Autolysosomal β-catenin degradation regulates Wnt-autophagy-p62 crosstalk

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Abstract

The Wnt/β-catenin signalling and autophagy pathways each play important roles during development, adult tissue homeostasis and tumorigenesis. Here we identify the Wnt/β-catenin signalling pathway as a negative regulator of both basal and stress-induced autophagy. Manipulation of β-catenin expression levels *in vitro* and *in vivo* revealed that β-catenin suppresses autophagosome formation and directly represses *p62/SQSTM1* (encoding the autophagy adaptor p62) via TCF4. Furthermore, we show that during nutrient deprivation β-catenin is selectively degraded via the formation of a β-catenin-LC3 complex, attenuating β-catenin/TCF-driven transcription and proliferation to favour adaptation during metabolic stress. Formation of the β-catenin-LC3 complex is mediated by a W/YXXI/L motif and LC3-interacting region (LIR) in β-catenin, which is required for interaction with LC3 and non-proteasomal degradation of β-catenin. Thus, Wnt/β-catenin represses autophagy and p62 expression, while β-catenin is itself targeted for autophagic clearance in autolysosomes upon autophagy induction. These findings reveal a regulatory feedback mechanism that place β-catenin at a key cellular integration point coordinating proliferation with autophagy, with implications for targeting these pathways for cancer therapy.

Introduction

The Wnt/β-catenin pathway maintains normal intestinal homeostasis and is aberrantly activated in the majority of colorectal tumours (Bienz & Clevers, 2000; Kinzler & Vogelstein, 1996). β-catenin is an essential transcriptional co-regulator in the canonical Wnt pathway, forming complexes with the TCF/LEF (T cell-specific transcription factor/lymphoid enhancer-binding factor 1) family of transcription factors to control target gene expression. In the absence of an exogenous Wnt signal, the pathway is inhibited through the tumour suppressor *Adenomatous Polyposis Coli* (APC) destruction complex that phosphorylates cytoplasmic β-catenin, leading to its ubiquitination and proteasomal degradation (Li et al, 2012). Mutations of Wnt pathway components prevent the destruction of β-catenin, leading to increased TCF-regulated transcription of Wnt/β-catenin target genes that increase cell proliferation, promoting tumorigenesis (Clevers & Nusse, 2012).

In addition to its role in cell proliferation, β-catenin is also known to facilitate adaptation to microenvironmental conditions, including hypoxia (Kaidi et al, 2007) and oxidative stress (Essers et al, 2005). This involves diverting β-catenin away from TCF to favour quiescence and adaptation-promoting transcription, mitigating the deleterious consequences that could occur if proliferation were allowed to continue unabated during stress. Another key adaptive response to microenvironmental stress is

macroautophagy (herein referred to as autophagy), a vital mechanism for recycling cellular components to sustain viability when cells have outstripped their exogenous nutrient supply (Kroemer et al, 2010). During autophagy, cellular components to be degraded are surrounded by autophagosomes and catabolised after fusion with lysosomes to provide precursors for synthetic processes and energy production, which can contribute to the survival of tumour cells during unfavourable microenvironmental conditions (Mathew et al, 2007). However, the role of autophagy in cancer is complex, as autophagy can also protect against tumour development through removal of reactive oxygen species (ROS), toxic protein aggregates and damaged organelles (Eng & Abraham, 2011). As such, tumours often exhibit reduced basal levels of autophagy, which can occur as a result of mutations in autophagy genes or down-regulation of autophagy-regulatory proteins (Mizushima et al, 2008). Despite the reduced basal activity in these tumours, autophagy can still be induced when necessary to assist tumour cell survival (White & DiPaola, 2009), highlighting the contextual roles of autophagy in cancer.

Maintaining cellular homeostasis requires a careful balance between proliferation and autophagy under normal physiological conditions as well as during microenvironmental stress, but the mechanisms by which cells achieve this remain unclear. Given the function of β-catenin in facilitating adaptation to hypoxia and oxidative stress, we asked whether Wnt/β-catenin signalling has a role in the regulation of autophagy. In this report, we provide the first evidence that β-catenin suppresses autophagy and represses the expression of the autophagy adaptor p62, while during nutrient deprivation β-catenin is itself subject to proteasome-independent degradation via its interaction with the autophagy protein LC3. These findings support a novel regulatory feedback model between Wnt signalling and autophagy, where β-catenin functions to integrate proliferative signalling with autophagy and p62 expression.

Results

β-catenin negatively regulates autophagy and p62 expression *in vitro* and *in vivo*

To determine whether β-catenin regulates autophagy, we first reduced β-catenin expression using siRNA in HT29 colorectal carcinoma cells stably expressing YFP-LC3 and examined the formation of LC3 puncta. The number of LC3 puncta per cell represents autophagosome number, serving as a marker for autophagic activity (Kabeya et al, 2000). We observed that autophagosome number increased both under normal conditions and during starvation following β-catenin knockdown (Figures 1A and 1B). Consistent with this, western blotting revealed an increase in lipid-bound LC3-II levels in β-catenin depleted cells, indicative of increased autophagosome number (Figure 1C). In complementary experiments, a stabilised mutant form of

β-catenin (β-catenin^{S33Y}) was introduced into HCT116 β-catenin$^{WT/-}$ colorectal carcinoma cells, in which the endogenous mutant β-catenin allele had been knocked out (Chan et al, 2002). In HCT116 β-catenin$^{WT/-}$ cells overexpressing β-catenin^{S33Y}, immunofluorescence revealed that the formation of endogenous LC3 puncta was reduced (Figure 1D), while western blotting showed a decrease in the LC3-II/β-actin ratio, suggestive of reduced autophagosome number (Figure 1E). In addition, comparison of LC3 protein expression between HCT116 β-catenin$^{WT/-}$, and their isogenic counterpart HCT116 β-catenin$^{-/\Delta S45}$ in which the endogenous wild-type β-catenin allele had been knocked out (Chan et al, 2002), showed that the LC3-II/β-actin ratio was lower in cells expressing endogenous stabilised mutant β-catenin$^{\Delta S45}$ (Figure 1F). Taken together, these data suggest that β-catenin negatively regulates autophagosome formation.

To further analyse the regulation of autophagy by β-catenin, we examined the expression of p62 (also known as SQSTM1 and A170) following β-catenin knockdown. p62 interacts with and is degraded alongside polyubiquitinated proteins destined for autophagosomes, therefore p62 protein levels decrease upon autophagy induction (Kirkin et al, 2009). Since knockdown of β-catenin increased autophagosome number, it was expected that p62 protein levels would decrease as a result of increased autophagic turnover. Surprisingly, β-catenin knockdown increased p62 protein expression (Figure 2A). In addition, p62 protein levels were higher in β-catenin$^{WT/-}$ cells compared to β-catenin$^{-/\Delta S45}$ cells (Figure 2B). Given that β-catenin knockdown increased both LC3 puncta and p62 levels, it raised the possibility that β-catenin siRNA blocked the completion of autophagy, thereby preventing the degradation of p62 and cellular components targeted to the autophagosome. To address whether reducing β-catenin expression inhibited autophagy-specific degradation, we monitored autophagic flux. Increased co-localisation of LC3 with the lysosome marker LAMP-1 (lysosomal-associated membrane protein-1) would indicate a block in flux due to inhibition of autolysosome degradation, as was observed when using the inhibitor of flux bafilomycin A1 (Figure S1A). However, we found that β-catenin knockdown did not increase the co-localisation of LC3 with LAMP-1 (Figure S1A). To confirm these data, we performed a starvation and nutrient add-back assay. If β-catenin knockdown did in fact inhibit autophagic flux, autophagosome number would be sustained with nutrient addition post-starvation. However, following nutrient add-back, LC3 puncta number returned to basal levels in both control and β-catenin knockdown cells (Figures 2C). Importantly, the lysosomal autophagy inhibitors chloroquine and bafilomycin A1 increased LC3-II and p62 accumulation in β-catenin silenced cells (Figure 2D), supporting the notion that β-catenin knockdown does not lead to a block in autophagic flux. Taken together, these data suggest that reduced β-catenin expression levels do not inhibit autophagic flux, but instead increase both autophagosome number and p62 protein levels.

Having shown that β-catenin negatively regulates autophagy and also reduces p62 expression *in vitro*, it was important to determine whether this was detectable *in vivo*. To do this, we used a mouse model where β-catenin can be inducibly deleted only in the intestinal epithelium (Figure S1B; Fevr et al, 2007). Immunofluorescence of LC3 and p62 protein was compared in the intestinal epithelium between tamoxifen treated control (β-catenin$^{+/lox}$-villin-creERT2) and β-catenin-deleted (β-catenin$^{-/lox}$-villin-creERT2) mice (Figure 3). LC3 puncta expression increased throughout the crypt-villus axis following β-catenin deletion (Figures 3A-D), indicative of up-regulated autophagy. In addition, deletion of β-catenin increased p62 protein expression throughout the crypt-villus axis (Figures 3E-H). Western blotting using extracts from mouse intestinal epithelial tissue confirmed increased levels of LC3-II and p62 following β-catenin deletion (Figure 3I and 3J). Therefore, both *in vitro* and *in vivo* data indicate that β-catenin inhibits both autophagosome formation and p62 expression.

Wnt/β-catenin represses p62 expression via TCF4

The negative regulation of p62 protein expression by β-catenin was an intriguing finding. Given that p62 protein levels were elevated by β-catenin knockdown, but autophagic flux was not inhibited, it raised the possibility of p62 transcriptional regulation by β-catenin. To address this, we measured p62 mRNA levels using qRT-PCR. β-catenin knockdown in HT29 cells increased p62 mRNA (Figure 4A), whereas over-expression of β-catenin^{S33Y} in HCT116 β-catenin$^{WT/-}$ cells decreased p62 mRNA (Figure 4B). To investigate whether elevated p62 protein expression following β-catenin knockdown was a consequence of increased p62 mRNA, we blocked translation using cycloheximide in HT29 cells. The increase in p62 protein observed following β-catenin knockdown was decreased by 8 and 24 hours translation inhibition (Figure 4C), with comparable results using the transcription inhibitor actinomycin D (Figure S1C). These results suggest that the increase in p62 protein following β-catenin knockdown is due, at least in part, to increased p62 mRNA levels. It has recently been reported that β-catenin knockdown reduces the binding of Nrf2 (an important positive regulator of *p62* gene expression) to E-cadherin, leading to nuclear translocation and activation of Nrf2 (Kim et al, 2012). To determine whether p62 expression was regulated by inhibition of the Wnt/β-catenin pathway independently of modulating β-catenin expression levels, we over-expressed the non-canonical Wnt receptor frizzled 6 (Fzd6), which blocks TCF binding to target promoters and thereby inhibits Wnt target gene transcription (Golan et al, 2004). In cells over-expressing Fzd6, we observed increased expression of p62 protein compared to cells that did not over-express Fzd6 (Figure 4D). To confirm this, we induced expression of a dominant negative form of TCF4 (DNTCF4) (van de Wetering et al, 2002) that is unable to bind β-

catenin. Induction of DNTCF4 with doxycycline increased p62 protein (Figure 4E) and mRNA (Figure 4F) expression, suggesting that β-catenin may repress p62 expression by binding to the transcription factor TCF4. Furthermore, similar to β-catenin siRNA, induction of DNTCF4 increased LC3-II expression, suggesting that inhibition of Wnt/β-catenin signalling induces autophagy (Figure 4E). Indeed, treatment of the Wnt-responsive HCT116 β-catenin$^{WT/-}$ with recombinant Wnt3a reduced p62 protein expression and decreased the LC3-II/β-actin ratio (Figure 4G). These data are suggestive of a transcriptional role for β-catenin/TCF in the repression of p62.

***p62/SQSTM1* is a physiological target of Wnt/β-catenin-mediated gene repression**

While β-catenin is well known for transcriptional co-activation, recent evidence indicates that β-catenin can also behave as a transcriptional co-repressor (Hoverter & Waterman, 2008; Smartt et al, 2012) and that gene suppression may form part of a global mechanism of regulation by Wnt signalling (Clevers & Nusse, 2012). To address the possibility that p62 represents a transcriptional target of β-catenin/TCF4, chromatin immunoprecipitation (ChIP) and qPCR was performed to determine whether β-catenin and TCF4 could be detected at the promoter region of the *p62*/*SQSTM1* gene. Under normal nutrient-rich growth conditions, we found that β-catenin and TCF4 associated with the *p62* promoter in HT29 cells (Figure 5A; Figure S2A). We also observed increased recruitment of RNA polymerase II to the *p62* promoter and up-regulation of p62 mRNA during starvation (Figures 5A and 5B), suggesting that increased p62 synthesis occurs during autophagy to replenish p62 protein degraded during autophagy. Given the substantial increase in p62 transcription accompanying decreased β-catenin occupancy in nutrient deprivation, we assessed the epigenetic status of the *p62* promoter region. Nutrient deprivation led to a ~3-fold increase in acetylated histone H3 at the *p62* promoter (Figure 5C), consistent with increased epigenetic permissivity of the *p62* gene. Importantly, association of β-catenin with the *p62* promoter was decreased following two hours of starvation (Figure 5D and Figure S2A). Taken together, these findings support the notion that β-catenin represses p62 transcription and that de-repression may occur during nutrient starvation when an increased amount of p62 is required for autophagy.

