The complexity of biological control systems: an autophagy case study

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Summary

Autophagy and YAP1-WWTR1/TAZ signalling are tightly linked in a complex control system of forward and feedback pathways which determine different cellular outcomes in differing cell types at different time-points after perturbations. Here we extend our previous experimental and modelling approaches to consider two possibilities. First, we have performed additional mathematical modelling to explore how the autophagy-YAP1 crosstalk may be controlled by posttranslational modifications of components of the pathways. Second, since analogous contrasting results have also been reported for autophagy as a regulator of other transduction pathways engaged in tumorigenesis (Wnt/β-catenin, TGFβ/Smads, NF-kB or XIAP/cIAPs), we have considered if such discrepancies may be explicable through situations involving competing pathways and feedback loops in different cell types, analogous to the autophagy-YAP/TAZ situation. Since distinct posttranslational modifications dominate those pathways in distinct cells, these need to be understood to enable appropriate cell type-specific therapeutic strategies for cancers and other diseases.

Keywords: autophagy, YAP1 signalling, transduction pathways, mathematical model, cell heterogeneity, precision medicine

Introduction

Autophagy is an evolutionarily conserved degradation process for cytoplasmic cargo (ranging from proteins, lipids, nucleotides to entire organelles) within the lysosomes,[1-3] which plays a pivotal role in the maintenance of both cellular quality control and energetic balance.[4,5] The physiological relevance of autophagy is related to the normal turnover of the cellular components and the clearance of misfolded long-lived proteins or damaged organelles.[4,6,7] Under stress conditions, such as starvation or oxidative stress, autophagy is upregulated to degrade dispensable macromolecules and restore nutrient balance.[8]

A series of autophagy-related (ATG) proteins participate in the main steps of the autophagy pathway: formation of autophagosomal membranes, maturation and final fusion with lysosomes.[4,5,9] Briefly, downstream of mTORC1 complex inhibition and other signalling cascades, several protein complexes (ULK1/ATG1 complex; class III phosphatidylinositol 3-kinase/VPS34 complex containing Beclin1/BECN1-p150-ATG14; ATG12-ATG5-ATG16L1 complex) are formed, and initiate the assembly of the autophagosomal membranes delivered by ATG 9.[4,5] The microtubule-associated protein 1 light chain 3 (MAP1-LC3 or simply LC3) family of proteins is conjugated to phosphatidylethanolamine in the nascent autophagosome membranes in a defining step in autophagosome biogenesis.[10] Fully formed autophagosomes finally fuse with lysosomes through a process mediated by SNAREs and other proteins proteins.[3,11]

The destinations, functions, and activities of ATGs or autophagic cargoes are mainly regulated by posttranslational modifications (PTMs) - such as phosphorylation, acetylation, O-GlcNAcylation, ubiquitination, lipidation, glycosylation, and proteolysis.[12] Monitoring the effects that these distinct PTMs have on the overall rate of autophagic degradation enables better definition of the general autophagy pathway control. For instance, phosphorylation of serine and threonine residues regulates the interaction between various ATG proteins to form complexes (e.g. ATG12-ATG5-ATG16L1, ULK1 or VPS34 complexes), modulates the catalytic activities of various kinases (e.g. AMPK, mTOR, ULK1, VPS34), or defines the cellular localisation (nuclear *vs.* cytoplasm) of the transcription factors involved in the positive (TFEB,[13] YAP1-TEAD[14]) or negative (ZKSCAN3[15]) transcriptional control of autophagy. However, phosphorylation may cause opposite outcomes on the autophagy pathway depending on the target: either induction (when AMPK, ULK1/ATG1, ATG9, or p62 are phosphorylated),[16-18] or repression of autophagy (p-BECN1)[19]. In addition to being modified by phosphorylation, serine and threonine residues of autophagy proteins can also be modified by the O-GlcNAcylation (O-linked attachment of β-N-acetyl-glucosamine) with similar contrasting effects: autophagy activation, when AMPK is O-GlcNAcylated,[20] or inhibition, when BECN1 is posttranslationally modified in this way.[21] Additionally, K63 poly-ubiquitination of aggregate-prone proteins is believed to dictate their degradation via the autophagic-lysosomal route, while the K48 or K11 polyubiquitinated proteins are rather delivered to the ubiquitin-proteasome system (UPS).[18] Adaptor molecules (such as p62/SQSTM1, NBR1, NDP52, optineurin, VCP) through their ability to bind both LC3/GABARAP (they have a LC3 Interacting Region - LIR domain) and K63 polyubiquitinated

proteins (often via an Ub-binding - UBA domain), manage to selectively target the cargo to the autophagy pathway.[22,23] Ultimately, the autophagy receptors are degraded together with their cargos in autolysosomes.[24,25]

