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Autophagy acts through TRAF3 and RELB to regulate gene expression via antagonism of SMAD proteins

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Macroautophagy can regulate cell signalling and tumorigenesis via elusive molecular mechanisms. We establish a RAS mutant cancer cell model where the autophagy gene ATG5 is dispensable in A549 cells in vitro, yet promotes tumorigenesis in mice. ATG5 represses transcriptional activation by the TGFβ-SMAD gene regulatory pathway. However, autophagy does not terminate cytosolic signal transduction by TGFβ. Instead, we use proteomics to identify selective degradation of the signalling scaffold TRAF3. TRAF3 autophagy is driven by RAS and results in activation of the NF-κB family member RELB. We show that RELB represses TGFβ target promoters independently of DNA binding at NF-κB recognition sequences, instead binding with SMAD family member(s) at SMAD-response elements. Thus, autophagy antagonises TGFβ gene expression. Finally, autophagy-deficient A549 cells regain tumorigenicity upon SMAD4 knockdown. Thus, at least in this setting, a physiologic function for autophagic regulation of gene expression is tumour growth.
Mac autophagy (hereafter autophagy) is a major cytosolic degradative pathway that participates in cellular metabolism, homeostasis and anti-microbial defence. Upstream stress signals converge on proteins involved in biogenesis of a double-membraned vesicle known as the autophagosome. This core autophagy machinery includes ATG5, which predominantly exists in a protein–protein conjugate with ATG12 (ATG5–12), and other key players such as the FIP200/ULK1 complex. These proteins act upstream of the recruitment of ATG8-family ubiquitin-like proteins, such as LC3B, to nascent autophagic membranes, via lipidation of their C-terminal glycine residues with phosphatidylethanolamine. Fully formed, enclosed autophagosomes sequester cytosolic cargo that is in turn degraded upon autophagosomal–lysosomal fusion.

Autophagic cargo can comprise general cytosol. However, autophagy pathways may also select specific cargoes for degradation, for example damaged mitochondria, bacteria or protein aggregates. Notably, termination of cystosolic signalling events by selective autophagy (signalphagy) is emerging as an important mediator of cell fate, although this has been less widely analysed. Selective autophagy is facilitated by bifunctional ‘cargo receptors’ that bind both to ATG8-family proteins, and, directly or indirectly, to selected ubiquitinated cargoes. The prototypical cargo receptor is p62 (SQSTM1). However, other, less well-characterised cargo receptors also participate, including nuclear dot protein 52 kDa (NDP52), which was identified first as a mediator of bacterial autophagy and latterly as a component of the mitochondrial autophagy apparatus.

A rich, yet complex, scenario for unravelling signalling functions of selective autophagy is tumorigenesis. RAS small GTPases are oncogenically activated in numerous cancers and generally drive elevated autophagy activity in order to support tumorigenesis, with some notable exceptions. Altered metabolism and mitophagy may have a role here. However, other molecular mechanisms remain to be identified. Hypothetically, these could encompass signalphagy events that would participate in signalling cross-talk downstream of RAS with other tumour-relevant pathways and consequently mediate reprogramming of gene expression. Indeed, some recent studies illustrate the potential for gene regulation by autophagy, such as inhibition of inflammatory gene expression via degradation of TBK1 and its substrate, the transcription factor IRF3, or, on senescence-associated degradation of the transcription factor GATA4.

Nonetheless, the prevalence of signalphagy-mediated transcriptional regulation is largely unexplored. We recently proposed that non-canonical (alternative) NF-κB signalling, involving the RELB transcription factor, may be dependent upon ATG5, presumably via an as-yet-undefined selective autophagy pathway. However, the mechanism and significance of this is unclear.

An important signalling molecule that regulates gene expression is transforming growth factor β (TGFβ). TGFβ ligates receptor serine–threonine kinases, ultimately resulting in cytosolic phosphorylation of selected transcription factors of the SMAD family, such as SMAD2 and SMAD3. Contingent upon this, heteromeric SMAD assemblies, such as SMAD 2/2/4, SMAD 3/3/4 and, possibly, SMAD2/3/4 complexes, translocate to the nucleus and bind SMAD–response element (SREs) at proximal promoters to drive transcription. The TGFβ transcriptome exerts pleiotropic effects on tumour biology. On the other hand, TGFβ-driven transcriptional changes also underpin epithelial–mesenchymal transition (EMT) and enhanced metastatic abilities of cancer cells. This latter occurs particularly during cancer progression when resistance or insensitivity to the anti-proliferative effects of TGFβ are evident. Such insensitivity may be acquired during the evolution of a tumour. Indeed, RAS mutant cancer cells commonly exhibit decreased sensitivity to the anti-tumorigenic effects of the TGFβ ligand. In certain settings, such as some pancreatic cancers, this may occur by mutation, for example deletion of SMAD4. However, resistance of RAS-driven cancer cells to anti-tumorigenic effects of TGFβ may occur via alternate, unknown mechanism(s) in other settings.

Here we show that autophagy is required for tumour formation in mice by RAS-mutant cancer cells. We identify transcriptional reprogramming via the SMAD proteins when autophagy is inhibited. We discover that a SMAD–RELB complex ordinarily represses transcription at TGFβ target genes. This is independent of the conventional DNA-recognition activity of RELB but requires indirect recruitment of RELB to genes via SMAD(s). Activation of RELB, and consequent antagonism of TGFβ, occurs specifically by RAS-mediated engagement of autophagy. This autophagy facilitates NDP52-mediated degradation of the signal terminator for the alternative NF-κB pathway, TRAF3. Finally, cross-talk with TGFβ via autophagy/RELB is required for the promotion of tumorigenesis in mice by A549 lung cancer cells.

Results

Autophagy promotes tumorigenesis. To investigate the effect of stable autophagy inhibition in RAS-mutated human cancer cells, we used CRISPR/Cas9 to ablate ATG5 expression in A549 lung adenocarcinoma cells (Fig. 1a, b). Inhibition of autophagy was confirmed by accumulation of unmodified LC3B-I protein at the expense of lipidated LC3B-II (Fig. 1a, ATG5Δ vs. wild-type, WT, controls). Surprisingly, ATG5Δ cells had no differences in growth kinetics compared to controls (Fig. 1c). This is in contrast to the reported anti-proliferative effects of acute autophagy inhibition in numerous RAS-mutated human cell types. Indeed, RNA interference (RNAi) against ATG5 and FIP200 inhibited proliferation of the parental A549 cell population (Supplementary Fig. 1a, b, but notably did not kill cells (Supplementary Movies 1–3). Thus, autophagy is not required for the long-term proliferative potential of cells in vitro.