Autophagy induction inhibits Wnt/β-catenin signalling via proteasome-independent β-catenin degradation

The above data suggest that the increase in p62 mRNA during starvation may be due to attenuated Wnt/β-catenin signalling when autophagy is induced. Previous reports have hinted at a role for autophagy in β-

catenin degradation (Korolchuk et al, 2009; Sukhdeo et al, 2012), although a mechanism has not yet been described. To investigate this, we monitored β-catenin protein levels upon autophagy induction and observed a clear decrease in β-catenin expression by 8 hours nutrient deprivation (Figure 6A). In addition, inhibition of autophagy by knockdown of Atg7 (an important gene in the canonical autophagy pathway) increased β-catenin protein expression under both normal and nutrient-deprived conditions (Figure 6B; Figure S2B). To determine whether the decrease in β-catenin levels observed during autophagy reduced the transcriptional output of the Wnt pathway, we used the TopFlash reporter system. TopFlash activity and the expression of Wnt-target genes *Axin2* and *Cyclin D1* decreased during both starvation and treatment with the autophagy-inducing mTOR inhibitor PP242 in HT29 cells (Figures 6C-F; Figures S2C and S2D). Furthermore, in findings similar to a previous report (Gao et al, 2010), induction of autophagy attenuated Wnt3a-stimulated TopFlash (Figure 6G and 6H) and *Cyclin D1* (Figure 6I) expression. In addition, Atg7 knockdown increased TopFlash activity under basal conditions in HT29 cells and upon Wnt3a stimulation in RKO cells (Figures 6J-L). Interestingly, *CTNNB1/β-catenin* mRNA expression did not decrease during 8 hours of starvation in HT29 cells (Figure 6M), suggesting that the observed reduction in β-catenin protein expression upon autophagy induction was through increased protein turnover rather than transcriptional regulation.

The decrease in β-catenin protein expression upon autophagy induction, in conjunction with increased β-catenin protein expression following autophagy inhibition, suggested that β-catenin may be subject to autophagic degradation To explore this possibility, we monitored β-catenin expression levels during nutrient deprivation in the presence and absence of lysosomal autophagy inhibitors (chloroquine and bafilomycin A1) or the phosphoinositide 3-kinase (PI3K) inhibitor wortmannin. Blockade of autophagy with lysosomal inhibitors (Figure 6N) or wortmannin (Figure 6O) attenuated the starvation-induced decrease in β-catenin protein expression (Figure 6N and 6O). Since autophagy inhibition can compromise the clearance of proteasome substrates (Korolchuk et al, 2009), we over-expressed a myc-tagged β-catenin mutant (S33A, S37A, T41A, S45A; termed myc-β-catenin^{AAAA}) resistant to proteasomal degradation (Cheon et al, 2002; Liu et al, 2002) and assessed its expression during starvation. Western blotting revealed that the expression of myc-β-catenin^{AAAA} decreased upon starvation (Figure 6P), suggestive of a proteasome-independent mechanism for β-catenin degradation. In support of this, β-catenin protein levels decreased in cells subjected to starvation in the presence of the proteasome inhibitor MG132 (Figure 6Q). These data show that the reduction in β-catenin protein expression during starvation does not proceed via the proteasome, but through an alternative mechanism sensitive to autophagy inhibition

The autophagy protein LC3 directly interacts with β-catenin and targets β-catenin for autophagic degradation

Having shown that the induction of autophagy reduces β-catenin protein expression independently of the proteasome, we investigated whether β-catenin is specifically targeted for autophagy-mediated degradation. As shown earlier (Figure 3), expression of LC3 puncta was induced in β-catenin$^{-/lox}$-villin-creERT2 mouse intestinal epithelium 2 days post tamoxifen treatment, a time point at which β-catenin protein expression is reduced, but not yet completely eliminated (compare Figure 7A control; 7B; β-catenin deleted). Immunofluorescence revealed co-localisation of β-catenin and the autophagosome component LC3 in mouse intestinal epithelium (Figure 7B, 7C and 7D), and linescan analysis confirmed co-localisation of cytoplasmic β-catenin and LC3 puncta (Figure 7E). Given that LC3 is known to bind cargoes targeted for selective turnover in autophagosomes, we performed co-immunoprecipitation experiments to determine whether β-catenin associates with LC3. In HT29 cells expressing YFP-LC3, endogenous β-catenin immunoprecipitated with YFP-LC3 and this was enhanced by nutrient deprivation and chloroquine treatment (Figure 8A), suggesting that β-catenin interacted with LC3 during autophagy induction. Nutrient deprivation also promoted the interaction between endogenous β-catenin and endogenous LC3 in HT29 cells (Figure 8B). LC3 can bind proteins at an LC3-interacting region (LIR), which contains the motif W/YXXI/L (Ichimura et al, 2008; Noda et al, 2008; Pankiv et al, 2007). Examination of the β-catenin amino acid sequence revealed a potential LIR, consisting of a W/YXXI/L motif at positions 504-507 within the armadillo repeat domains of the protein. To determine whether this sequence was important for the association between LC3 and β-catenin, we used site-directed mutagenesis to generate a W504A and I507A β-catenin double mutant (termed β-catenin$^{W504A/I507A}$) (Figure 8C). Pulldown assays using recombinant GST-LC3 and lysates from cells expressing HA-tagged wild-type β-catenin (HA-β-catenin^{WT}) or W504A/I507A β-catenin (HA-β-catenin$^{W504A/I507A}$) revealed that the β-catenin/LC3 interaction was diminished when the putative LIR was mutated (Figure 8D). In complementary *in vitro* experiments, we found that recombinant myc-tagged His-LC3 (His-LC3-myc) directly interacted with recombinant GST-β-catenin^{WT}, but the interaction of His-LC3-myc with GST-β-catenin$^{W504A/I507A}$ was reduced (Figure 8E). In addition, *in vivo* co-immunoprecipitation experiments in cells transiently expressing YFP-LC3 with β-catenin^{WT} or β-catenin$^{W504A/I507A}$ demonstrated that while β-catenin^{WT} immunoprecipitated with YFP-LC3, the interaction was abolished by the β-catenin$^{W504A/I507A}$ mutation (Figure 8F; Figure S2E).

Having previously shown (Figure 6P) that the expression of a β-catenin mutant resistant to proteasomal degradation (myc-β-catenin^{AAAA}) decreased during nutrient deprivation, we made a β-

catenin$^{S33A/S37A/T41A/S45A/W504A/I507A}$ mutant (termed HA-β-catenin^{6A}) to distinguish between non-selective and selective autophagic β-catenin degradation during nutrient deprivation. Whereas expression of proteasome resistant myc-β-catenin^{AAAA} (Figure 6P) and HA-β-catenin^{S33Y} (Figure 8G) mutants decreased during nutrient deprivation, HA-β-catenin^{6A} expression was less sensitive to starvation-mediated β-catenin downregulation (Figure 8G). Taken together, these data show that β-catenin directly interacts with LC3 and hence is specifically targeted for degradation by autophagy.

Autophagy is up-regulated during conditions of stress for cell survival. However, excessive or unrestrained autophagy can eventually lead to the loss of cell viability (White & DiPaola, 2009). Interestingly, we found that inhibition of autophagy by Atg7 knockdown increased cell viability in HT29 cells subjected to starvation for 24 hours (Figure 8H). This suggests that, under these conditions, the inhibition of autophagy promotes survival in colorectal tumour cells. As β-catenin protein levels also increased following Atg7 knockdown (see Figures 6B and 6L), and as β-catenin has been linked to cell survival during conditions of stress (Essers et al, 2005; Kaidi et al, 2007), we reasoned that the increased levels of β-catenin may, at least in part, be responsible for the observed increase in cell survival. To examine this, we targeted both Atg7 and β-catenin simultaneously with siRNA. Indeed, the increase in cell viability afforded by Atg7 knockdown was partially abrogated when β-catenin was also depleted (Figure 8H). This suggests that β-catenin can promote cell survival in conditions of nutrient stress.

Discussion

During development and in the context of tumorigenesis, growing cells quickly outstrip their supply of oxygen and nutrients. Handling fluctuating microenvironmental conditions effectively requires precise regulation of transcriptional programs to properly equip cells for particular circumstances and ensure their survival. An important question that remains unanswered is how cells 'switch gear' to resolve the conflict between proliferative signals and autophagy to maintain cellular homeostasis, both under normal physiological conditions and during microenvironmental stress.

In this study, we demonstrate for the first time that β-catenin functions as a key cellular integration point to coordinate proliferative signalling with autophagy. Our data indicate that during nutrient stress, β-catenin interacts with LC3 and is directly targeted for autophagic degradation, providing a proliferative 'pit stop' and favouring adaptation by inhibiting β-catenin/TCF signalling. This represents a new paradigm for β-catenin protein turnover and Wnt signal regulation, where β-catenin is degraded via autophagy in a proteasome-independent manner. Furthermore, our *in vitro* and *in vivo* data provide the first evidence that

the Wnt/β-catenin pathway is a critical negative regulator of autophagy and repressor of p62 expression. These findings support a novel regulatory feedback model between the Wnt/β-catenin and autophagy pathways where growth and stress signals converge upon β-catenin to balance proliferation with autophagy and p62 expression (Figure 9).

Autophagy plays important roles in intracellular quality control and homeostasis under normal conditions, and has a dual role in cytoprotection and cell death during stress. Therefore, careful regulation of basal and induced levels of autophagy is important because either insufficient or excessive autophagy can be deleterious. For this reason, it is notable that the suppression of autophagy by Wnt/β-catenin reported in our study was apparent in both normal and starvation conditions, indicating a global mechanism of autophagy regulation by Wnt/β-catenin rather than solely during conditions of stress. This suggests that crosstalk between autophagy and Wnt/β-catenin signalling may be important not only during cellular adaptation to microenvironmental stress, but also during adult tissue homeostasis. As autophagy has roles in differentiation (Mizushima & Levine, 2010), and a major role of Wnt/β-catenin signalling may be to block differentiation (Clevers & Nusse, 2012), it is interesting to speculate that one mechanism by which Wnt/β-catenin contributes to the inhibition of differentiation is by limiting the capacity for autophagy.

What are the consequences of our findings in terms of colorectal tumorigenesis? Given that high levels of autophagy are thought to be incompatible with cell proliferation (Mizushima et al, 2008), Wnt/β-catenin-mediated lowering of autophagy levels may be advantageous for rapidly dividing cells such as tumour cells. Our data support this notion, as inhibition of autophagy by Atg7 knockdown in HT29 cells promoted cell survival, at least in part through increasing β-catenin levels. Given that mutational activation of Wnt/β-catenin signalling initiates intestinal tumorigenesis, this may reduce the capacity for autophagy at the earliest stages of tumour development, thus contributing to deregulated proliferation and tumour cell survival. Indeed, mutant β-catenin deletion has been reported to decrease colony forming efficiency in HCT116 cells (Chan et al, 2002) and our data would indicate that this could be due to increased levels of autophagy.

β-catenin has long been known as a transcriptional co-activator, while only few examples of Wnt/β-catenin-mediated gene repression have been reported. However, this underappreciated function has recently come to prominence with the proposal that gene suppression may form part of a global mechanism of regulation by Wnt/β-catenin signalling (Clevers & Nusse, 2012). This highlights the relevance of our data showing dynamic regulation of β-catenin at the *p62* promoter; mediating *p62* repression under normal conditions and *p62* derepression during nutrient stress. One consequence of Wnt/β-catenin-mediated p62 and autophagy suppression may be the accumulation of toxic misfolded proteins, as p62 is known to

scavenge soluble misfolded proteins for removal in autophagosomes. Indeed, it has previously been reported that depletion of p62 can inhibit the recruitment of LC3 to autophagosomes under starvation conditions and that the background level of LC3-II is higher in cells overexpressing p62, which may suggest that autophagic activity is greater when p62 levels are higher (Bjorkoy et al, 2005). Hence while p62 may not be absolutely required for autophagy per se (Komatsu et al, 2007), our finding that Wnt/β-catenin represses p62 expression may interfere with the capacity to degrade toxic substrates normally targeted by p62 for clearance by autophagy. This could have harmful consequences such as genome damage, leading to tumour progression. Interestingly, p62 accumulation has been reported in late stage colorectal tumours (Gao et al, 2010) and is known to promote tumorigenesis via activation of NF-κB signalling. How can this be reconciled with our finding that Wnt/β-catenin signalling represses p62 expression? One possibility is that increased p62 protein levels occur as a result of *p62* derepression during hypoxia when tumours outgrow their blood supply and β-catenin/TCF signalling is inhibited (Kaidi et al, 2007). Alternatively, mutational activation of the Ras pathway, which occurs commonly during colorectal tumour progression, could account for the observed increases in p62 expression in late stage tumours, since Ras has been reported to transcriptionally induce p62 expression via a MEK/PI3K-dependent mechanism (Duran et al, 2008). In addition, NF-κB signalling has been shown to transcriptionally induce *p62* downstream of Ras in pancreatic cancer, leading to a feed forward loop that increases NF-κB activity and p62 expression (Ling et al, 2012). Similarly, elevated p62 levels lead to hyperactivation of Nrf2, resulting in a positive feedback loop that drives further expression of p62 (Jain et al, 2010; Komatsu et al, 2010), which can contribute to the development of hepatocellular carcinoma (Inami et al, 2011). In future investigations it will be important to fully understand the roles of p62 in colorectal tumorigenesis and the mechanisms controlling its regulation during the adenoma-carcinoma sequence. Since p62 deregulation is observed in Parkinson's disease, insulin resistance and Paget's disease of bone (Geetha et al, 2012), our finding that the Wnt/β-catenin pathway regulates p62 could have implications for a number of human diseases.