PTMs impact differently on the autophagy route and can influence the pool sizes of intermediates (including phagophores, autophagosomes, autolysosomes and lysosomes) and/or alter their rates of synthesis, fusion or degradation. These autophagy perturbations further signal to control the transcription of genes, cell growth and proliferation or the balance between cell survival and apoptosis *via* various transduction pathways. The tight interconnections between autophagy and various signalling routes ultimately dictate cell fate and often serve as the main control systems that defines cell identity.[5,26]

Autophagy-YAP1 signalling crosstalk: the balance between multiple forward and feedback loops dictates the cell fate

We have recently shown that the complex interconnection between the YAP1-WWTR1(TAZ) signalling and autophagy controls cell proliferation and survival with opposite outputs in different cell lines, but also at different time points of autophagy perturbation. More precisely, we identified α-catenin, the endogenous inhibitor of the Hippo pathway effector YAP1,[27-29] to be a direct autophagy substrate (via two newly described LIR motifs). Consequently, autophagy positively regulates YAP1 protein levels and activity in a number of cell lines with high basal levels of α-catenins, like the non-malignant mammary epithelial (MFC10A) cells, human embryonic kidney (HEK293T) cells, human cervical epithelium (HeLa) cells, primary mouse embryonic fibroblasts (pMEFs) or primary mammary epithelial cells (pMECs).[26,30] On the other hand, previous studies have identified that Yap1 itself is an autophagy substrate (independently of p62 expression), and have further shown, using *in vivo* models (Atg7KO mice) or hepatocyte cell lines (Atg7-deficient murine AML12, or human THLE5B), that autophagy negatively impacts Yap1 activity, as Atg7-deficiency leads to the accumulation of active Yap1 that increases liver size, causing progenitor cell expansion with hepatocarcinogenesis.[31] This result was also confirmed by us using various hepatocyte cell lines (THLE2, HepG2, Huh7 cells) or non-small cell lung cancer cells (A549 cells), as all these cells have a low basal protein expression of α-catenins.[26,30] In other words, one may conclude that cells that have the capacity to bind a significant portion of the YAP1 pool by α-

catenins will respond to autophagy inhibition by reducing cell proliferation, size and migration capacities, while cells that have transcriptional changes or PTMs that overcome or lower this interaction will behave in completely opposite way, as the direct ability of autophagy to degrade YAP1 will be dominant: autophagy inhibition would activate YAP1 (Figure 1A). From a general perspective, one may summarize that the initial posttranslational status is crucial in defining the direction of response to any external/internal perturbation (of autophagy or other metabolic pathways).

The autophagy-YAP1 picture is further entangled by a feedback path, since YAP1 regulates autophagy. We previously also showed that YAP1/TAZ positively modulates autophagy by upregulating the transcriptional expression of myosin-II genes in a series of cell lines, like MFC10A, HeLa cells, human keratinocyte (HaCaT) cells, primary mouse embryonic fibroblasts (pMEFs) or primary mammary epithelial cells (pMECs).[14] Other studies have confirmed this general observation that YAP1 is required for proper autophagy, but suggest various distinct mechanisms. YAP1 transcriptional targets include Armus, a protein of the RAB-GAP family, which is required for the proper fusion of autophagosomal vesicles with lysosomes,[32] and HMGB1, the well-known activator of Beclin-1 (which displaces the inhibitor protein Bcl-2 from its interaction with Beclin-1)[33,34] in human glioma (U251 and U87)[35] cell lines. Autophagy flux is further increased by the interaction of YAP1 with the master transcription factor EB (TFEB)[36] or TEAD[37] and co-transcriptional regulation of autophagy and lysosomal genes in neonatal rat cardiomyocytes and human breast cancer cells (MCF7 or MDA-MB-231). This feedback loop (of YAP1 controlling autophagy) is important, as mathematical modelling suggests that the strength of the effect exerted by YAP1 on autophagy controls the magnitude of the YAP activity outcome after autophagy perturbations (Figure 1B). For instance, in cells with low basal α-catenin protein levels, events that enhance the control of YAP over the autophagy pathway will lead to a lesser increase in YAP activity than the cases with negligible feedback effects upon autophagy inhibition. In cells with high basal α-catenin expression, a high feedback effect (of YAP1 controlling autophagy) will cause a lesser reduction in the YAP activity output upon autophagy compromise, but only at earlier time points.[26,30] For cancer cells,[38] the typically heightened autophagy flux is expected to cause an increase in the magnitude of the observed effect only for the low α-catenin