We next investigated in vivo tumorigenicity, using a subcutaneous xenograft model. To ensure that differences between WT and ATG5Δ cells were attributable to autophagy, we stably expressed GFP-ATG5 in one clone of ATG5Δ cells, rescuing LC3B lipidation and p62 regulation (Fig. 1d). Using these cell lines (Fig. 1e) or an alternate pair of wild-type and ATG5Δ-deleted clones (Fig. 1f), we observed that loss of ATG5 markedly diminished tumour growth kinetics in vivo. Consistent with the in vitro analyses, impairment of autophagy was detected in ATG5Δ tumours, as shown by a loss of LC3B puncta by immunohistochemistry (Fig. 1g).

The above data show that sustained and complete loss of autophagy does not necessarily impede RAS-mutant cancer cell proliferation in vitro. However, autophagy can be a contributor to physiological tumour growth in this same cell type.

Autophagy represses TGFβ-driven gene expression. We next sought to identify mechanism(s) by which autophagy was promoting tumorigenesis. We performed global gene expression analysis of A549 cells after RNAi against core autophagy genes ATG5 or ULK1 (Supplementary Data set 1). These siRNA reagents have previously been validated in these cells. We aimed to extract differentially expressed, ontologically coherent transcript sets that would illuminate transcriptional reprogramming events regulating tumorigenesis. Accordingly, computational gene set enrichment analysis (GSEA) was performed. Changes in transcript levels upon autophagy inhibition were compared with known oncogenic and tumour suppressive pathway readouts. Surprisingly, the highest scoring correlation was with transcripts...
that are upregulated by the growth factor TGFβ (Fig. 2a). Indeed, two-fifths of the 50 most autophagy-repressed transcripts were identified by manual curation as either direct or indirect targets of transcriptional upregulation by TGFβ, referred to collectively hereafter as ‘TGFβ-driven genes’ (Fig. 2b, consult Supplementary Table 1 for a detailed justification of gene classification).

We selected a subset of the transcripts identified in Fig. 2b for further analyses, in order to elucidate the molecular mechanisms underlying the apparent antagonism of TGFβ function by autophagy. Firstly, quantitative real-time PCR (qRT-PCR) after ATG5 RNAi confirmed repression of TGFβ-driven genes by autophagy, either under basal growth conditions or upon stimulation by exogenous TGFβ ligand (Fig. 2c). ULK1 RNAi also enhanced gene expression to a comparable extent (Fig. 2d).

The persistent autophagy defect in non-tumorigenic A549 ΔATG5 cells was also associated with upregulation of such transcripts (Fig. 2e). Repression of TGFβ-driven genes by autophagy was also evident upon analysis of orthologous transcripts, basally or after TGFβ treatment, in RAS-transformed mouse embryonic fibroblasts (MEFs) where Atg5 was deleted (Fig. 2f, MEF KRAS-V12 cells, congenic WT or Atg5−/−). Thus, the apparent TGFβ-inhibitory function of autophagy is manifest in different cell lineages and across species.

We inferred from the above data that autophagy antagonises TGFβ signalling, at least at the level of target gene output. Indeed, we confirmed that the basal levels of known TGFβ-driven transcripts in our data were dependent upon tonic TGFβ-dependent signal transduction, using an ALK2/4/5 receptor serine-threonine kinase inhibitor (ALKi) (Supplementary Fig. 2b). The cytosolic signalling events in the TGFβ pathway can be read out in phosphorylation of SMAD2 and SMAD3. However, no increases in basal or exogenous TGFβ-stimulated phospho-SMAD species were detected in A549 ΔATG5 or MEF KRAS-V12 Atg5−/− cells (Fig. 2g, h). These data raise the question of a non-cytosolic site of convergence between molecular events downstream of autophagy and TGFβ.

RAS drives RELB signalling via autophagy of TRAF3. We hypothesised that autophagy might selectively target cytosolic proteins with hitherto unknown functions in regulating the nuclear output of TGFβ signalling. To identify such proteins, we performed mass spectrometric screening for interactors of known cargo receptors. Of note, putative NDP52 interactors included a number of signal transduction mediators (Fig. 3a, Supplementary Data set 2). In particular, we identified tumour necrosis factor receptor-associated factor 3 (TRAF3), a cytosolic scaffold that

Fig. 1 Autophagy promotes tumorigenesis in vivo. a A549 cells deficient in ATG5 protein expression were generated by CRISPR/Cas9-mediated genome editing. Each of the two wild-type control clones (WT and WT-2) and ΔATG5 clones (ΔATG5 and ΔATG5-2) were immunoblotted for the indicated proteins. b Genomic DNA from A549 ΔATG5 and ΔATG5-2 cells was PCR amplified. The amplicon encompassed the sequence to which Cas9 had been targeted (PAM in bold) and different indels were identified in both clones via Sanger sequencing. c A549 WT and ΔATG5 cells were plated at low confluency for time-lapse phase-contrast videomicroscopy using an Incucyte microscope and cell proliferation was monitored by automated confluence analysis at set intervals post plating (means, n = 9 wells, ±S.D.). d A pooled derivative of ΔATG5 cells was generated by stable transduction with GFP-ATG5 retrovirus (rescue). The indicated cell lines were immunoblotted as shown. e WT, ΔATG5 and rescue cells were subcutaneously injected into immunocompromised mice and tumour volume was measured longitudinally (means, n = 10 flanks, ±S.E.M., *P < 0.05 vs. WT, two-tailed t-test). f WT-2 and ΔATG5-2 cells were compared for tumour growth after subcutaneous injection into immunocompromised mice (means, n = 12 flanks, ±S.E.M., *P < 0.05 or **P < 0.01 vs. WT-2; two-tailed t-test). g At the end of tumour growth in e, control tumours and sufficiently large ΔATG5 tumours were fixed and stained for LC3B via immunohistochemistry (DAF stain, arrows indicate regions of LC3B puncta, scale bar = 10 μm). Representative images are shown here. Uncropped blots are available in Supplementary Fig. 10.
**Fig. 2** Autophagy suppresses TGFβ target gene expression. **a** siRNA oligonucleotides targeting ATG5 and ULK1, or two sequence-unrelated non-targeting controls, were transfected in A549 cells in each of the three independent replicates. Expression profiles were obtained from an Illumina bead array, summarised in Supplementary Data set 1. Gene set enrichment analysis was performed, comparing genes differentially expressed in the autophagy inhibited cells with known oncogene- and tumour suppressor-driven expression profiles. The comparison yielding the greatest normalised enrichment score (NES), 'TGFβ upregulated genes', is shown here. **b** Fold-change heat map showing the top 50 upregulated genes after RNAi. Mean siATG5 or siULK1 siRNA values are normalised to the averaged means of the two non-targeting controls (siCtrl s). Blue text indicates genes that are known to be transcriptionally upregulated by TGFβ (Supplementary Table 1). **c-f** qRT-PCR was performed for the indicated transcripts after the following treatment regimens. **c** A549 cells were transfected with siCtrl or siATG5 for 72 h. Cells were either left untreated (Ctrl) or treated with 5 ng/ml TGFβ1 for the final 16 h of transfection. **d** A549 cells were transfected with siCtrl, siATG5 or siULK1 for 72 h. **e** Exponentially dividing A549 WT and ΔATG5 cells were compared. **f** Congenic wild-type (WT) or ΔATG5 null (ΔATG5−/−) MEF KRAS-V12 cells were left untreated (Ctrl) or treated with 5 ng/ml TGFβ1 for 16 h (mean, n = 3, ± S.D., *P < 0.05 or **P < 0.01 vs. siCtrl or similarly treated WT cells, two-tailed t-test). **g** A549 WT and ΔATG5 cells and WT and ΔATG5−/− MEF KRAS-V12 cells, with (+) or without (−) prior treatment with 5 ng/ml TGFβ1 for 16 h, were lysed and immunoblotted (P-SMAD2 = phospho-S465/467 SMAD2, P-SMAD3 = phospho-S423/425 SMAD3). Uncropped blots are available in Supplementary Fig. 10.
represses the activation and nuclear translocation of the alternative NF-kB transcription factor, v-rel avian reticuloendotheliosis viral oncogene homolog B (RELB)33, 36, 37, yet has unclear functions or transcriptional activity26, 36, 37. RELB is emerging as an important player in various non-
leukaemic cancers, including those dependent upon RAS signalling26, 36, 37, yet has unclear functions or transcriptional targets in non-haematopoietic lineages35, 38, 39. Thus, we decided to prioritise investigation of the NDP52-TRAF3 interaction.