Autophagy has also been reported to regulate turnover of dishevelled (Dvl) (Gao et al, 2010), an important component of the Wnt signalling pathway that transmits an inhibitory signal to the APC-containing β-catenin destruction complex. This regulation of Dvl likely to play important roles in tissue development and homeostasis. In contrast, the Wnt/β-catenin pathway is activated downstream of Dvl in the majority of colorectal tumours (most commonly through APC mutation or deletion), and therefore our finding that β-catenin/TCF activity is directly regulated by the autophagic degradation of β-catenin may be particularly important in the context of tumorigenesis. Our study indicates that therapeutic augmentation of autophagy

may be effective in preventing the growth of colorectal tumours, where aberrantly high levels of β-catenin (due to defective clearance by the proteasome) could be targeted for degradation by autophagy, thereby inhibiting oncogenic Wnt/β-catenin pathway activity.

Given that autophagy occurs physiologically during mammalian development and differentiation (Mizushima & Levine, 2010), where Wnt/β-catenin signalling is also known to have important roles, the novel crosstalk between autophagy and β-catenin reported here may have broad functional implications during tumorigenesis and beyond. Indeed, aberrant Wnt/β-catenin signalling and disturbances to autophagy are known to underlie not only tumorigenesis but a wide range of important pathologies in humans. Therefore, our study may present new opportunities to further our understanding of key cellular processes influenced by Wnt/β-catenin signalling and autophagy, providing new therapeutic avenues for exploration in a range of important diseases.

Materials and methods

Cell culture

HT29 (APC mutant/β-catenin wild-type) and RKO (APC wild-type/β-catenin wild-type) carcinoma-derived cell lines and HEK293T cells were maintained as described previously (Smartt et al, 2012). Isogenic HCT116 cells carrying a mutant or wild-type *CTNNB1* allele (a kind gift from Bert Vogelstein) have been described previously (Chan et al, 2002) and are referred to as β-catenin$^{WT/-}$ (mutant *CTNNB1* allele deleted) and β-catenin$^{-/\Delta S45}$ (wild-type *CTNNB1* allele deleted). HCT116 cells were maintained in McCoy's medium (Gibco) containing 10% FBS, supplemented with penicillin (100 units/ml), streptomycin (100µg/ml) and glutamine (2mM). The doxycycline-inducible DNTCF4-expressing LS174T cell line (a kind gift from Hans Clevers) was maintained in RPMI (Gibco) containing 5% tetracycline-free FBS, supplemented with glutamine (2mM). HT29 cells engineered to stably express YFP-LC3B were selected with G418 before experiments were set up.

Treatments

For starvation conditions, cells were grown in ddH_2O containing 140 mM $NaCl_2$, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, 20 mM HEPES, 1% BSA, pH 7.4 (Axe et al, 2008). Treatments with the following reagents were performed as described: mTOR inhibitor PP242 (100nM) Sigma (P0037); bafilomycin A1 (100nM) Sigma (B1793); chloroquine diphosphate (10µM) Sigma (C6628); cycloheximide (10µg/µl) Sigma (C4859); actinomycin D (8µM) Sigma (A9415); Wnt3a (100-200ng/ml) R&D (5036-WN), MG132 (10µM) Calbiochem (474790), wortmannin (50nM) Sigma (W1628).

Plasmids and cloning

The following mammalian plasmid expression constructs were used: β-catenin^{WT} and β-catenin^{S33Y} were kind gifts from Eric Fearon; myc-tagged β-catenin^{AAAA} (S33A, S37A, T41A, S45A) was a kind gift from Benjamin Alman (Cheon et al, 2002). Site-directed mutagenesis (Genewiz) was used to generate β-catenin$^{W504A/I507A}$ and β-catenin$^{S33A/S37A/T41A/S45A/W504A/I507A}$ (termed β-catenin^{6A}). β-catenin^{WT}, β-catenin$^{W504A/I507A}$ and β-catenin^{6A} were directionally subcloned into HA-pcDNA3.1 (a kind gift from Ian McGough and Pete Cullen, University of Bristol) using BamH1 and Not1 restriction sites.

Transfection, siRNA and TopFlash/FopFlash reporter assays

All transfections were performed using Lipofectamine 2000 (Invitrogen) in Opti-MEM (Gibco). For knockdown experiments, cells were reverse transfected with siRNA sequences to human β-catenin, Atg7 or validated non-targeting negative control siRNA, as described previously (Greenhough et al, 2010). Briefly, cells were transfected overnight (18h) and normal growth medium was replaced the next day; 24h later cells were treated as indicated. The following siRNAs were used (Ambion): β-catenin ID: 42816 (10nM unless otherwise stated), Atg7 pool ID: 20650/20651/20652 (20nM unless otherwise stated). Luciferase TopFlash/FopFlash reporter assay constructs were co-transfected with TK renilla luciferase reporter. After recovery, cells were treated as described in figure legends. Activity was determined using a dual luciferase reporter system (Promega).

Recombinant protein expression and GST pulldown assays

GST (12-350) and human GST-β-catenin (12-537) proteins were purchased from Millipore; human GST-LC3B (BML-UW1155-0500) was purchased from Enzo Life Sciences. His-LC3B-myc was produced as described previously (Betin & Lane, 2009). β-catenin$^{W504A/I507A}$ cDNA was directionally subcloned into the GST vector pGEX-6P1 (GE Healthcare) using BamH1 and Not1 restriction sites and expressed in BL21 cells. GST protein expression was induced by 0.3mM IPTG (Sigma, I6758) overnight at 15°C. GST-β-catenin$^{W504A/I507A}$ was purified on glutathione sepharose 4B beads (GE healthcare), eluted (50mM Tris pH8, 25mM reduced glutathione) and subject to diafiltration (Amicon Ultra, Millipore). GST pulldown assays were performed as described previously (Nguyen & Goodrich, 2006; Pankiv et al, 2007). Briefly, for GST pulldowns with His-LC3B-myc, 2μg GST-tagged proteins was bound to 10μL glutathione sepharose 4B beads and incubated with 0.5μg His-LC3B-myc in 40μL NETN-E buffer (50 mM Tris pH8, 100-500mM NaCl, 6mM EDTA, 6mM EGTA, 0.5% NP40) supplemented with 1mM dithiothreitol and complete-mini protease

inhibitor cocktail tablet (Roche) for 1h at 4°C, washed five times in ten bead volumes NETN-E buffer, and boiled in 2x Laemmli buffer prior to loading onto SDS-PAGE gels for analysis. For GST pulldowns using cell lysates, HEK293 cells were transfected for 6h with constructs encoding proteins of interest. 24h later cells were washed with ice-cold PBS and lysed on ice for 10 minutes with Cell Signaling Technology lysis buffer (9803) supplemented with protease inhibitors. Following protein quantitation (Bio-Rad DC protein assay), 500ug protein lysate was incubated with 5µg GST-protein bound to glutathione sepharose 4B beads for 1h at 4°C, prior to washing and preparation for SDS-PAGE as described above.

Western blotting

Western blot analysis was performed are described previously (Greenhough et al, 2010) using the following antibodies: LC3B (Sigma, 17453), p62 (BD, 610832), β-actin (Sigma, A5316), β-catenin (Cell Signaling Technology, 9587 and BD, 610154), TCF4 (Sigma, T5187), Lgr5 (Abcam, ab75850) Atg7 (Cell Signaling Technology, 2631), Phospho-p70 S6K (Cell Signaling Technology, 9205), p-4E-BP1 (Cell Signaling Technology, 2855), HA (Covance, HA.11), GFP (Covance, MMS-118R). Cells were washed with ice-cold PBS and lysed on ice for 10 minutes with Cell Signaling Technology lysis buffer (9803) supplemented with protease inhibitors. Equal concentrations of protein were resolved using SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to an Immobilon-P polyvinylidene difluoride membrane (Millipore). Densitometric analysis was performed using ImageJ software.

Immunofluorescence

Treated cells grown on cover slips were fixed and, if required, stained with primary and secondary antibodies prior to DAPI nuclear staining and mounted on to slides. The following antibodies were used for immunofluorescence: β-catenin (BD, 610154), p62 (BD, 610832), LC3B (MBL, PM036), LAMP-1 (Abcam, 24170). Widefield and Confocal microscopes were used as described previously (Betin et al, 2012). Images were processed in Adobe Photoshop (as described in corresponding legends).

Real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

Following treatment, RNA was extracted using Tri-reagent (Sigma), chloroform and isopropanol. After purification using the Turbo DNase kit (Ambion), complementary DNA was produced using the moloney murine leukaemia virus reverse transcriptase kit (Promega). qRT-PCR was performed using a SYBR Green

PCR kit (Qiagen) and primers for β-catenin and p62 (Qiagen) in a Stratagene MX3005P QPCR cycler (La Jolla).

Chromatin Immunoprecipitation (ChIP)

Following treatment, cells were fixed in 1% paraformaldehyde for 10 minutes and processed using EZ-Magna ChIP Protein G immunoprecipitation kit (Millipore). Briefly, chromatin from nuclear lysates was sheared to a 500bp average by sonication (Diagenode Bioruptor), pre-cleared and subject to immunoprecipitation overnight at 4°C with antibodies to normal mouse IgG (Millipore, 12-371), RNA polymerase II (Millipore), β-catenin (BD, 610154), TCF4 (Sigma, T5187) or acetylated histone H3 (Millipore, 06-599). Samples were incubated for a further 1 hour at 4°C with Protein G Magnabeads (Millipore) and processed using a DynaMag-2 magnetic particle separator (Invitrogen). qPCR was performed as above with the following primers: *p62* promoter, forward 5'-ATGGGACGCTGACTCACTGC-3', reverse 5'-GAAGCACAGAAGAGGGAGTCT-3' to amplify a 212bp product. *GAPDH* promoter primers (Millipore) were used to amplify PCR product of 166bp.

Animal models

Intraperitoneal injection of 1mg per 20g of body weight of tamoxifen for 2-4 consecutive days induced β-catenin deletion in 10 week old β-catenin$^{-/lox}$-villin-creERT2 mice (Fevr et al, 2007) compared to similarly treated control β-catenin$^{+/lox}$-villin-creERT2 mice. Immunofluorescence confirmed β-catenin deletion. All animal procedures were performed in accordance with the Swiss legislation on animal experimentation.

Immunoprecipitation

GFP-Trap (ChromoTek, gta-20) was used to pull-down tagged YFP-LC3. 400μg of whole cell lysate was used per condition and treated as per manufacturer's instructions with the addition of high salt washes (500mM NaCl). Bound and unbound protein was assessed by western blotting. A YFP-only construct was used as a control.

Cell death quantification

Cell death was determined as described previously (Greenhough et al, 2010; Kaidi et al, 2007).

Statistical analyses

Student's t-test and ANOVA were performed and expressed as * P < 0.05, ** P < 0.01, *** P < 0.001, NS: not significant.

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Author contributions

KJP, CP and AG designed the project. CP and AG supervised the project. ACW, JL, CP and AG monitored the project. KJP, AG, PO-M, TJC, HJMS and JB carried out experiments. JH was responsible for animal studies. JL, JH, HJMS and KM gave technical support and conceptual advice. KJP, AG, PO-M and TJC collected and analysed data. KJP, CP and AG prepared the manuscript and KJP, ACW, JL, HJMS, TJC, PO-M, JH, KM, CP and AG revised the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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Figure Legends

Figure 1. Modulation of β-catenin expression regulates autophagosome number

(A-C) Measurement of autophagy during nutrient starvation in HT29 cells stably expressing YFP-LC3. (A) Increased LC3 puncta per cell following β-catenin knockdown with siRNA compared with a non-targeting (NT) control siRNA. Columns show autophagosome numbers (mean ± SEM, n>200 cells in >20 fields of view per experiment of 3 independent treatments) assessed under normal and starved (2h) conditions with β-catenin or non-targeting (NT) siRNA. Representative images (B) of YFP-LC3 puncta (green) and DAPI nuclei staining (blue) are shown. (C) Upper panel: Western blotting showed increased LC3-II expression in HT29 cells with β-catenin knockdown compared to control cells after 2h starvation. Lower panel: Quantification of the LC3-II/β-actin ratio by densitometry (mean ± SEM of 4 independent treatments, * P=0.011 in nutrient conditions, * P=0.035 under starvation). (D-E) HCT116 β-catenin$^{WT/-}$ cells overexpressing a control or β-catenin^{S33Y} plasmid nutrient starved for 24h. (D) Immunostaining of LC3 (green) with DAPI nuclei staining (blue) showed decreased LC3 puncta with β-catenin^{S33Y} overexpression. (E) By western blotting, LC3-II decreased with β-catenin^{S33Y} overexpression (Upper panel). Lower panel: Quantification of the LC3-II/β-actin ratio by densitometry (mean ± SEM of 4 independent treatments, * P=0.015). (F) Comparison of isogenic β-catenin$^{WT/-}$ and β-catenin$^{-/\Delta S45}$ HCT116 cells. Upper panel: Western blotting showed decreased LC3-II in HCT116 β-catenin$^{-/\Delta S45}$ cells. Lower panel: Western blotting confirmed by quantification using densitometry of the LC3-II/β-actin ratio (mean ± SEM of 3 independent treatments, * P=0.046).