cases. A second general observation could be made at this point: the feedback paths control the magnitude of the investigated effects in a time-dependent manner.

Proposed mathematical model for the autophagy-YAP1 signalling crosstalk

One may ask how these apparently contradicting observations (positive and negative controls) can be integrated to define a general model for a particular pathway (e.g. autophagy-YAP/TAZ) to enable a better understanding of the outputs empirically observed after various perturbations, and to allow prediction of the cellular behaviour in new situations.

To answer this question, we previously developed a mathematical model of three interconnected differential equations that describe the dynamics of the three key players (autophagy, YAP1 activity, and α-catenin levels) in a time-dependent manner, starting from the three main constituent processes (Figure S1, Supporting Information):[26] i) autophagy positively modulates YAP1 activity by degrading its inhibitor, α-catenin (so-called *positive forward path;* the α-catenin that accumulates upon autophagy inhibition interacts with YAP1 and sequesters it into the cytosoplasm);[27] ii) autophagy negatively modulates YAP1 activity by facilitating its direct degradation (so-called *negative forward path;* active-YAP1 accumulates upon autophagy inhibition);[31] iii) the feedback path of YAP1 positively controlling autophagy (by monitoring autophagosome formation, maturation, and fusion with lysosomes).[14,36] Thus, during autophagy inhibition, the rate of autophagy decrease is controlled by both the decay rate due to the applied perturbation (parameter c),[26,39] and the strength of the feedback path (parameter v_Y). The rate of YAP1 variation relies on the balance between the strengths of the two, *positive* (controlled by parameter r1, which defines the strength of the cytosolic-sequestration rate of YAP1 by α-catenins) and *negative* (controlled by parameter r2, which defines the strength of active-YAP1 accumulation rate upon autophagy inhibition) *forward paths*, while the rate of α-catenin accumulation depends on its degradation rate by autophagy (controlled by parameter r3). It is also important to note that the intracellular compartmentalisation of various key players may vary, based on the cell type and nutrient/environmental conditions, and thus will influence the values of r1, r2 and r3 parameters. For example, nutrient rich conditions activate mTOR on peripheral lysosomes, which inhibits autophagy and promotes YAP1 activation (increased r2 value) [40,41] or α-catenin accumulation (increased r3 value).

In our previous study, using this mathematical model, we were able to reconcile the controversial observations present in the field of YAP1 and autophagy research and identify the basal α-catenin levels (by appropriate experimental validation) as the main driver for the direction of YAP output (activation/ inhibition) upon autophagy perturbation with considerable input in the magnitude of the effect offered by the strength of the feedback path (so called parameter v_Y). Additionally, the other parameters (c, r1, r2, and r3) were assumed as constants for the numerical simulations, their values being extrapolated from experimental observations.

However, if one wants to extend the model to a broader range of cell types and systems, the variations in these parameters (c, r1, r2, and r3) should be carefully considered. Thus, we considered possible scenarios that would lead to variations in order to understand, at least theoretically, their potential impact over the outputs.