In HEK293T cells, FLAG-tagged TRAF3 co-immunoprecipitated with NDP52, via the cargo-binding zinc finger (ZnF) domain of the latter (Fig. 3b). Furthermore, an endogenous NDP52-TRAF3 complex could be immunoprecipitated from A549 cells (Fig. 3c). Thus, TRAF3 binds NDP52 in the mode of an autophagic cargo13. We next sought to determine via immunofluorescence whether the NDP52-TRAF3 complex was targeted to autophagic intermediates. As expected40, most TRAF3 was found to target to the Golgi (Supplementary Fig. 3a). However, distinct TRAF3 foci localised with NDP52 and LC3B puncta, indicative of autophagosomal targeting (Fig. 3d). Furthermore, RNAi-mediated silencing of NDP52 both abrogated the localisation of TRAF3 with LC3B foci (Fig. 3e) and increased endogenous TRAF3 protein levels (representative blot in Fig. 3f, quantified in Supplementary Fig. 3b). Pooled derivatives of A549 cells were generated where NDP52 was eliminated via CRISPR/Cas9. These also had elevated TRAF3 levels (Fig. 3g). Furthermore, TRAF3 targeting to lysosomes, the end-point of autophagy pathway, was supported by the colocalisation of TRAF3 foci with the lysosomal marker LAMP2 after a brief treatment with the vacuolar H+ ATPase inhibitor, Bafilomycin A1 (BafA1) (Supplementary Fig. 3c). The above data suggest that TRAF3 is degraded by autophagy.

Readouts of low TRAF3 function include stabilisation of NF-κB (NIK), the subsequent processing of p100 to p52, which heterodimerises with RELB, and the eventual nuclear translocation of RELB (Supplementary Fig. 4). Supporting the hypothesis of TRAF3 degradation, TRAF3 protein levels were increased post-transcriptionally, and all of the above readouts were diminished, when autophagy was inhibited by RNAi against ATG5 or FIP200 in A549 cells (Supplementary Fig. 5a, Supplementary Fig. 5b, c). The same differences were seen when comparing A549 ΔATG5 cells with control WT cells (Fig. 4b, c). In addition, similar increases in TRAF3 levels and loss of p100 processing were also observed after ULK1 RNAi (Supplementary Fig. 5c–e) or in FIP200 deleted cells (Fig. 4d). NDP52 or FIP200 deletion also phenocopied ATG5 loss in elevation of TGFβ-target gene transcript levels (Supplementary Fig. 5f) and inhibition of tumour growth in vivo (Fig. 4e). The ATG5-dependency of TRAF3 levels and transcriptional events were also demonstrated by CRISPR-Cas9 deletion of ATG5 in a second RAS-mutant lung cancer line, NCI-H23 (Fig. 4f, Supplementary Fig. 6a). Furthermore, autophagy-dependent p100 processing, nuclear RELB, and the autophagic dependency of

Fig. 3 Autophagic degradation of TRAF3 via the cargo receptor protein NDP52. a FLAG-HA-NDP52 was used as bait for co-immunoprecipitation LC-MS/MS from A549 cells. Hits were identified using CompPASS thresholding for high-confidence interacting proteins (HCIPs) and by inclusion of immediate subthreshold interactors on the proviso of known secondary interaction with at least two HCIPs (BioGRID). Known interactors of NDP52 were equally distributed among HCIP and subthreshold hits. b HEK293T cells were co-transfected with FLAG-TRAF3 and either empty vector (–) or myc-tagged forms of NDP52 (full-length, FL, or deluents of the N-terminal SKICH domain, ΔSKICH, or C-terminal Zinc Fingers, ΔZnF). Anti-myc immunoprecipitation (IP) was performed followed by immunoblotting. c A549 lysates were immunoprecipitated with anti-NDP52 antibody-conjugated beads or control rabbit IgG beads and immunoblotted. d A549 cells stably expressing FLAG-HA-TRAF3 were stained for indicated epitopes and analysed by confocal microscopy (scale bar = 10 μm). LC3B and NDP52 are both false-coloured green and are overlaid separately with magenta HA (TRAF3) in merge panels. Arrowheads indicate co-localising foci. Boxes correspond to zoomed insets. e A549 cells expressing FLAG-HA-TRAF3 were transfected with the indicated siRNA for 72 h and then cells were stained for confocal microscopy (scale bar = 10 μm). Arrowheads indicate dual foci of TRAF3 and LC3B, quantified on a per cell basis (means, n = 3, ±s.E.M., *P < 0.05, two-tailed t-test). f A549 cells were transfected with siCtrl or siNDP52 for 72 h and immunoblotted as shown. g A549-Cas9 control cells or pooled ΔNDP52 counterparts were immunoblotted as shown. Uncropped blots are available in Supplementary Fig. 10.
TRAF3 regulation, were all apparent in MEFs, but only when these cells were transfected with RAS (Fig. 4g, h, Supplementary Fig. 6b, c). Thus, autophagy-mediated TRAF3 regulation and consequent RELB activity are strongly linked to RAS activation. To show that autophagy-mediated turnover of TRAF3 is indeed the mechanism of regulation of NF-κB, we reverted TRAF3 levels using RNAi in Atg5−/− MEF KRAS V12 cells (Fig. 4i). This rescued p100 processing, indicating that blockade of NF-κB signalling due to loss of ATG5 was overcome. Knockdown of TRAF3 in A549 ΔATG5 cells also partially rescued growth in vivo (Supplementary Fig. 6d), although tumours did eventually regress. The eventual regression is in line with an additional observation made that complete loss of TRAF3 actually impairs tumorigenicity of A549 cells (Supplementary Fig. 6e). These data suggest that the elevated TRAF3 level in autophagy-deficient cells does suppress tumorigenicity. However, TRAF3 has other functions that contribute to long-term tumour growth. Notably, in non-RAS mutant murine cells there was minimal evidence of TRAF3 turnover by potent autophagy stimuli such as amino-acid starvation (Supplementary Fig. 6f).