Figure 2. β-catenin negatively regulates p62 expression but does not block autophagic flux

(A) Protein expression of p62 increased following β-catenin knockdown under normal and starved conditions in HT29 cells. (B) Comparison of p62 expression in isogenic HCT116 β-catenin$^{WT/-}$ and HCT116 β-catenin$^{-/\Delta S45}$ cells by western blotting showed decreased p62 expression in β-catenin$^{-/\Delta S45}$ cells. (C) LC3 puncta number per cell during starvation and nutrient addback post starvation. Left panel: LC3 puncta number reduces in non-targeting (NT) siRNA control with nutrient addback. Right panel: LC3 puncta number reduces in β-catenin knockdown cells with nutrient addback (mean ± SEM of 3 independent treatments; n>200 cells in >20 fields of view per experiment). Western blotting for β-catenin confirmed knockdown. (D) Western blotting of LC3 and p62 in HT29 cells following β-catenin knockdown and starvation with lysosomal inhibitors. LC3-II and p62 protein expression showed an increase with β-catenin siRNA in the presence of autophagy

flux inhibitors chloroquine (10μM) or bafilomycin A1 (100nM) (applied for the final 30 minutes of the 8 hours) compared to β-catenin knockdown alone. Quantification by densitometry of the LC3-II/β-actin ratio (mean ± SEM of 4 independent experiments). See also Figure S1A.

Figure 3. β-catenin deletion increases LC3 puncta and p62 expression in mouse intestinal epithelium *in vivo*

(A-D) LC3 staining (red) in mouse intestinal crypt and villus epithelium increased (white arrowheads) two days post β-catenin deletion in β-catenin$^{-/lox}$-villin-creERT2 mice (C and D) compared to control β-catenin$^{+/lox}$-villin-creERT2 mice (A and B). Further magnification of the areas marked by white squares is shown (lower panels). (E-H) p62 staining (green) increased in the crypt and villus epithelium two days after β-catenin deletion in β-catenin$^{-/lox}$-villin-creERT2 mice (G and H) compared to control in β-catenin$^{+/lox}$-villin-creERT2 mice (E and F). DAPI staining (blue) identifies nuclei. β-catenin deletion was confirmed by immunofluorescence (Figure S1B). Red and green channel levels were adjusted post acquisition for clarity (equal level changes applied across the entire figure). Further magnification of the areas marked by white squares is shown (lower panels). (I and J) Western blotting on extracts from mouse intestinal epithelial tissue demonstrated increased p62 and LC3-II protein expression two (I) and four (J) days post β-catenin deletion in β-catenin$^{-/lox}$-villin-creERT2 mice compared to control β-catenin$^{+/lox}$-villin-creERT2 mice.

Figure 4. The Wnt/β-catenin/TCF pathway controls p62 expression

(A) Relative *p62/SQSTM1* mRNA levels by quantitative real time-PCR (qRT-PCR) increased following β-catenin knockdown in HT29 cells (mean ± SEM, 3 independent experiments performed in triplicate, *** $P<0.001$). β-catenin knockdown was confirmed by western blotting. (B) Relative *p62* mRNA levels by qRT-PCR decreased with β-catenin^{S33Y} overexpression after 24h of starvation in HCT116 β-catenin$^{WT/-}$ cells (mean ± SEM, 3 independent experiments performed in triplicate, ***$P<0.001$). β-catenin^{S33Y} overexpression was confirmed by western blotting. (C) The increase in p62 protein expression induced by β-catenin siRNA was attenuated in HT29 cells treated with 10μg/μl cycloheximide and starvation for 8h and 24h compared to vehicle control. A complementary experiment using the transcription inhibitor actinomycin D is shown in Figure S1C. (D) Overexpression of Fzd6 (green, left panel) increased p62 (red, right panel) protein expression in HEK293T cells. As an internal control, cells not overexpressing Fzd6 are shown in the field of view. (E) Doxycycline-induction (1μg/mL) of DNTCF4 increased p62 and LC3-II protein expression in doxycycline-inducible DNTCF4 LS174T-L8 cells. LGR5 downregulation by DNTCF4 confirmed inhibition of β-catenin/TCF4 signalling. (F) Relative *p62* mRNA levels (48h) by qRT-PCR increased following doxycycline induction of DNTCF4 in LS174T-L8 cells (mean ± SEM, 3 independent experiments performed in triplicate, ***$P<0.001$). (G) Upper panel: Wnt3a (200ng/mL) treatment of HCT116 β-catenin$^{WT/-}$ cells (24h) decreased LC3-II and p62 protein expression by western blotting. Lower panel: Quantification by densitometry of the LC3-II/β-actin ratio (mean ± SEM of 3 independent experiments).

Figure 5. β-catenin and TCF4 associate with the *p62/SQSTM1* gene promoter

(A) Chromatin immunoprecipitation (ChIP) demonstrates β-catenin and TCF4 binding to the p62 promoter. HT29 cells were grown in normal or starvation conditions for 2h and subjected to ChIP analysis with the indicated antibodies (IgG was used as a negative control). Binding of RNA Pol II, β-catenin and TCF4 to the

p62 promoter region was measured by quantitative PCR and expressed as percent enrichment relative to the input chromatin. During nutrient deprivation, binding of RNA Pol II to the *p62* promoter increased; binding of β-catenin to the *p62* promoter decreased; TCF4 binding did not change (data are from one representative experiment of at least three independent experiments performed in triplicate). PCR products subjected to agarose gel electrophoresis are shown in Figure S2A. B) Relative p62 mRNA expression by qRT-PCR increases ~14 fold after 24h starvation in HT29 cells (mean ± SEM, 3 independent experiments performed in triplicate, *** P<0.001). (C) Binding of acetyl-Histone H3 to the *p62* promoter increased under starvation conditions, suggesting *p62* gene derepression (data are from one representative experiment of at least three independent experiments performed in triplicate). (D) Binding of β-catenin to the *p62* promoter (relative to β-catenin binding to a non-target gene promoter, *GAPDH*) significantly decreased under starvation conditions (mean ± SEM, 3 independent experiments performed in triplicate, ** P<0.01).

Figure 6. Autophagy induction reduces Wnt/β-catenin signalling

(A) Western blotting showing that autophagy was induced after 2h starvation, as evidenced by decreased p62 protein and increased LC3-II protein expression. β-catenin protein expression decreased over a 24h period of starvation in HT29 cells. (B) Autophagy decreased following Atg7 knockdown as shown by increased p62 and decreased LC3-II protein expression. β-catenin protein expression increased following Atg7 siRNA. Reduction of LC3 puncta with Atg7 siRNA was confirmed by immunofluorescence and is shown in Figure S2B. (C-D) Reduction of TopFlash activity in HT29 cells after 24h of autophagy induction using (C) starvation or (D) 100nM mTOR inhibitor PP242 (C and D, mean ± SEM, 3 independent experiments performed in triplicate, *** *P*<0.001). Autophagy induction was confirmed by western blotting and is shown in Figures S2C and S2D). (E-F) qRT-PCR shows reduction of Wnt target gene expression (E) *Axin2* (* *P*=0.017) and (F) *Cyclin D1* (** *P*=0.0036) after 8h starvation (mean ± SEM, 3 (Axin2) or 6 (Cyclin D1) independent experiments performed in triplicate). (G-I) Inhibition of Wnt-induced TopFlash activity by autophagy induction in (G) HCT116 β-catenin$^{WT/-}$ cells (mean ± SEM, 3 independent experiments performed in triplicate, ** *P*<0.01) and (H) RKO cells (mean ± SEM, 3 independent experiments performed in triplicate, * *P*<0.05). (I) Reduction of Wnt3a-induced *Cyclin D1* gene expression RKO cells after 12h treatment with autophagy induction using starvation or PP242 (mean ± SEM, 2 independent experiments). (J-K) TopFlash activity following Atg7 knockdown in (J) HT29 cells (mean ± SEM, 4 independent experiments performed in triplicate, * *P*=0.0488) and (K) RKO cells with 24h Wnt3a treatment (mean ± SEM, 3 independent experiments performed in triplicate, * *P*=0.0282). (L) A representative western blot of RKO cells following Wnt3a treatment and Atg7 siRNA. (M) Relative β-catenin mRNA levels did not change after 8h starvation by qRT-PCR (mean ± SEM, 3 independent experiments performed in triplicate). (N) Western blot showing prevented β-catenin protein degradation during starvation in the presence of lysosomal autophagy inhibitors chloroquine (10μM) and bafilomycin A1 (100nM) compared to starvation alone. (O) Inhibition of autophagy using wortmannin (50nM) prevented β-catenin protein degradation during starvation. (P) Western blot analysis of RKO cells expressing a myc-tagged β-catenin mutant (S33A, S37A, T41A, S45A) resistant to proteasomal degradation (myc-β-catenin^{AAAA}). RKO cells were subject to nutrient starvation for 2h, 8h and 24h, and autophagy induction was confirmed by increased LC3-II and decreased p62 protein expression. Both endogenous β-catenin and myc-β-catenin^{AAAA} protein expression decreased during starvation. (Q)

Proteasome inhibition with MG132 (10μM) did not prevent starvation-induced decrease in β-catenin protein expression.

Figure 7. LC3 and β-catenin co-localise in the mouse intestinal epithelium

(A and B) Immunofluorescence of LC3 (red) and β-catenin (green) expression in the intestinal epithelium following 2 days tamoxifen treatment in control (A: β-catenin$^{+/lox}$-villin-creERT2) and β-catenin deleted (B: β-catenin$^{-/lox}$-villin-creERT2) mice. (C and D) Magnified areas from (B) revealing co-localisation of LC3 (red) and β-catenin (green). Arrowheads indicate co-localisation of LC3 and β-catenin. (E) Line scan analyses from (C) and (D) showing staining intensity of indicated co-localised puncta. Red and green channel levels were adjusted post acquisition (equal changes applied across the entire figure) and the blue channel was removed for clarity.

Figure 8. β-catenin directly interacts with the autophagy protein LC3

(A) Co-immunoprecipitation of YFP-LC3 or negative control YFP in HT29 cells. Binding of endogenous β-catenin to YFP-LC3 was detected after 8h of autophagy induction by starvation and starvation in the presence of lysosomal autophagy flux inhibitor chloroquine (10μM). Input and immunodepleted lysates are shown. (B) Immunoprecipitation of endogenous LC3 in HT29 cells. IgG was used as a negative control. (C) β-catenin contains a W/YXXI/L motif (putative LC3-interacting region) at amino acid positions 504-507. β-catenin wild-type (green highlight) and W504A/I507A point mutant (blue highlight) sequences used in (D-F) are shown. (D) Pulldown assays using recombinant GST or GST-LC3B and lysates from HEK293 cells expressing HA-tagged wild-type β-catenin (HA-β-catenin^{WT}) or W504A/I507A β-catenin (HA-β-catenin$^{W504A/I507A}$). HA-β-catenin$^{W504A/I507A}$ exhibited reduced GST-LC3B binding compared to HA-β-catenin^{WT}. (E) Recombinant myc-tagged His-LC3 (His-LC3-myc) interacted with recombinant GST-β-catenin^{WT} *in vitro* but binding to GST-β-catenin$^{W504A/I507A}$ was reduced (upper panel). Input recombinant proteins visualised with coomassie blue are shown (lower panels). (F) Co-immunoprecipitation experiments using lysates from HEK293 cells transiently expressing YFP-LC3 with β-catenin^{WT} or β-catenin$^{W504A/I507A}$ starved for 8h with chloroquine (10μM). YFP-LC3 immunoprecipitated β-catenin^{WT} but not β-catenin$^{W504A/I507A}$. Input lysates are shown and immunoprecipitated p62 and YFP-LC3 served as positive controls. (G) HA-β-catenin$^{S33A/S37A/T41A/S45A/W504A/I507A}$ (HA-β-catenin^{6A}) was more resistant to the starvation-induced reduction in β-catenin protein expression than HA-β-catenin^{S33Y}. (H) Relative cell death in HT29 cells subject to nutrient starvation for 24h. Atg7 knockdown increased cell survival. The increase in cell survival following Atg7 knockdown was significantly reversed by simultaneously depleting β-catenin (double knockdown of Atg7 and β-catenin). Data is the mean ± SEM of 3 independent experiments performed in triplicate, *** P<0.001; ** *P*<0.01.

