose gradient (representative of $n = 4$ biologically independent experiments). (D) Scatterplots showing Log2 fold change ($\log_2FC$) total mRNA vs polysome-bound (translated) mRNA in CP-B cells ($n = 4$), (left) *SMAD4*^{-/-} (KO) versus Cas9 parental (CAS), (centre) *SMAD4*^{-/-} tumor derived (TD) versus Cas9 parental (CAS) and (right) upper right quadrant of TDvsCAS blot showing upregulated mTOR-sensitive mRNA. (E) Plots depicting mRNA counts of whole cell and polysome-bound CDK11B mRNA and (F) translation efficiency of CDK11B in CP-B cells. (G) Raw sgRNA counts for CDK11B in CP-B cells at day 0 (T0) and 6 (T6) in genome-wide CRISPR-Cas9 screen. (H) Schematic illustrating protein isoforms of CDK11B.

Figure 6

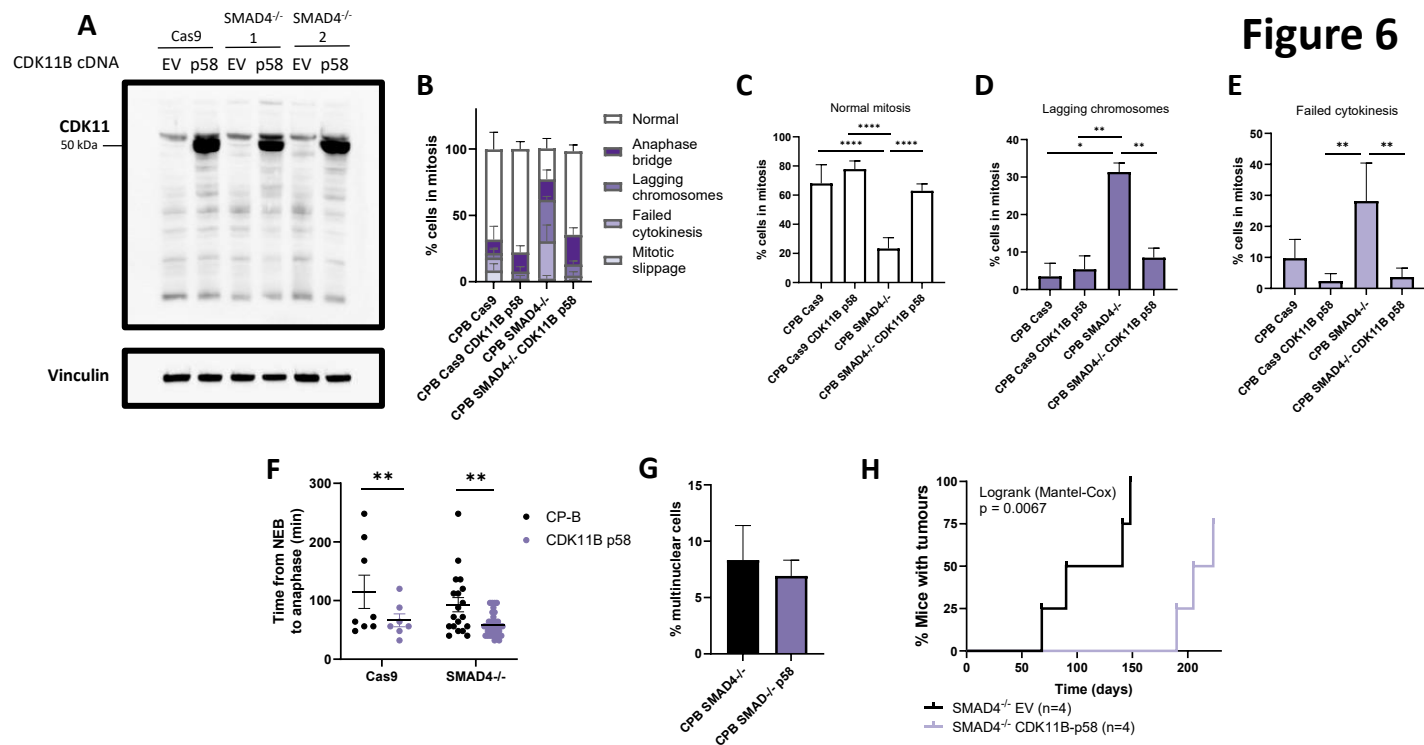


Figure 6 Re-introduction of p58 isoform of CDK11B rescues mitosis defects and suppresses tumorigenesis.

(A) Immunoblot showing CDK11 protein expression in CP-B cells following ectopic expression of CDK11B-p58 cDNA construct. Bar charts showing percentage of (B) mitotic cells with mitosis defects (range 9-38 cells in mitosis visualised per cell line), percentage of cells with (C) normal mitosis, (D) lagging chromosomes, and (E) failed cytokinesis. (F) Plot showing time from nuclear envelope breakdown (NEB) to anaphase in CP-B cells following ectopic expression of CDK11B-p58 cDNA construct. (G) Bar chart showing percentage of multinuclear cells detected via live cell imaging of CP-B cells following ectopic expression of CDK11B-p58 cDNA construct. (H) Kaplan-Meier plot depicting percentage of mice with tumors over time in CP-B *SMAD4*^{-/-} cells following ectopic expression of CDK11B-p58 cDNA construct or empty vector (EV). NB: "CPB Cas9" and "CPB *SMAD4*^{-/-}" data (i.e. untransduced controls) are repeated from Figure 2.

Supplementary Files

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