Taken together, the above data show that autophagy is important in TRAF3 turnover downstream of RAS, which links to activation of nuclear RELB and tumorigenesis.

**RELB represses TGFβ-driven gene transcription.** Deletion of RELB phenocopies inhibition of autophagy in preventing growth of A549 cells in vivo (Fig. 5a, b). We speculated that RELB might be responsible for both the maintenance of tumorigenicity and the suppression of TGFβ downstream of autophagy. We further hypothesised that nuclear RELB might, directly or indirectly, repress transcription of TGFβ-driven genes. Thus, we performed gene expression profiling of A549 cells after silencing of RELB (Fig. 5c, Supplementary Data set 1). Strikingly, GSEA identified that, just as with inhibition of autophagy, the top-ranking correlation of the transcriptional signature of RELB deficiency was...
Fig. 5 RELB promotes tumorigenesis and suppresses TGFβ target gene expression. a A549 ΔRELB cell clones (ΔRELB and ΔRELB-2) generated using CRISPR/Cas9 and two independent gRNA sequences were immunoblotted as shown. b A549 WT, ΔRELB and ΔRELB-2 cells were subcutaneously injected into immunocompromised mice and tumour volume was measured longitudinally (means, n = 12 flanks, ± S.E.M., *P < 0.05 or **P < 0.01 vs. WT, two-tailed t-test). c Two sequence-unrelated siRNA oligonucleotides targeting RELB were transfected in A549 cells (knockdown confirmed by immunoblotting). Expression profiling was performed vs. two sequence-unrelated non-targeting controls (Supplementary Data set 1). GSEA was performed, comparing genes differentially expressed in RELB knockdown cells with known oncogene and tumour suppressor profiles. The comparison yielding the greatest normalised enrichment score (NES), ‘TGFβ upregulated genes’, is shown here. d Fold-change heat map showing top 50 upregulated genes after RELB RNAi. Individual mean RELB siRNA values are normalised to the averaged means of the controls (siCtls). Blue text indicates genes known to be transcriptionally upregulated downstream of TGFβ (Supplementary Table 2). e, f qRT-PCR was performed for the indicated transcripts after the following treatment regimens in A549 cells. e A549 cells were transfected with siCtrl or siRELB for 72 h. Cells were either left untreated (Ctrl) or treated with 5 ng/ml TGFβ for the final 16 h of transfection. f Exponentially dividing A549 WT and ΔRELB cells were compared. (means, n = 3, ± S.D., *P < 0.05 or **P < 0.01 vs. similarly treated siCtrl or WT cells, two-tailed t-test). g A549 cells were transfected with the indicated siRNA for 72 h, stained for paxillin and then imaged by widefield microscopy (scale bar = 20 μm). As a positive control, siCtrl cells were treated with 5 ng/ml TGFβ for 8 h prior to fixation (+TGFβ). Paxillin foci were quantified on a per cell basis by image analysis (means, n = 3, ± S.E.M., *P < 0.05, two-tailed t-tests vs. siCtrl). h A549 WT or ΔRELB cells, with (+) or without (−) prior treatment with 5 ng/ml TGFβ for 16 h, were immunoblotted as shown (P-SMAD2 = phospho-S465/467 SMAD2, P-SMAD3 = phospho-S423/425 SMAD3). Uncropped blots are available in Supplementary Fig. 10.
with that of TGFβ-driven gene activity (Fig. 5c). Furthermore, concordance between the transcriptional changes seen upon autophagy inhibition and those evoked by RELB inhibition was evident upon heat map comparison of these expression profiles (Supplementary Fig. 7). Also consistent with the regulation of TGFβ output by RELB, 31 of the top 50 upregulated transcripts upon RELB inhibition were categorised as genes that are known to be upregulated upon TGFβ treatment (Fig. 5d, please consult Supplementary Table 2 for detailed categorisation). qRT-PCR experiments directly confirmed the repression of many of these transcripts by RELB (Fig. 5c and Supplementary Fig. 8). TGFβ-driven genes thus identified were also upregulated upon ablation of RELB by CRISPR/Cas9 (Fig. 5f, ΔRELB cells). Finally, cytoskeletal responses characteristic of TGFβ activation were observed upon RELB silencing, namely the increased abundance of paxillin adhesion foci (Fig. 5g).

Fig. 6 Autophagy/RELB suppress activation of TGFβ gene promoters independent of NF-κB binding elements. a WT, ΔAT5G and ΔRELB A549 cells were subjected to chromatin immunoprecipitation (ChIP) for dimethyl-K4-histone H3 (H3Me2K4). Precipitating proximal promoter DNA was quantified by qRT-PCR, expressed as a percentage of input (means, n = 3, ±S.D., *P < 0.05 or **P < 0.01 vs. WT cells, two-tailed t-test). Gene names are shown above the charts. b–e HEK239T cells were co-transfected for 30 h with a firefly luciferase reporter driven by b–d a SMAD-response element (SRE) or e an NF-κB binding consensus element (NBE), and a constitutive Renilla luciferase control plasmid, along with expression vectors for the indicated forms of epitope-tagged RELB (wild-type, WT, or R141A Y142A, AA), SMAD2, SMAD3 and/or SMAD4 or an empty vector (EV) control. Luciferase assays were then performed as described in Methods (left, firefly/Renilla ratios expressed in arbitrary units, means, n = 3, ±S.D., *P < 0.05 or **P < 0.01 vs. EV, two-tailed t-test). Immunoblotting was performed to assess transfected factor expression for each replicate (right, representative blots). Uncropped blots are available in Supplementary Fig. 10.
The above data show that RELB is a repressor of TGFβ-driven gene activity. Importantly, similar to autophagy inhibition, RELB loss does not modulate the cytosolic TGFβ signalling pathway (Fig. 5h).