Figure 9. Working model summarising the crosstalk between Wnt/β-catenin signalling and autophagy described in this study

β-catenin is a cellular integration point coordinating proliferative signalling with autophagy. Under normal physiological conditions (nutrient rich) when autophagy is required at basal levels only, β-catenin limits autophagy and functions as a transcriptional co-repressor of p62. During nutrient deprivation (starvation), the

inhibitory function of β-catenin on autophagy is reduced, *p62* becomes derepressed, and β-catenin is targeted for autophagic degradation.

Figure 1

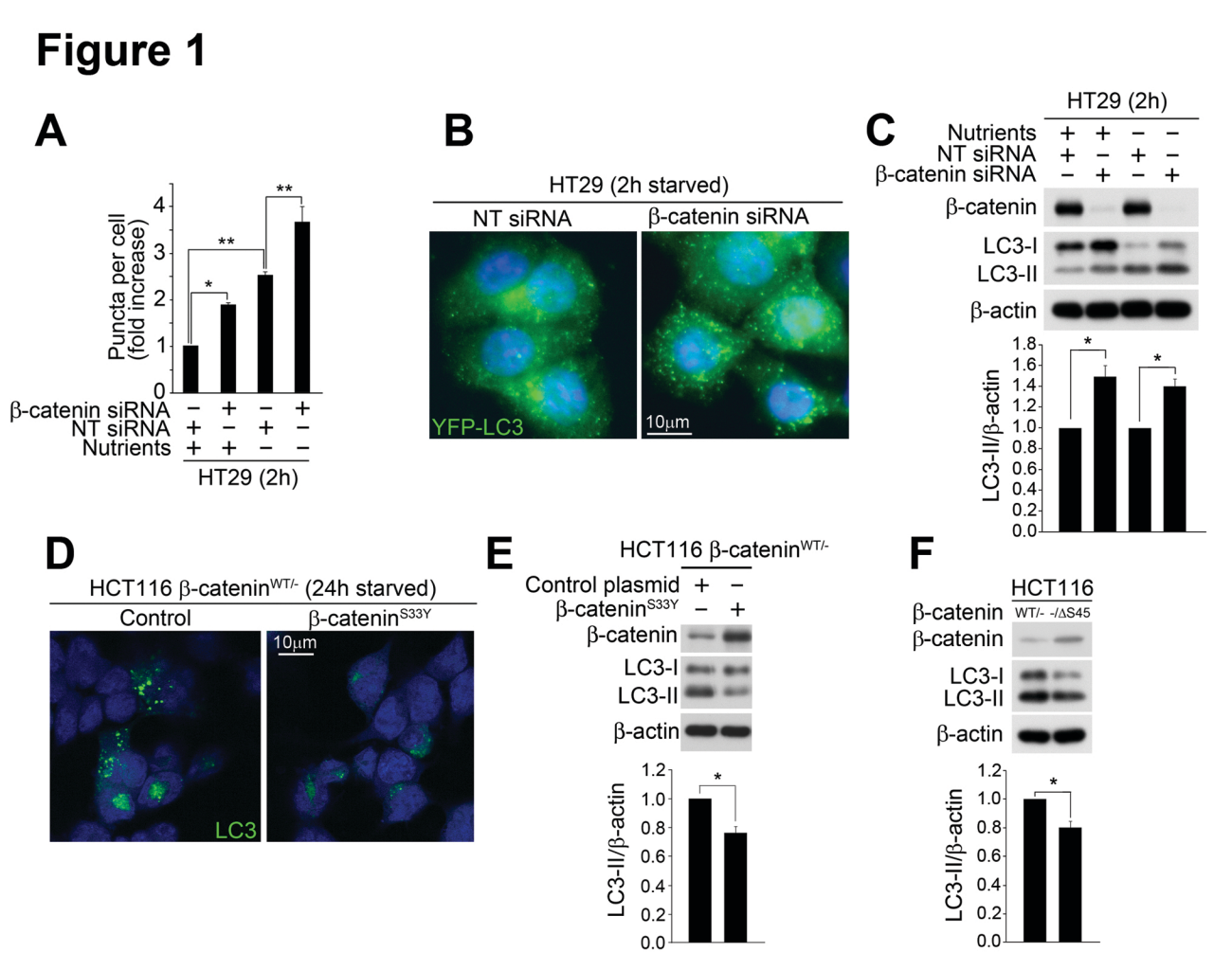

A
Puncta per cell (fold increase)
β-catenin siRNA
NT siRNA
Nutrients
HT29 (2h)
B
HT29 (2h starved)
NT siRNA
β-catenin siRNA
YFP-LC3
10μm
C
HT29 (2h)
Nutrients
NT siRNA
β-catenin siRNA
β-catenin
LC3-I
LC3-II
β-actin
LC3-II/β-actin
D
HCT116 β-catenin WT/- (24h starved)
Control
β-catenin S33Y
10μm
LC3
E
HCT116 β-catenin WT/-
Control plasmid
β-catenin S33Y
β-catenin
LC3-I
LC3-II
β-actin
LC3-II/β-actin
F
HCT116
β-catenin
WT/-
-/ΔS45
β-catenin
LC3-I
LC3-II
β-actin
LC3-II/β-actin

Figure 2

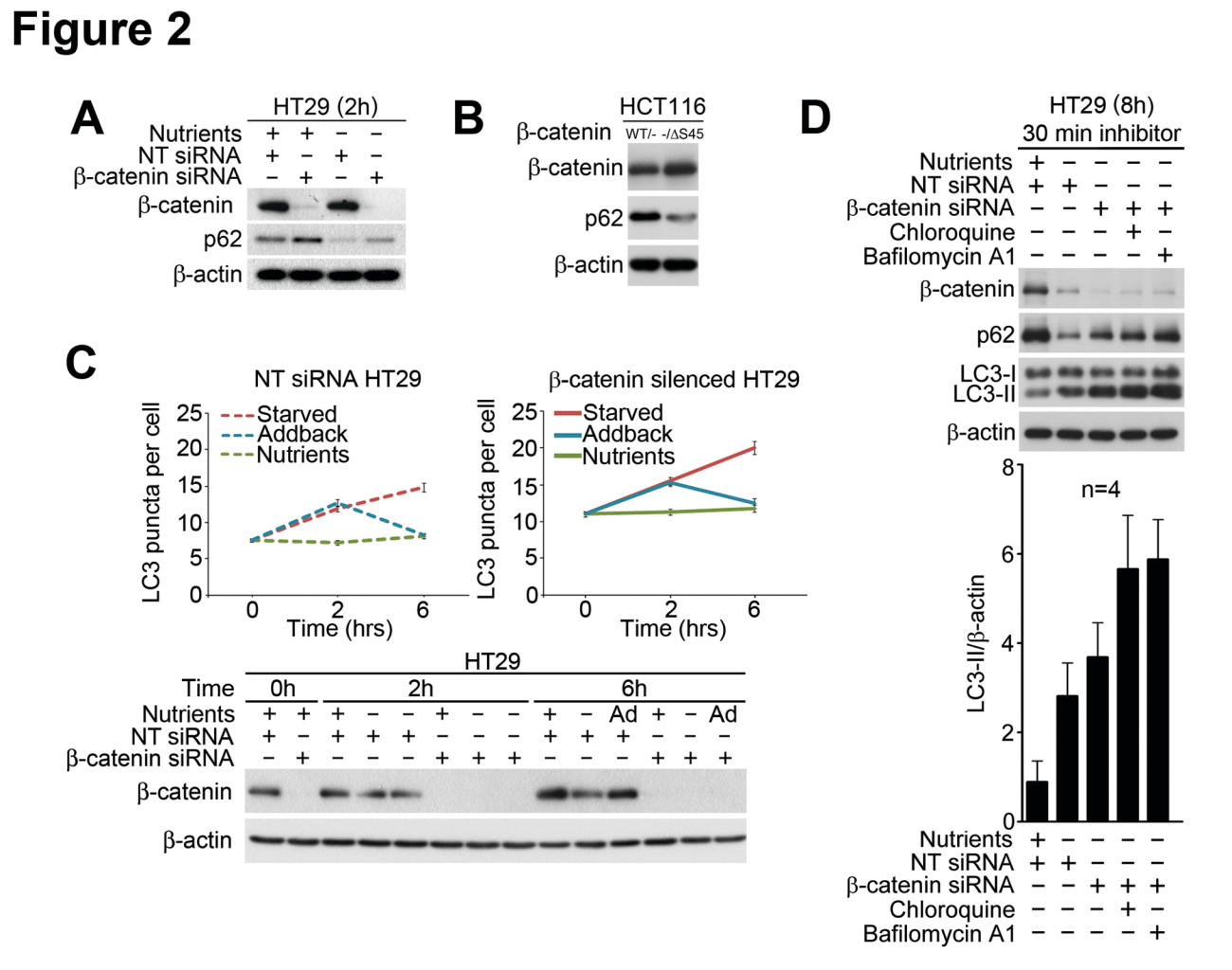
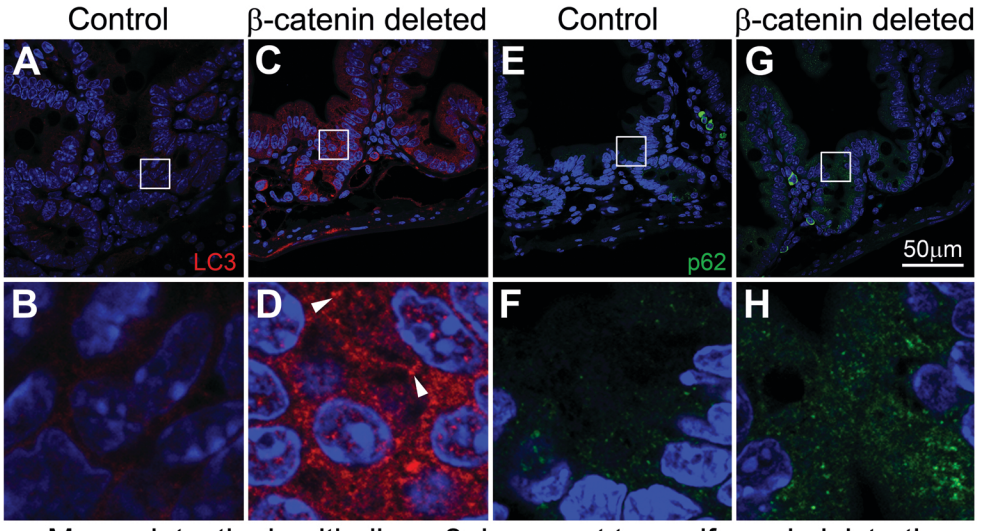

A
HT29 (2h)
Nutrients + + − −
NT siRNA + − + −
β-catenin siRNA − + − +
β-catenin
p62
β-actin
B
HCT116
β-catenin WT/- -/ΔS45
β-catenin
p62
β-actin
C
NT siRNA HT29
β-catenin silenced HT29
LC3 puncta per cell
Starved
Addback
Nutrients
Time (hrs)
HT29
Time 0h 2h 6h
Nutrients + + + − − + − − + − Ad + − Ad
NT siRNA + − + + + − − − + + + − − −
β-catenin siRNA − + − − − + + + − − − + + +
β-catenin
β-actin
D
HT29 (8h)
30 min inhibitor
Nutrients + − − − −
NT siRNA + + − − −
β-catenin siRNA − − + + +
Chloroquine − − − + −
Bafilomycin A1 − − − − +
β-catenin
p62
LC3-I
LC3-II
β-actin
n=4
LC3-II/β-actin
Nutrients + − − − −
NT siRNA + + − − −
β-catenin siRNA − − + + +
Chloroquine − − − + −
Bafilomycin A1 − − − − +