The autophagy decay rate, modelled by parameter c, dictates the YAP1 output in low basal α-catenin cells and/or low feedback strength conditions

The autophagy decay rate (parameter c) may vary, as it depends on the nature of the perturbation stimulus: if there is a chemical (inhibitors/activators that may require minutes-hours to impact autophagy) or genetic manipulation (siRNA knockdown experiments with visible effects in hours-days, or knockout cell lines – days-weeks). As most of our experiments were siRNA knockdown experiments and had prolonged treatments, the autophagy decay rate was relatively slow. Interestingly, increasing the value of parameter c (as would occur in biological systems exposed to a rapid perturbation decay rate) can change the outcome of the output effect (e.g. YAP1 activity) in cells characterized by low basal α-catenin and/or little feedback effect (low v_Y): switching from YAP1 activation (what we observed in our recent publications)[26,30] to YAP1 inhibition – Figure 2A. Conversely, the effect exerted by the autophagy decay rate in biological systems characterized by both high α-catenin levels and a strong feedback effect (high v_Y), is almost neglectable – Figure 2A and Figure S2, Supporting Information. The initial levels of autophagy and YAP1 activity only impact on the magnitude/extent of the final outcome (e.g. reducing the basal autophagy levels or initial YAP1 activity will produce the same effect - of either activation or inhibition, but to a lower extent) –Figure S3, Supporting Information.

The strength of the positive forward path, modelled by parameter r1, dictates the YAP1 output in low basal α-catenin cells

Regarding the cases where the strength of the cytosolic interaction between YAP1 and α-catenin varies, which influences the strength of the *positive* forward path, it is worth considering the possibilities of PTMs that facilitate (e.g. phosphorylation of YAP1) or disrupt this protein-protein interaction. The disruption may be caused by PTMs like ubiquitination, that amplifies the proteasomal-mediated degradation of YAP1 and/or α-catenins and/or 14-3-3 proteins (which are the molecules that intermediate the interaction between YAP1 and α-catenin), or acetylation of 14-3-3 proteins that shut-off their functions as intermediary binding partners.[42,43] The strength of the *positive* forward path is controlled by the value of parameter r1. For instance, a relatively moderate increase in parameter r1 (e.g. doubling its value) would cause a decrease in the YAP1 activity in low basal α-catenin cells, even reversing the final output effect at later time points of autophagy perturbation (switching from the previously expected YAP1 activation to YAP1 inhibition) – Figure 2B. The YAP1 activity in cells with high basal α-catenins is not sensitive to moderate changes (up to two folds increase) of this parameter – Figure 2B and Figure S4, Supporting Information.

The strength of the negative forward path, modelled by parameter r2, dictates the YAP1 output in low basal α-catenin cells

When considering the effect caused by the strength of active-YAP1 accumulation rate upon autophagy inhibition (controlled by parameter r2 – Figure S1, Supporting Information), over final YAP1 activity, it is important to understand the dynamics of the process. For instance, a system characterized by an increased cytosolic YAP1 delivery rate into autophagosomes (and followed by an accelerated autophagosome-lysosome fusion rate) would actually show *a reduction in the active-YAP1 accumulation rate* at earlier time points of autophagy inhibition using siRNA experiments targeting key ATGs: while the number of newly forming autophagosomes decreases (as this is not an instant process, rather a prolonged one that takes hours-days), the remaining autophagosomes are trying to maximize the cytosolic YAP1 degradation, being still able to deliver it into lysosomes, at the extent of active-YAP1 accumulation rate. While the YAP1 delivery rate

into autophagosomes depends rather on the cytoplasmic pool of YAP1 and its autophagy adaptors, the YAP1 clearance is rather linked to the autolysosome formation and degradation capacity.