**RELB represses the activation of TGFβ target genes.** We next sought to identify a molecular mechanism by which RELB could reduce the abundance of TGFβ-driven transcripts. Firstly, we noted that the dimethyl-K4-histone H3 (H3Me2K4) chromatin
mark was upregulated at promoters for these genes in both ΔATG5 and ΔRELB cells (Fig. 6a), suggesting increased transcriptionally active or poised chromatin 46.

SMAD3-binding at SREs occurs via protein–DNA interaction 47. Similarly, REL subunits of NF-kB classically bind to NF-kB-binding consensus elements (NBEs) 48, 49. REL is an outlier in the REL family, with a notably small set of bona fide target genes identified to date, particular in epithelial lineages 45. Also, RELB can act as a transcriptional repressor 45–47. Only a small subset of the RELB-regulated genes we identified, such as IL8 and IL11, contained validated NBEs for REL-family recruitment 46, 49. Thus, we first tested the possibility that RELB might instead act on SREs to repress transcription, using luciferase reporter assays. Indeed, SMAD2- and SMAD4-driven activation of minimal SRE elements (bound directly by endogenous SMAD3) was diminished by transfection of wild-type RELB (Fig. 6b), as was direct SMAD3-driven transactivation (Fig. 6c). Strikingly, a RELB mutant deficient in DNA binding (AA, R141A Y141A) was also effective in suppressing SRE activity (Fig. 6d) although, as expected, could not activate an NBE-driven reporter (Fig. 6e).

Taking the above data together, we conclude that RELB acts to inhibit promoter activity where SREs are present and undergoes an unconventional mode of recruitment that is independent of DNA-binding and NBE recognition.

RELB interacts with SMADs in order to repress transcription.

We next hypothesised that RELB might form a protein–protein complex with SMAD(s). Confirming the potential for this, HEK293 cross-linking co-immunoprecipitation experiments detected the formation of RELB complexes containing SMAD2, SMAD3 or SMAD4 (Fig. 7a). We also assayed recruitment of endogenous RELB and SMAD4 to known SMAD-binding promoters using chromatin immunoprecipitation (ChIP). Indeed, we observed RELB occupancy of test promoters (Fig. 7b). SMAD4 also bound at these sites, as expected (Fig. 7b). Importantly, however, knockdown of SMAD4 reduced the promoter occupancy of both SMAD4 and RELB (Fig. 7b). This implied that recruitment of RELB was mediated via the protein–protein interaction with SMAD(s). Strengthening this, similar reductions in RELB binding were observed when promoter occupancy by SMAD4 was blocked with ALKi (Supplementary Fig. 9a). Conversely, RELB promoter occupancy was stimulated by exogenous TGFβ (Fig. 7c), which increases SMAD-2 and -4 binding (Supplementary Fig. 9b). As with SMAD4, knockdown of SMAD2 inhibited RELB binding to chromatin (Supplementary Fig. 9c). However, SMAD3 knockdown did not diminish RELB binding to promoters, in fact producing an apparent increase in recruitment, albeit statistically insignificant (Fig. 7d). This suggests that SMAD2 and SMAD4 may be the key molecules for recruitment of RELB to endogenous promoters. Finally, re-ChIP (sequential ChIP) experiments showed that endogenous RELB and SMAD4 protein molecules co-occupied promoter DNA, consistent with recruitment of RELB via SMAD interaction (Fig. 7e). Last, we observed that indirect loss of RELB function in ΔATG5 cells also abrogated RELB recruitment to promoters (Fig. 7f).

Taking these data together, we conclude that SMAD protein complexes act as chromatin recruitment factors for RELB. Thus, RELB facilitates negative feedback on TGFβ-driven gene expression. Antagonism of SMAD-driven transcription occurs maximally when autophagy is active and acting to stimulate RELB function.

SMAD4 inhibition rescues effects of autophagy loss in vivo.

TGFβ-driven transcription has anti-tumorigenic 27, 29 and pro-tumorigenic functions, depending upon context. Herein, we have discovered that autophagy antagonises SMAD-mediated transcription. In order to examine this in the context of a physiological readout, we employed the A549 ΔATG5 model. We stably suppressed SMAD4 expression using a short-hairpin RNA (shSMAD4) in both wild-type and ΔATG5 cells (Fig. 8a). This had no impact on autophagy, as assessed by LC3B lipidation status (Fig. 8a), or on cell proliferation in vitro (Fig. 8b). However, tumour growth kinetics were restored in vivo (Fig. 8c). Similar SMAD4-dependent suppression of tumour growth was seen upon FIP200 deletion in A549 cells (Fig. 8d, e). Thus, the suppression of TGFβ transcriptional output by autophagy and RELB/NF-kB is a component of the pro-tumorigenic effect of autophagy, at least in A549 cells. This provides a proof-of-principle that the capacity for regulation of transcriptional output by autophagy, seen in several cell lines, can affect cell behaviour in vivo. However, given the pleotropic effects of TGFβ, different aspects of tumour biology might be predominantly affected in other settings or models.

Discussion

We aimed to uncover new selective autophagy-based mechanisms acting to modify cytosolic signalling and cell fate. We focused on a model for RAS mutant cancer. In many such cancers, autophagy promotes tumorigenesis by mechanisms that are, at best, partially understood. Our findings are summarised in Fig. 9. A key component of the mechanism is that autophagy specifies transcriptional output. Previously, it has been shown that
transcriptional responses to interferon pathway stimuli are exaggerated when autophagy is inhibited. However, the most penetrant effect we observed upon autophagy inhibition was upregulation of the transcriptional response to TGFβ signalling.