Figure 3



Mouse intestinal epithelium: 2 days post tamoxifen administration

Figure 4

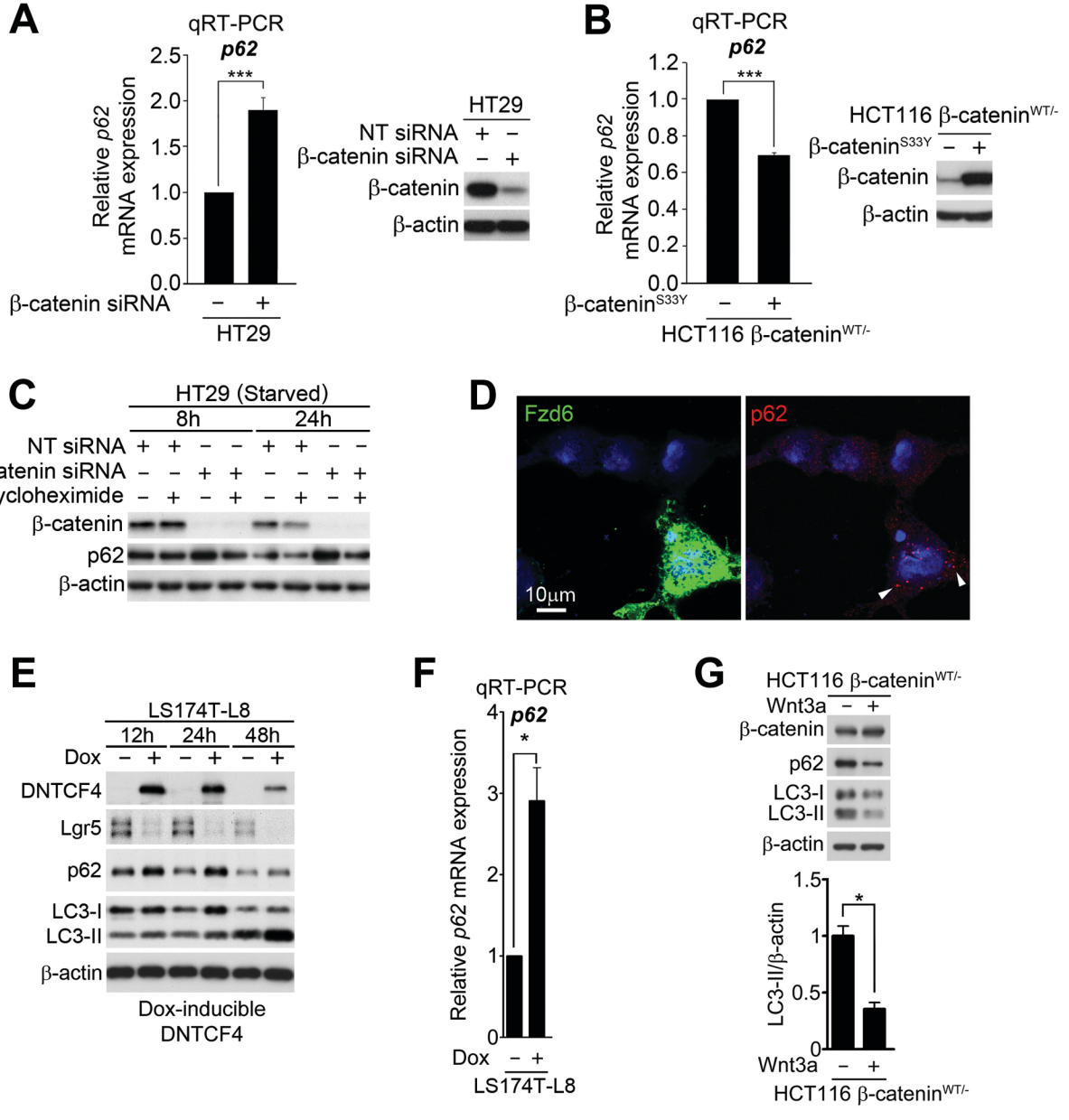

A
qRT-PCR
p62
***
Relative p62 mRNA expression
2.5
2.0
1.5
1.0
0.5
0.0
β-catenin siRNA – +
HT29
HT29
NT siRNA + –
β-catenin siRNA – +
β-catenin
β-actin
B
qRT-PCR
p62
***
Relative p62 mRNA expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
β-catenin S33Y – +
HCT116 β-catenin WT/-
HCT116 β-catenin WT/-
β-catenin S33Y – +
β-catenin
β-actin
C
HT29 (Starved)
8h
24h
NT siRNA + + – – + + – –
β-catenin siRNA – – + + – – + +
Cycloheximide – + – + – + – +
β-catenin
p62
β-actin
D
Fzd6
p62
10μm
E
LS174T-L8
12h
24h
48h
Dox – + – + – +
DNTCF4
Lgr5
p62
LC3-I
LC3-II
β-actin
Dox-inducible DNTCF4
F
qRT-PCR
p62
*
Relative p62 mRNA expression
4
3
2
1
0
Dox – +
LS174T-L8
G
HCT116 β-catenin WT/-
Wnt3a – +
β-catenin
p62
LC3-I
LC3-II
β-actin
LC3-II/β-actin
1.5
1
0.5
0
*
Wnt3a – +
HCT116 β-catenin WT/-

Figure 5

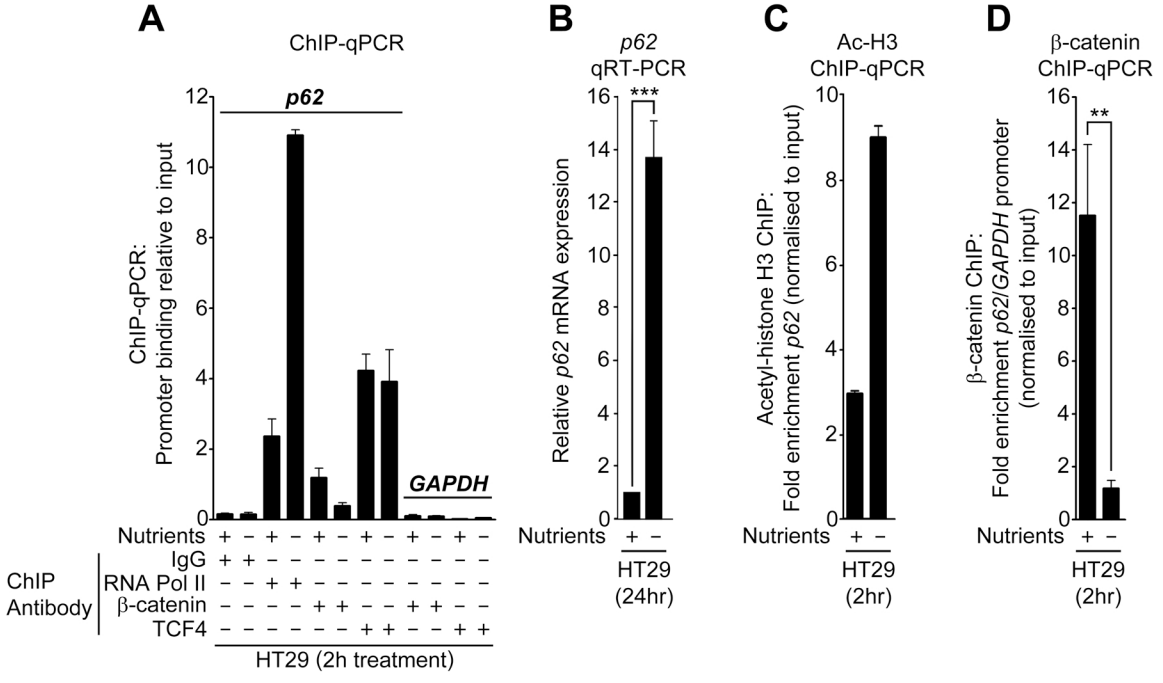

A
ChIP-qPCR
p62
GAPDH
ChIP-qPCR: Promoter binding relative to input
Nutrients
ChIP Antibody
IgG
RNA Pol II
β-catenin
TCF4
HT29 (2h treatment)
B
p62 qRT-PCR
***
Relative p62 mRNA expression
Nutrients
HT29 (24hr)
C
Ac-H3 ChIP-qPCR
Acetyl-histone H3 ChIP: Fold enrichment p62 (normalised to input)
Nutrients
HT29 (2hr)
D
β-catenin ChIP-qPCR
**
β-catenin ChIP: Fold enrichment p62/GAPDH promoter (normalised to input)
Nutrients
HT29 (2hr)

Figure 6

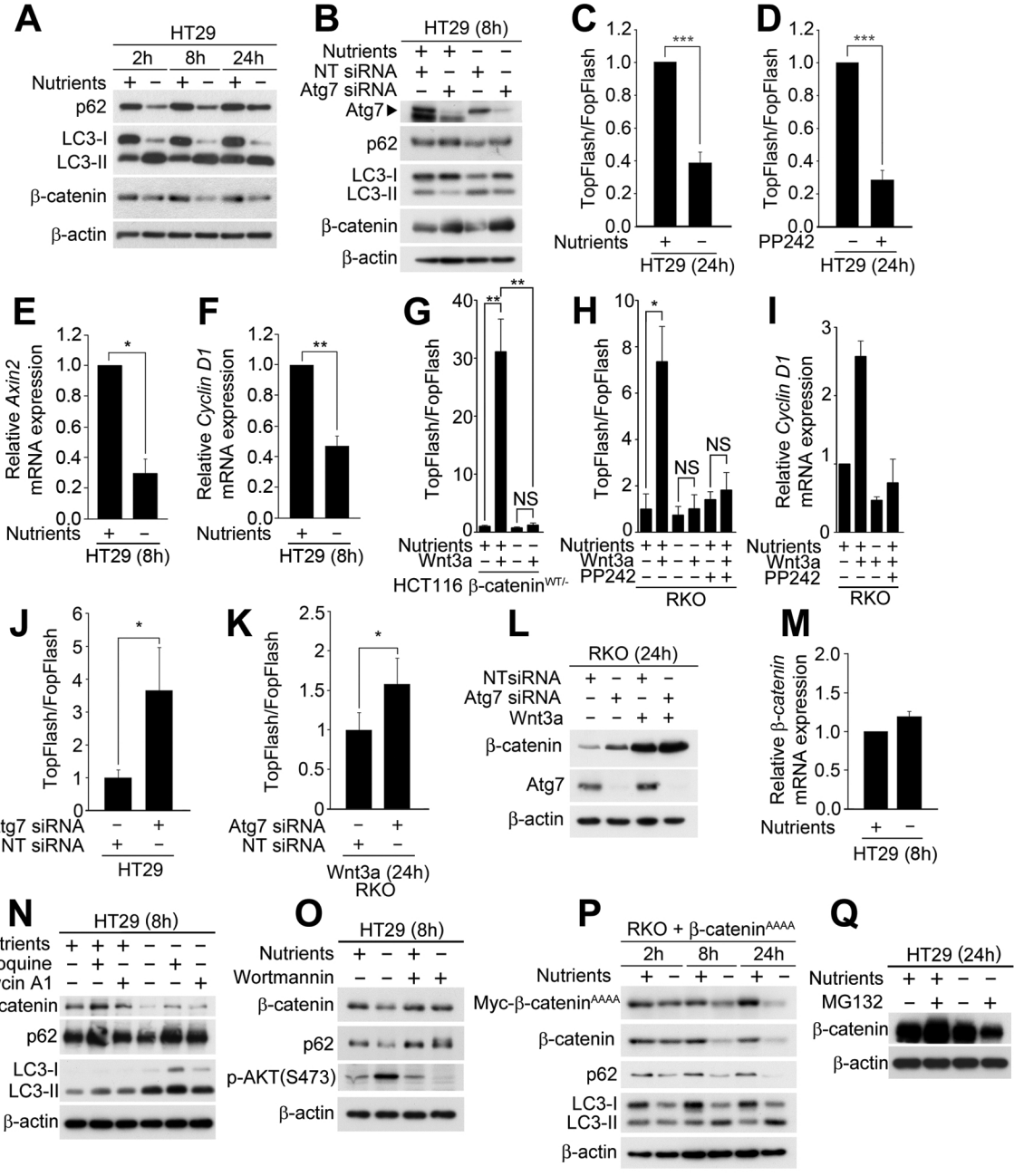

A
HT29
2h
8h
24h
Nutrients + − + − + −
p62
LC3-I
LC3-II
β-catenin
β-actin
B
HT29 (8h)
Nutrients + + − −
NT siRNA + − + −
Atg7 siRNA − + − +
Atg7▶
p62
LC3-I
LC3-II
β-catenin
β-actin
C
TopFlash/FopFlash
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
Nutrients + −
HT29 (24h)
D
TopFlash/FopFlash
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
PP242 − +
HT29 (24h)
E
Relative Axin2 mRNA expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Nutrients + −
HT29 (8h)
F
Relative Cyclin D1 mRNA expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
Nutrients + −
HT29 (8h)
G
TopFlash/FopFlash
40
30
20
10
0
**
**
NS
Nutrients + + − −
Wnt3a − + − +
HCT116 β-catenin WT/-
H
TopFlash/FopFlash
10
8
6
4
2
0
*
NS
NS
Nutrients + + − − + +
Wnt3a − + − + − +
PP242 − − − − + +
RKO
I
Relative Cyclin D1 mRNA expression
3
2
1
0
Nutrients + + − +
Wnt3a − + + +
PP242 − − − +
RKO
J
TopFlash/FopFlash
6
5
4
3
2
1
0
*
Atg7 siRNA − +
NT siRNA + −
HT29
K
TopFlash/FopFlash
2.5
2
1.5
1
0.5
0
*
Atg7 siRNA − +
NT siRNA + −
Wnt3a (24h)
RKO
L
RKO (24h)
NTsiRNA + − + −
Atg7 siRNA − + − +
Wnt3a − − + +
β-catenin
Atg7
β-actin
M
Relative β-catenin mRNA expression
2.0
1.5
1.0
0.5
0.0
Nutrients + −
HT29 (8h)
N
HT29 (8h)
Nutrients + + + − − −
Chloroquine − + − − + −
Bafilomycin A1 − − + − − +
β-catenin
p62
LC3-I
LC3-II
β-actin
O
HT29 (8h)
Nutrients + + − −
Wortmannin − − + +
β-catenin
p62
p-AKT(S473)
β-actin
P
RKO + β-catenin AAAA
2h
8h
24h
Nutrients + − + − + −
Myc-β-catenin AAAA
β-catenin
p62
LC3-I
LC3-II
β-actin
Q
HT29 (24h)
Nutrients + + − −
MG132 − + − +
β-catenin
β-actin