In situations characterised by a decreased accumulation rate of active-YAP1 (which is defined by a decreased value of parameter r2) it is worth mentioning the types of PTMs that facilitate the proper cytosolic YAP1 delivery into autophagosomes by promoting its interaction with various cytosolic autophagy adaptor proteins like p62, NBR1, co-chaperone BAG3 (phosphorylation of YAP1 and various components of the autophagy machinery – LC3, GABARAP, GABARAPL1, ATG9, ATG31-ATG29 complex; YAP1 ubiquitination linked to K63 or K27;[12] or decreased YAP1 O-GlcNAcylation – that causes YAP1 instability and promotes its degradation),[44] or increased autophagosome-lysosome fusion and recycling (eg. dephosphorylation of TFEB).[13] The r2 decrease displays a similar trend as r1 increase over the outcome. For instance, a relatively moderate reduction in parameter r2 (e.g. decrease by half) would lessen the YAP1 activity, even reversing the expected output phenotype in low basal α-catenin cells at later time points of autophagy perturbation (switching from the previously expected YAP1 activation to YAP1 inhibition) – Figure 2C and Figure S5, Supporting Information. Changes in the initial levels of autophagy, YAP1 or the strength of the feedback path only impact the magnitude of the output measures – Figure S6, Supporting Information. Interestingly, if there is a high discrepancy between the strengths of the two *positive* (defined by parameter r1) and *negative* (defined by parameter r2) forward paths, the effect caused by distinct basal α-catenin levels is only minor. For instance, if the strength of the direct YAP1 autophagic accumulation rate (*negative forward path*) is 5 times higher than the strength of theYAP1 cytosolic sequestration rate by α-catenins (*positive forward path*) (ratio of r2/r1 – Figure 3), one may observe that the cells with high basal α-catenin expression start to behave similarly to those with initially low α-catenins levels at early-time points: they increase the YAP1 activity output – Figure 3 and Figure S7, Supporting Information. The effect of variations in parameter r3 (the rate of α-catenin accumulation) over the final YAP1 activity levels is only minor if compared to the r1 and r2 parameters – Figure S8, Supporting Information. Importantly, the autophagic flux is additionally regulated, at least in yeast, by the number and size of the forming autophagosomes, which directly correlates with the cellular pool of Atg8, the ortholog of the mammalian LC3 protein.[45-48]

To summarize what we generally learnt from our proposed model:

i) distinct basal expression of one single protein (e.g. α-catenins) is sufficient to define the cell fate upon external/internal perturbations;

ii) distinct time periods of applied perturbations may cause contradictory outcomes in the same cellular system;

iii) the strength of the feedback path (e.g. YAP1 controlling autophagy) impacts on the magnitude of the outcome, but has only minor effects on the cell fate (i.e. directionality of outcome);

iv) PTMs (e.g. responsible for controlling the strength of the positive and negative forward paths exerted by autophagy over YAP1 activity) may influence the cell fate in cells with low expression of inhibitors;

v) if one of the contrasting effects is predominant, it may override the outcome irrespective of any other basal conditions.

Crosstalk between autophagy and other intracellular transduction pathways: forward and feedback paths

The aforementioned posttranslational and transcriptional interconnections between YAP1/TAZ (Hippo signalling) and autophagy with opposite outputs in different cell lines, at distinct time-points of autophagy perturbation, may reflect processes that underlie some of the apparent cell-type specific discrepancies in cellular responses to autophagy, involving other pathways.

The previous mathematical-numerical model used for explaining the autophagy-YAP1 link might serve as an exemplar that could be extended to autophagy and its cross-talk with other transduction pathways (Wnt/β-catenin signalling, TGF-β/Smads signalling, NF-kB or XIAP/cIAP-mediated cell survival) where similar major axes have been identified: a) a *positive* forward path, when autophagy indirectly up-regulates that particular signalling pathway (e.g. autophagy degrades the pathway's inhibitors); b) a *negative* forward path, when autophagy directly inhibits the signalling (e.g. autophagy directly degrades the pathway's components); c) a feedback regulatory path exerted by the transduction pathway over autophagy. Further insights that may connect the contrasting, but extremely valuable observations from the literature in the field of autophagy and intracellular signalling is indeed required. In the following sections, we consider some possibilities that may inform future efforts aiming to reconcile apparent contradictions in the literature.