TGFβ-driven transcription acts to suppress tumour establishment and growth in a number of cancer types, via a range of modalities encompassing senescence, inhibition of proliferation and engagement of cell death. Indeed, components of the TGFβ pathway such as SMAD4 and the ligand receptor TGFBR2 are encoded by classic tumour suppressor genes, which undergo direct loss-of-function mutation during tumorigenesis in certain cancers. We reveal an alternate mechanism for suppression of TGFβ function. RAS engages selective autophagy, which in turn mediates signalling events resulting in antagonistic cross-talk with TGFβ, dampening its tumour-suppressive transcriptional output and thus permitting tumorigenesis in vivo. The molecular basis via which upregulated TGFβ signalling inhibits tumour growth remains to be explored. It is likely well-understood mechanisms such as inhibition of cell-cycle progression and apoptosis play a role. In RAS-driven cancers, cell-autonomous autophagy function can promote lipid catabolism and regulate the balance between oxidative phosphorylation and aerobic usage of glucose. It is possible that regulation of TGFβ output could affect metabolism, although this remains to be investigated.

In certain contexts, TGFβ/SMAD can promote EMT and increased tumour aggression. Some EMT-implicated genes are upregulated when autophagy is inhibited in the system described herein, including CDH2 (N-Cadherin) and SNAI2 (SLUG) (Supplementary Fig. 7). This is also thematically consistent with reports that autophagy can post-transcriptionally repress the levels of transcription factors that would otherwise promote EMT, such as TWIST and SNAIL. Although, on the other hand, autophagy has been shown in HRAS-transformed mammary epithelial cells to promote the secretion of factors that facilitate matrix invasion. Thus, it is possible that other in vivo model systems will reveal a more complex relationship between autophagy and the outcomes of TGFβ signalling, including regulation of pro-tumorigenic EMT events.

The mechanism of selective autophagy involvement in regulation of gene expression was revealed as termination of the activity of a new cargo, TRAF3. This culminates in nuclear translocation of the transcription factor RELB. There is a growing consensus that such ‘signalphagy’-type responses will prove important in physiological autophagy function. Indeed, TRAF3 degradation joins recent examples such as degradation of GSK3β and β-catenin, TBK1 and IRF3, by autophagy in various systems.

We uncovered a new function for RELB in repression of TGFβ transcriptional output, wherein RELB operates directly upon
physiology. Increased comprehension of signal regulatory functions of autophagy will lead to future improvements in understanding and targeting autophagy outcomes in health and disease.

**Methods**

**Cells and materials.** ALK2/4/5 inhibitor, 2-(3-(6-methylpyridin-2-yl)-1H-pyrazolo[4-1]yl)-1.5-naphthyridine was from Calbiochem (#616452). Recombinant human TGFβ ligand was from AbD Serotec. All cell lines were cultured in standard DMEM supplemented with 10% foetal calf serum and penicillin/streptomycin, at 37 °C and 5% CO2. A549-EcoR cells are A549 cells expressing the Ecotropic receptor for retroviral infection and G418 resistance marker. They were obtained from the laboratory of Chris Marshall (Institute of Cancer Research, London). A549-EcoR cells were identity checked by microsatellite genotyping. NCI-H23 parental cells were obtained from ATCC and derivatised to express Ecotropic receptor. Neither A549 nor NCI-H23 cell lines are commonly misidentified (source: ICLAC database v6, updated December 2016). Phoenix-Eco cells were lysates for Kevin Ryan, Beatson Institute, UK. NKX cells are keratinocytes in culture, which were used. A549-Cas9 cells were generated by infection of A549 cells with lentivirus expressing NTAP-NDP52 were washed and harvested with ice-cold PBS followed by storage at -80 °C or immediate lysis in 4 ml MCLB buffer. Cell debris was removed from the lysates by centrifugation and supernatants were passed through 0.45 μm spin filters (Millipore). Anti-HA-agarose (60 μl), Sigma) was added to lysates or immunoprecipitation overnight at 4 °C, rotating. Samples were washed five times with 1 ml MCLB followed by five washes with PBS and elution with 150 μl HA peptide (250 μg/ml, Sigma). Eluted immune complexes were essentially processed in a similar manner to those in published studies65,68. Briefly, proteins were precipitated with trichloroacetic acid (Sigma) followed by digestion with trypsin (Promega) and desalting by stage tips. Samples were in technical duplicates on a LTQ Velos (Thermo Scientific). Spectra were identified by Sequest searches followed by target-decoy filtering and linear discriminant analysis66. Peptides that could be assigned to more than one protein in the database were assembled into proteins according to parsimony principles. For CompgPass analysis, we employed 34 unrelated bait proteins that were all previously processed in the same way in A549 cells. Weighted and normalised D-scores (WDN-score) were calculated based on average peptide spectral matches (APSMs). Proteins with WDN ≥ 1 and APSM ≥ 3 were considered as high-confidence candidate interacting proteins (HCIPs). Proteins with APSM ≥ 2 and that had interactions documented in BioGrid with at least two of the HCIPs and/or NDP52 were also considered candidate interactors (subthreshold).

**Luciferase assays.** HEK293T cells were transfected in triplicate with Lipofectamine 2000 according to the manufacturer’s instructions. After 36 h, cells were lysed in Passive Lysis Buffer (Promega) for 15 min at room temperature and both firefly and Renilla luciferase activity measured using the Dual-Luciferase® Reporter Assay System (Promega) and a Fluoroskan Ascent FL plate reader (Labsystems), following manufacturer’s instructions. Cells were transfected with plasmids described in Figure legends as well as an identical replicate set in which pGL3 basic was.

**Fig. 9** Model of the molecular events comprising an autophagy-mediated, inhibitory cross-talk with the TGFβ pathway in RAS-transformed cells. Selective autophagy of TRAF3 via cargo receptors, such as NDP52, terminates the tonic inhibition of the alternative NF-κB pathway that is ordinarily observed in unstimulated primary cells. This results in nuclear accumulation of RELB. Downstream of TGFβ, frequently present in the tumour milieu, DNA binding by SMADs drives gene transcription. Directly TGFβ-activated promoters host SRE-containing SMAD-response elements (SREs). However, RELB has the ability to repress SRE-containing gene promoters. This occurs not via interaction with NF-κB consensus sites but instead via recruitment to chromatin by protein-protein interaction with active SMAD complexed. Thus, transcriptionally repressive RELB effectively ‘hijacks’ SMAD promoters to exert negative feedback on TGFβ-mediated transcription. In the absence of autophagy, the above events involving RELB are not engaged. Thus, in vivo, autocrine and/or paracrine sources of TGFβ repress tumorigenesis when autophagy is ablated. Please note though, that in vivo models other than A549 cells, it is possible that altered sensitivity to TGFβ could potentially have other phenotypic outcomes.
substituted for the reporter construct to confirm negligible firefly luciferase activity in the absence of specific binding elements from the reporters.