Figure 7

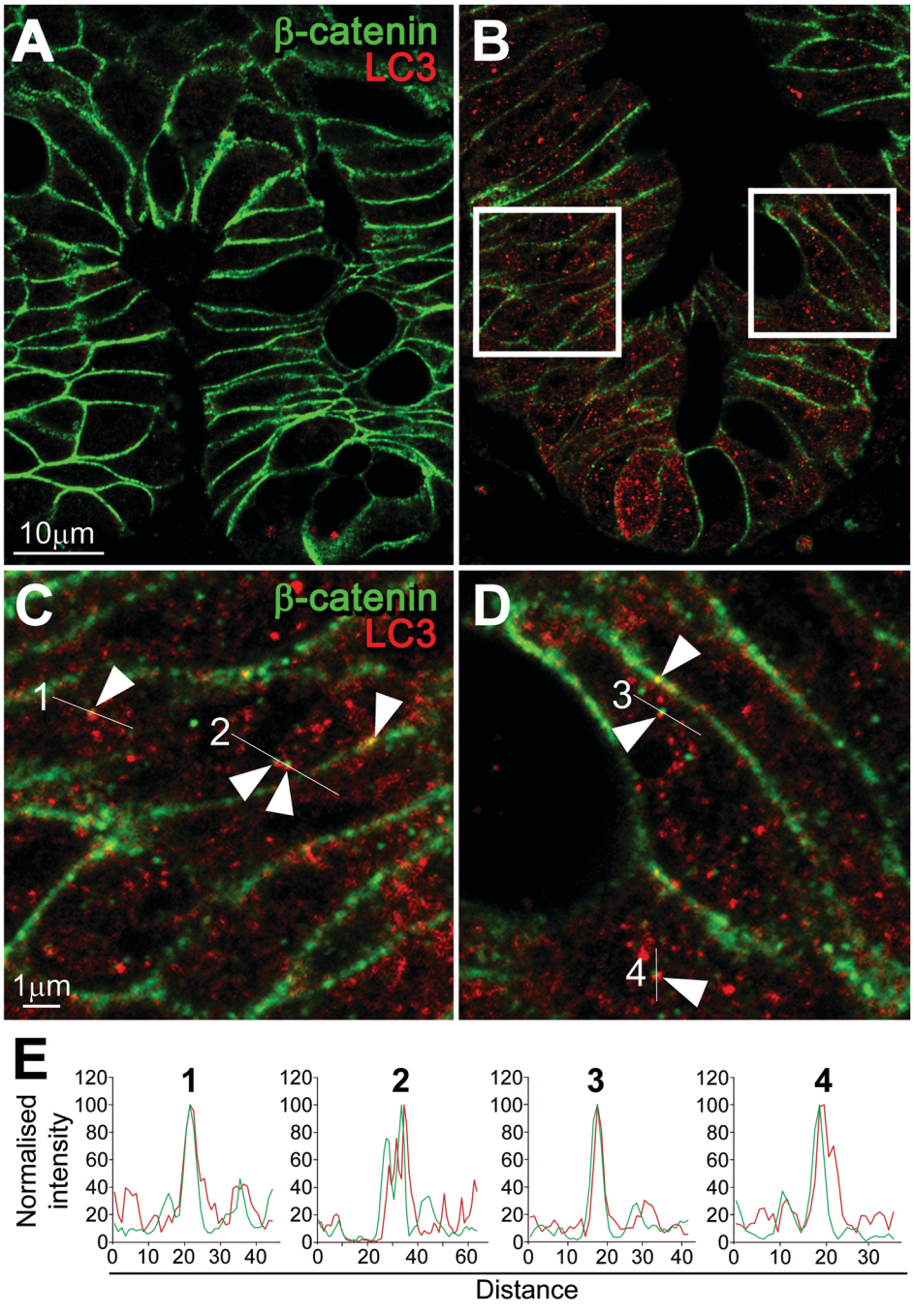

A
β-catenin
LC3
10μm
B
C
β-catenin
LC3
1
2
1μm
D
3
4
E
Normalised intensity
1
2
3
4
120
100
80
60
40
20
0
0 10 20 30 40
0 20 40 60
0 10 20 30 40
0 10 20 30
Distance

Figure 8

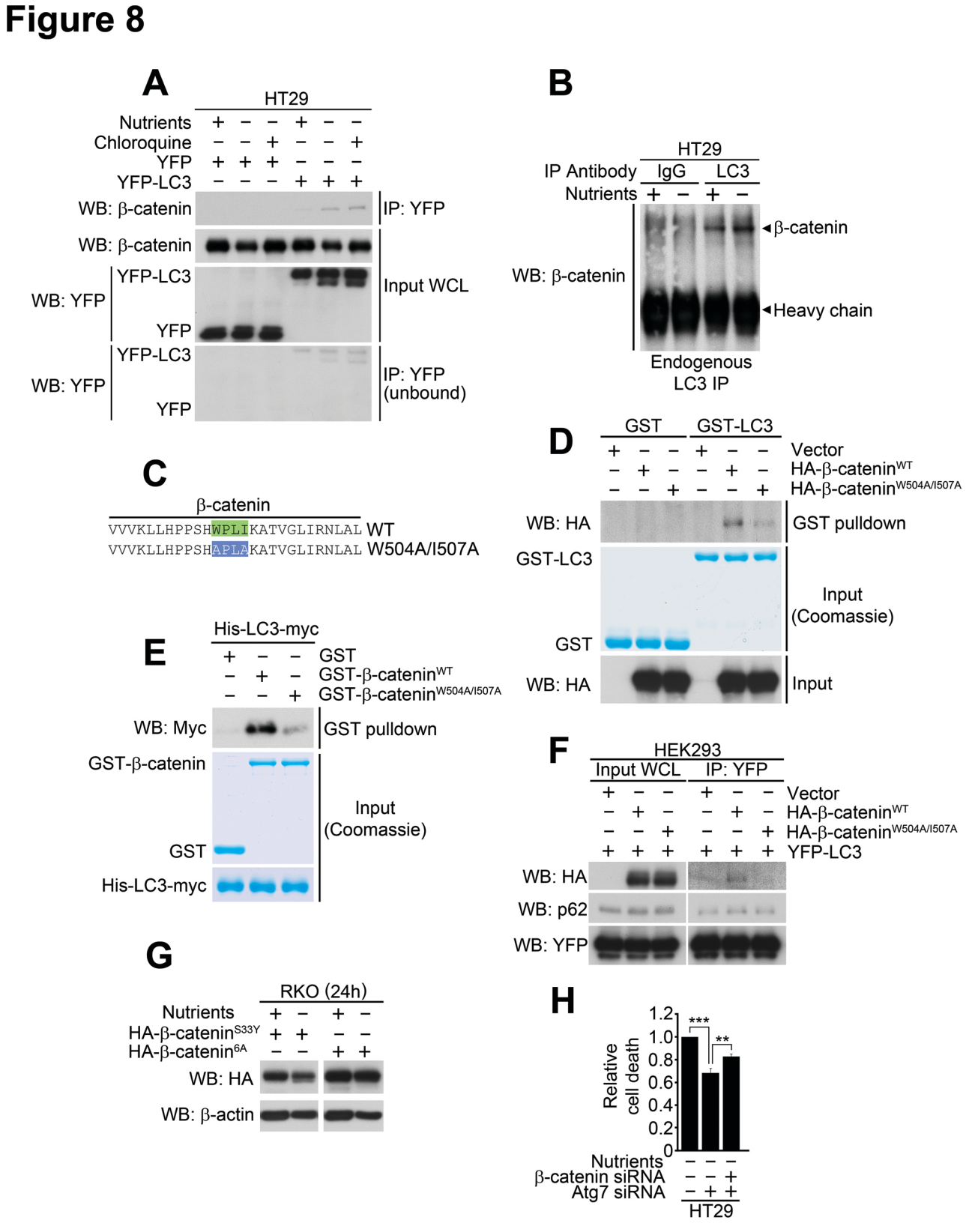
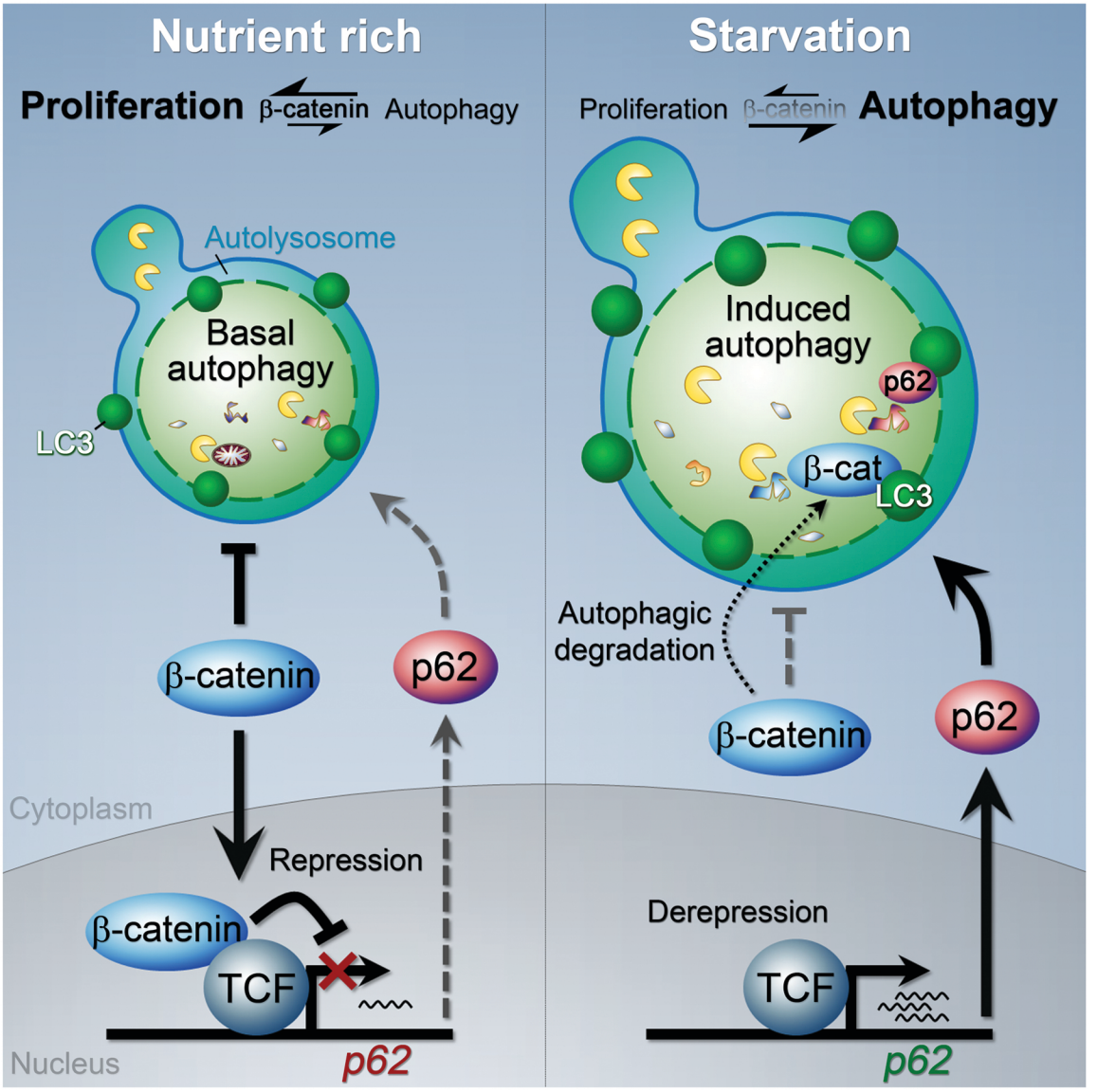