Autophagy and Wnt/ β-catenin signalling

Autophagy was shown to positively regulate the activation of Wnt/β-catenin signalling in hepatocellular carcinoma cells, causing increased cancer cell glycolysis.[49] On the other hand, it has been reported that autophagy induction was able to repress the Wnt/β-catenin signalling pathway by stimulating the autolysosomal degradation of key pathway-components, like β-catenin[50] and Dishevelled (Dvl)[51] – Figure 4A. If the β-catenin is directly delivered to the forming autophagosomal membranes (a LIR-mediated process) in a series of cells lines (HT29 and RKO carcinoma-derived cell lines, HEK293T cells, HCT116 cells, intestinal epithelial mouse cells), Dvl2 first requires PTMs (ubiquitylation mediated by the von Hippel-Lindau/VHL protein) that would favour its aggregation and binding to p62, which further mediates the delivery of Dvl2 to the autophagy-lysosomal system in cell lines like HeLa, HEK293T, SW480, and immortalized mouse embryonic fibroblasts (MEFs) or biopsy pieces from human colon carcinoma tissues.[51] The feedback loop caused by Wnt/β-catenin controlling autophagy complicates the picture, as Wnt/ β-catenin signalling (activated through the Wnt3 ligand) positively regulated autophagy in squamous cell carcinoma of the head and neck, promoting the cancer cell radioresistance.[52] Conversely, other studies have shown that the Wnt/ β-catenin pathway negatively modulates autophagy: activation of this signalling route attenuated Beclin-1-dependent autophagy in human osteosarcoma cells, while inhibition led to increased expression of key autophagy genes (eg. *LC3B*, *BECN1*, *P62*), and downregulation of autophagy inhibitory proteins (e.g. Bcl-2) in multiple myeloma cells,[53] glioblastoma cells,[54] or mammary epithelial cells.[55]

Autophagy and TGFβ/Smads signalling

For the TGFβ signalling and autophagy link, two distinct studies, one from Javad Alizadeh and co-workers studying TGFβ1, and another one by Yan Sun et al focusing on TGFβ2, have shown that autophagy positively modulates the TGB-beta/Smads signalling-induced epithelial to mesenchymal transition (EMT), cellular migration and contraction in non-small cell lung cancer cells (NSCLC – A549, H1975 cell lines),[56] and primary rabbit lens epithelial cells by controlling the phosphorylation status of Smad2/3 proteins.[57] A recent study further reinforces these observations, supporting the role of autophagy in mediating the TGFβ/Smads-induced fibrosis in human trabecular meshwork cells (specialised ocular tissue which maintains intraocular pressure)

by controlling the transcription of the TGFβ antagonist, BMP and activin membrane bound inhibitor (BAMBI).[58] With relevance to the innate immune antiviral response, autophagy also triggers the activation of TGFβ production and Smad2/3 signalling in Human respiratory syncytial virus (RSV)-infected primary mouse macrophages and -RAW 264.7 (mouse macrophage cell line) cells to induce the production of optimal IFNβ[59] – Figure 4B.

This biology appears to include a negative regulation loop, as it has been reported that autophagy degrades TGFβ, reducing the protein levels without alterations in the mRNA levels, in primary mouse renal tubular epithelial cells (RTEC) and human HK-2 (human proximal RTEC) cells.[60] For the feedback loop (how the TGFβ signalling controls autophagy), multiple studies have shown that TGFβ signals by upregulating the expression of key autophagy genes (*LC3B*, *BECN1*, *ATG5*, *ATG7*), and thus induces autophagy, which, in turn, facilitates the phosphorylation status of Smad2/3 to control fibrosis in primary human atrial myofibroblasts[61] or cell growth in HuH7 (human hepatocellular carcinoma) cells, MDA-MB-231 (mammary carcinoma) cells,[62] or normal bovine mammary epithelial BME-UV1 cells.[63] Interestingly, a recent study described that TGFβ signals through Smad proteins to induce TFEB expression and facilitate the TFEB-driven autophagy in a panel of pancreatic cancer cell lines (eg. MIA PaCa-2, PANC-1, Panc03.27 lines) and patients' tissues.[64]

Autophagy and NF-kB signalling

NF-kB transcription factors are key regulators of cell survival and aberrant NF-κB signalling has been involved in the pathogenesis of most human malignancies. The cross-talk between NF-kB signalling and autophagy is also complex. For instance, autophagy positively regulates NF-kB signalling by promoting the degradation of its well-known inhibitor IkBα in a set of intestinal epithelial cell lines (HT29, HCT116, HCT15, HCA7, SW48, RKO, and HCT8) and MEFs.[65]