**Immunoblotting.** Cells were routinely lysed in RIPA lysis buffer (50 mM Tris-HCl, pH 7.5, 0.5% Na deoxycholate, 1% Triton-X-100, 150 mM NaCl, complete protease inhibitors + 1 mM EDTA (Roche #01645001), 2 mM Na fumarate, 25 mM Na β-glycerophosphate, 10 mM NaF, 10 mM Na pyrophosphate). Cells were washed briefly in ice-cold PBS and pre-incubated with ice-cold RIPA lysis buffer for 5 min prior to scraping and homogenisation. Scraped and homogenised lysates were incubated on ice for a further 10 min before clarification by centrifugation in a benchtop centrifuge at full-speed for 15 min at 4 °C. The supernatant was removed and quantified by Pierce BCA Protein Assay (Life Technologies #23225). For immunoblotting to detect NIK, RIPA lysis buffer was supplemented with 20 μM MG132 to stabilise NIK levels during lysis. Gel electrophoresis was performed using 4–12% NuPAGE Novex Bis-Tris gels, or for LC3B immunoblotting, 4–20% NuPAGE Tris-Glycine gels. Nitrocellulose membranes, or PVDF membranes for LC3B immunoblotting, were probed according to the standard methods and visualised using enhanced chemiluminescence.

**Immunoprecipitations.** For tagged protein immunoprecipitation, HEK293T cells were transfected with Lipofectamine 2000 according to the manufacturer’s instructions. After 24 h, cells were lysed in IGEPAAL buffer (50 mM Tris-HCl, pH 7.5, 0.5% deoxycholate, 150 mM NaCl, complete protease inhibitors + 1 mM EDTA (Roche), 2 mM activated orthovanadate, 25 mM Na β-glycerophosphate, 10 mM NaF, 10 mM Na pyrophosphate). For cross-linking immunoprecipitation experiments, 24 h after transfection, cells were cross-linked with 1% paraformaldehyde in DMEM for 10 min at room temperature, and quenched by 0.125 M glycine for 10 min. Cells were washed twice with ice-cold PBS, scraped, pelleted and lysed in 1% SDS, 10 mM EDTA and 50 mM Tris, pH 8.0 + protease inhibitors, then sonicated. Samples were then clarified by centrifugation and supernatants diluted with IGEPAL buffer to 0.1% SDS. For both standard and cross-linking immunoprecipitations, lysates were incubated with rabbit anti-myc-conjugated agarose beads (Sigma) for 4 h and then beads were washed three times in IGEPAAL and eluted in Laemmli sample buffer.

For endogenous co-immunoprecipitation, 4 × 10^6 A549 cells were seeded overnight in 15 cm dishes and lysed in IGEPAL buffer. Magnetic beads were conjugated with antibody using the Dynabeads coupling kit (Invitrogen #14311d), following manufacturer's instructions. Lysates were incubated with these beads for 4 h, washed three times in IGEPAL buffer and eluted in 4% SDS (no reducing agent).

**Light microscopy.** For immunofluorescence, cells were grown on glass coverslips and fixed in 4% paraformaldehyde for 10 min at room temperature and permeabilised with 0.25% Triton X-100 for 20 min at room temperature, or, for LC3B immunofluorescence, with methanol for 5 min at ~20 °C. Cells were incubated with primary antibodies overnight at 4 °C and secondary antibodies and DAPI for 1 h at room temperature. All antibodies were incubated using DAKO fluorescent mounting medium. Secondary antibodies were Invitrogen goat anti-mouse, anti-rat and anti-rabbit antibodies conjugated to Alexa Fluor 488, 594 or 647. Widefield fluorescence images were captured with an Olympus BX51 microscope and an Olympus DP71 camera using Olympus Soft Imaging Solutions Solutions Software. Acquisition time and illumination intensity was consistent across experimental conditions. RELB nuclear localisation was quantified by single blind scoring. Confocal microscopy was performed using an Olympus FV1000 confocal microscope using Olympus proprietary software (Fluoview). Images were viewed in Image J using the Bio-Formats v5.1 plugin. Acquisition parameters were consistent across experimental conditions. Brightness and contrast were adjusted consistently across experimental conditions using Image J software. For merge images for colocalisation, Paint.NET software (v 3.3x, 3.5) was used to adjust the relative level and curves for red, green or blue channels and channels applied to the whole image, the same channels were applied to all images across an individual experiment. Colocalisation of punctate signals in different channels was quantified by single blind scoring. For quantification of Paxillin foici, the Foci Picker 3D plugin for Image J was used. The number of foci was normalised to cell number and the criteria for scoring was unchanged across experimental conditions. In all experiments where quantification was performed, a minimum of 100 cells were scored for each condition and biological replicate.

**Immunohistochemistry.** For immunohistochemistry, xenograft tumours were dissected from the mouse immediately after killing and fixed in 10% neutral-buffered formalin overnight. Tumours were transferred into 70% EtOH before embedding in paraffin and sectioning. All further processing was performed using a BOND III immunostainer (Leica Biosystems). Dewaxing was performed using dewaxing solution (Leica #AR0222), epitope retrieval was performed using solution 1 (Leica #AR9996) for 10 min, followed by 10 min in solution 2 (Leica #AR9996) for 10 min, followed by 10 min in solution 3 (Leica #AR9996). Tumours were subjected to DAK immunohistochemistry using the BOND Refine Kit (Leica #AR922) with the exception of blocking, which was performed using the mouse IgG blocking solution (Vector #MKB2213).

**siRNA transfection.** Total RNA was extracted from cells 72 h after transfection with siRNA, and total RNA was purified using the RNeasy mini kit (Qiagen), including optional DNA digestion. Biotin-labelled cRNA was prepared using the TotalPrep RNA Amplification Kit (Ambion, #AM1770), according to the manufacturer’s instructions, and hybridised to the Illumina HT-12 human array array v4.0. The array was scanned using the Illumina HiScan platform. Raw data was processed using VST transformation and subsequent RSN normalisation, using the lumi package in R.63 64 65

**Transcriptomic expression profiling.** RNA was harvested from cells 72 h after transfection with siRNA, and total RNA was purified using the RNeasy mini kit (Qiagen), including optional DNA digestion. Biotin-labelled cRNA was prepared using the TotalPrep RNA Amplification Kit (Ambion, #AM1770), according to the manufacturer’s instructions, and hybridised to the Illumina HT-12 human array array v4.0. The array was scanned using the Illumina HiScan platform. Raw data was processed using VST transformation and subsequent RSN normalisation, using the lumi package in R.63 64 65

**Gene set enrichment analysis.** We acknowledge our use of the gene set enrichment analysis, GSEA, software and the Molecular Signature Database (MSigDB) at http://www.broad.mit.edu/gsea/32. Gene set enrichment was performed using normalised, reverse transformed data sets with low-intensity probes filtered out, as described above. Six control replicates were compared against six ATG5/ULK1 siRNA replicates or six RELB replicates in each of two sets of analyses. The Gene Set used for comparison was ‘oncogenic signatures’ from MSigDB. The analysis was performed with the following parameters: probes collapsed to single-gene identities and 1000 permutations, permuting on gene set and Signal2Noise selected.