A
HT29
Nutrients + − − + − −
Chloroquine − − + − − +
YFP + + + − − −
YFP-LC3 − − − + + +
WB: β-catenin
IP: YFP
WB: β-catenin
Input WCL
WB: YFP
YFP-LC3
YFP
IP: YFP (unbound)
B
HT29
IP Antibody IgG LC3
Nutrients + − + −
WB: β-catenin
β-catenin
Heavy chain
Endogenous LC3 IP
C
β-catenin
VVVKLLHPPSHWPLIKATVGLIRNLAL WT
VVVKLLHPPSHAPLAKATVGLIRNLAL W504A/I507A
D
GST GST-LC3
Vector
HA-β-catenin^WT
HA-β-catenin^W504A/I507A
WB: HA
GST pulldown
GST-LC3
GST
Input (Coomassie)
WB: HA
Input
E
His-LC3-myc
GST
GST-β-catenin^WT
GST-β-catenin^W504A/I507A
WB: Myc
GST pulldown
GST-β-catenin
GST
His-LC3-myc
Input (Coomassie)
F
HEK293
Input WCL IP: YFP
Vector
HA-β-catenin^WT
HA-β-catenin^W504A/I507A
YFP-LC3
WB: HA
WB: p62
WB: YFP
G
RKO (24h)
Nutrients + − + −
HA-β-catenin^S33Y + + − −
HA-β-catenin^6A − − + +
WB: HA
WB: β-actin
H
Relative cell death
1.2
1.0
0.8
0.6
0.4
0.2
0
***
**
Nutrients − − −
β-catenin siRNA − − +
Atg7 siRNA − + +
HT29

Figure 9


Nutrient rich
Proliferation
β-catenin
Autophagy
Autolysosome
Basal autophagy
LC3
β-catenin
p62
Cytoplasm
Repression
β-catenin
TCF
Nucleus
p62
Starvation
Proliferation
β-catenin
Autophagy
Induced autophagy
p62
β-cat
LC3
Autophagic degradation
β-catenin
p62
Derepression
TCF
p62

Supplementary Information

Autolysosomal β-catenin degradation regulates Wnt-autophagy-p62 crosstalk

Katy J. Petherick, Ann C. Williams, Jon D. Lane, Paloma Ordóñez-Morán, Joerg Huelsken, Tracey J. Collard, Helena J. M. Smartt, Jennifer Batson, Karim Malik, Chris Paraskeva and Alexander Greenhough

Figure S1

(A) Immunostaining of LC3 (green) with LAMP-1 (red) after 2h treatment with autophagy flux inhibitor Bafilomycin A1 (left panels) and β-catenin or non-targeting (NT) siRNA after 8h starvation (right panels). Increased co-localisation was apparent after addition of Bafilomycin A1, indicative of blocked autophagic flux. No increase in LC3/LAMP-1 co-localisation was observed between control and β-catenin siRNA, suggesting flux was not inhibited by β-catenin depletion. (B) Inducible β-catenin deletion in mouse intestinal epithelium over four days of tamoxifen treatment. By two days, β-catenin staining is reduced in β-catenin$^{-/lox}$-villin-creERT2 mice, notably from the crypt, compared to control β-catenin$^{+/lox}$-villin-creERT2 mice. After four days of tamoxifen treatment, the majority of β-catenin protein expression had disappeared from β-catenin$^{-/lox}$-villin-creERT2 mouse intestinal epithelium. (C) Western blotting for protein expression of p62 after β-catenin siRNA in HT29 cells treated with 8μM actinomycin D and starvation for 8h. The increase in p62 protein after β-catenin siRNA was decreased upon addition of actinomycin D.

Figure S2

(A) Association of β-catenin with the p62 promoter by ChIP. PCR products from ChIP-qPCR analysis (Figure 5) visualised by agarose gel electrophoresis and ethidium bromide staining under UV light (data are from one representative experiment of at least three independent experiments). (B) Immunofluorescence of HT29 following Atg7 knockdown demonstrating reduced YFP-LC3 (green) expression compared to non-targeting (NT) siRNA (C) Western blotting of HT29 cells treated for the TopFlash reporter system demonstrating increased autophagy after 24h starvation by increased LC3-II protein and decreased p62. (D) Western blotting for HT29 cells treated for the TopFlash reporter system demonstrating inhibition of mTOR activity by decreased phospho-p70 S6K and phospho-4E-BP1 protein expression, and subsequent increased autophagy by increased LC3-II protein and decreased p62. (E) Overexpression of β-cateninWT, β-cateninS33Y or -catenin$^{W504A/I507A}$ in RKO cells. Immunoprecipitation of YFPLC3followed by detection of β-catenin by western blotting. β-catenin^{WT} and β-catenin^{S33Y} immunoprecipitated with LC3, while β-catenin$^{W504A/I507A}$ did not (Related to Figure 8F).

Figure S1

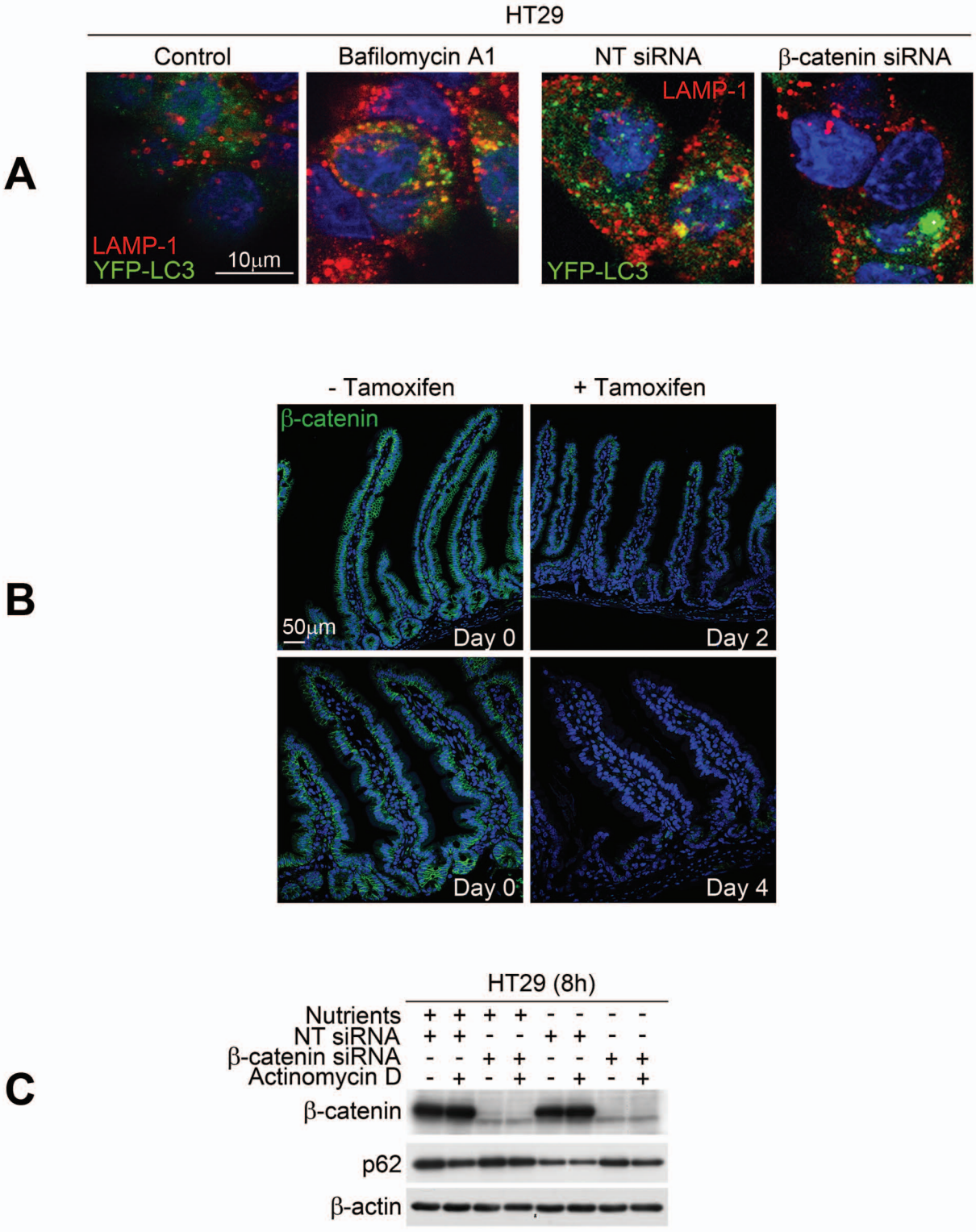
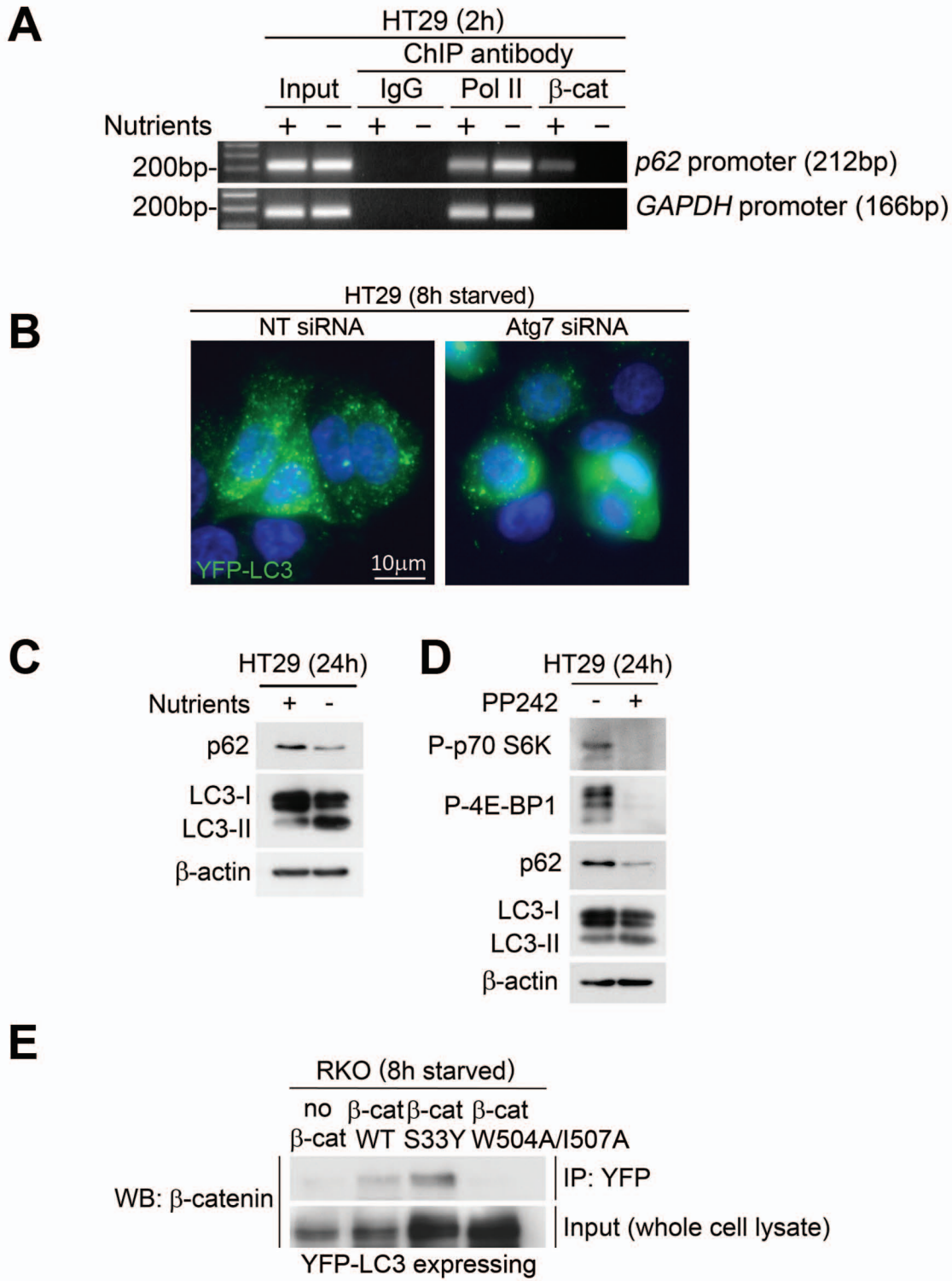

A
HT29
Control
Bafilomycin A1
NT siRNA
β-catenin siRNA
LAMP-1
YFP-LC3
10μm
LAMP-1
YFP-LC3
B
- Tamoxifen
+ Tamoxifen
β-catenin
50μm
Day 0
Day 2
Day 0
Day 4
C
HT29 (8h)
Nutrients + + + + - - - -
NT siRNA + + - - + + - -
β-catenin siRNA - - + + - - + +
Actinomycin D - + - + - + - +
β-catenin
p62
β-actin

Figure S2


A
HT29 (2h)
ChIP antibody
Input
IgG
Pol II
β-cat
Nutrients
+ − + − + − + −
200bp-
p62 promoter (212bp)
200bp-
GAPDH promoter (166bp)
B
HT29 (8h starved)
NT siRNA
Atg7 siRNA
YFP-LC3
10μm
C
HT29 (24h)
Nutrients + -
p62
LC3-I
LC3-II
β-actin
D
HT29 (24h)
PP242 - +
P-p70 S6K
P-4E-BP1
p62
LC3-I
LC3-II
β-actin
E
RKO (8h starved)
no β-cat β-cat β-cat
β-cat WT S33Y W504A/I507A
WB: β-catenin
IP: YFP
Input (whole cell lysate)
YFP-LC3 expressing