**qRT-PCR.** Total RNA was extracted from cells using the RNeasy mini kit (Qiagen) with QiAshedders columns, following manufacturer’s instructions. cdNA was synthesised using 1–5 μg template RNA using the First Strand cDNA synthesis kit (Applied Biosystems). qRT-PCR was performed with DyNaMo HS SYBR Green qPCR mastermix (Thermo Scientific–F-410) on aRotor-Gene RG2000 (Corbett Research) or a StepOne Plus Real-Time qPCR machines and analysed with the corresponding software. All experiments were quantified in relation to standard curves where presented with an individual gene per chart, and readings were normalised to 18S levels. In higher-throughput analyses, multiple genes are pre-selected. Six control replicates were compared against six ATG5/ULK1 siRNA replicates or six RELB replicates in each of two sets of analyses. The Gene Set used for comparison was ‘oncogenic signatures’ from MSigDB. The analysis was performed with the following parameters: probes collapsed to single-gene identities and 1000 permutations, permuting on gene set and Signal2Noise selected.

**Chromatin immunoprecipitation.** Samples were cross-linked with 1% paraformaldehyde in DMEM for 10 min at room temperature and quenched by 0.125 M glycine for 10 min. Cells were washed twice with ice-cold PBS, scraped, pelleted
and lysed in 1% SDS, 10 mM EDTA and 50 mM Tris, pH 8.0 + protease inhibitor tablets (Roche). Then supernatants were sonicated uniformly to generate fragments ranging from 200 to 1000 bp. A sample was stored as input. Sonicated supernatants were pre-cleared with Protein A Dynabeads (Life Technologies) bound with 5 μg rabbit IgG (Cell Signaling Technology) in the presence of 2 μg salmon sperm DNA. Pre-cleared lysates were incubated with Protein A Dynabeads (Invitrogen) and 5 μg of immunoprecipitating antibody or rabbit IgG control, overnight at 4 °C under rotation. Washes were performed in each of the buffers sequentially: low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris, 200 mM NaCl, pH 8.1), high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris, 550 mM NaCl, pH 8.1), LiCl buffer (250 mM LiCl, 1% IGEPAL, 1% sodium deoxycholate, 1 mM EDTA and 10 mM Tris, pH 8.1) and twice in Triton-X-100 (10 mM Tris-HCl 1 mM EDTA, pH 8.1). Beads were incubated and then vortexed, twice sequentially, in elution buffer (100 mM NaHCO₃ and 1% SDS). 200 mM NaCl was added to eluted, precipitated chromatim, or to input samples, and crosslinking was reversed by incubation at 65 °C overnight. The sample was then adjusted to 40 μg/ml Proteinase K, 40 mM Tris, 10 mM EDTA and digestion was performed for 4 h at 37 °C. DNA was purified with a QIA-gen PCR clean-up kit according to the manufacturer’s instructions, and samples were quantified for specific DNA species by SYBR-green qRT-PCR as described above, using the tabulated primers (Supplementary Table 5).

For re-ChIP assays, following the first immunoprecipitation (as above), beads were washed three times with re-ChIP wash buffer (2 mM EDTA, 200 mM NaCl, 0.1% SDS, 1% NP-40) and twice with Triton-X-100 DNA. Bacteria were eluted in Re-ChIP elution buffer (10 mM Tris-HCl, 1 mM EDTA, 2% SDS) for 30 min at 37 °C. Following elution, the supernatant was diluted to a concentration of 0.1% SDS with ChIP dilution buffer (1% Triton-X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris, pH 8.1). CRISPR/Cas9-mediated gene editing in A549 cells

CRISPR/Cas9-mediated gene editing in A549 cells. For clonal cell cultures, cells were transfected with a 50:50 ratio of gRNA plasmid and Cas9PotreoZA by nucleofection with Lonza Nucleofector kit T, according to the manufacturer’s instructions for A549 cells. Cells were selected for successful transient transfection with 2.5 μg/ml puromycin, 24 h post transfection for a further 24 h duration, and then grown in regular medium as single cell colonies. Control colonies were derived after transfection of empty gRNA plasmid. Then grown in regular medium as single cell colonies. Control colonies were obtained by injection in order to achieve sufficient experimental power, based upon prior experience of the model. Mice were from Charles River Laboratories and in all experiments were allocated to groups from purchased stocks at random. Mice were housed in individually ventilated cages and tumour measurements thereafter taken using calipers. Tumours were excised and paraffin embedded. All animal studies were performed after University of Edinburgh local ethical review and under the authority of a UK Home Office project licence.

Statistics. All replicates are biological replicates unless explicitly indicated otherwise in Figure legends. All statistical tests were based upon estimates of variation relating to presumed normal distribution (standard deviation and/or standard error of the mean). Unless stated otherwise in the Figure legend, for example where multiple testing was performed or stated otherwise, the Student’s t-test was two-tailed. As a special instance, treatment of gene expression profiling is described in a dedicated Method section, above.

Blinding was performed where practicable and in such instances is described under individual methodology descriptions (for example, immunofluorescence scoring).

Code availability. R scripts used to process array data are available from the authors.

Data availability. A MIAME-compliant data set encompassing all the array-based expression profiling raw data, processed data, and further experimental detail, has been deposited in NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) (supe-ass-000158. Raw data for NDP52 affinity purification mass spectrometry have been deposited at MassIVE (http://massive.ucsd.edu) with accession number MSV000081221. Other data are available from the authors upon reasonable request.

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Author contributions
A.C.N., A.I.K. and S.W. designed and interpreted the expression array analyses. C.B. and S.W. designed and analysed the mass spectrometry screen. C.B. performed the mass spectrometry. A.C.N. and S.W. designed and interpreted all other experiments. A.C.N. and S.W. performed xenograft analyses. Y.D. performed the ChiP analyses.

Additional information
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