Interestingly, autophagy also negatively impacts NF-kB signalling by promoting the degradation of key activators (IKK alpha, beta, gama, and NIK – the activator of IKK) by several mechanisms – Figure 5. One mechanism implies that the autophagic degradation of IKK is accelerated in the absence of its binding-partner Hsp90 and independently of its ubiquitination status in HEK293, Jurkat cells, Human B-cell line Ramos RG69, mouse fibroblasts ts20, and MEFs.[66] Kelch-like

ECH-associated protein 1 (KEAP1), an E3 ubiquitin ligase, further promoted the delivery of IKKbeta to the autolysosomal system for degradation by competing with HSP90 for the direct binding to IKKbeta. KEAP1, in addition to diminishing the expression of IKKbeta, also causes inactivation of IKKbeta by reducing its phosphorylation status in HEK293 and HeLa cells.[67] Another E3 ubiquitin ligase, Ro52, facilitates the monoubiquitination of the phosphorylated active form of IKKbeta induced by the Tax oncoprotein of HTLV-1, a PTM involved in the subcellular translocation of the active IKKbeta to autophagosomes with subsequent lysosomal degradation, leading to the inactivation of NF-kB pathway in HEK293 and HeLa cells.[68] The active IKKbeta may also be delivered to autophagosomes via an adaptor protein, an F-box protein, S-phase kinase associated protein 2 (SKP2), that bridges IKKbeta and the autophagic cargo receptor p62, thus promoting p62-mediated selective autophagic degradation of IKKbeta followed by NF-Kb inhibition in HEK293 and HeLa cells.[69] With relevance to the innate immune anti-bacterial response, Atg7 was found as a binding partner for the phosphorylated IkBα. Thus, the loss of Atg7 (independently of autophagy) led to the p-IkBα release from the interaction promoting its ubiquitination and UPS-mediated degradation, and ultimately triggering the NF-kB activation in murine macrophages.[70] ATG5 deficiency also augmented NF-kB-mediated inflammation in proximal tubular epithelial cells.[71]

This pathway may also involve a feedback loop (how NF-kB impacts on autophagy pathway?), as several studies have confirmed that NF-kB/IKK signals to stimulate autophagy by up-regulating the expression of several genes involved in the formation and maturation of the autophagic machinery (eg. *BECN1*, *ATG5*, *LC3*, *LAMP1*, *RAB7*) in a set of cell lines including MEFs,[72] HeLa, and MCF10A cells.[73] However, NF-kB also suppresses autophagy, by upregulating the expression of several well-known autophagy repressors (A20, Bcl-2 family members, phosphatase and tensin homolog/mammalian target of rapamycin (PTEN/mTOR) and nitric oxide (NO)), and/or by suppressing some autophagy inducers (Bcl-2 interacting protein 3 (BNIP3), JNK1, p53 and ROS) in distinct cell lines and time points after perturbation/signalling (rat primary cortical neurons, MEFs, HeLa, immature B cell lymphoma WEHI 231 cells, and sarcoma cells).[74-77]

NF-kB signalling has been associated to the upregulation of several inhibitors of apoptosis (IAPs) family members, including cellular inhibitor of apoptosis 1 (cIAP1) and 2 (cIAP2), X chromosome-linked inhibitor of apoptosis (XIAP).[78-82]

Autophagy and XIAP/cIAP-mediated cell survival

Controversial literature also exists for the role of autophagy in controlling the balance between cell survival and apoptosis. If one searches for the links between the E3 ubiquitin ligases, XIAP/cIAPs, and autophagy, one may observe that short periods of autophagy activation promote cell survival via the NF-kB mediated upregulation of anti-apoptotic genes (Bcl-2,[83] Bcl-XL, XIAP, cIAP1, cIAP2…) in various cancer cell types: human glioma cell lines (A172, U87, U251 cell lines),[84] fibrosarcoma cell lines, or human diffuse large B cell lymphoma cell lines (DLBCL - RIVA, OCI-LY3, SUDHL-2, HBL-1 and SUDHL-5).[78,85-87] However, induction of autophagy by Timosaponin AIII caused the lysosomal degradation of ubiquitinated XIAP and induced apoptosis in hepatocarcinoma cells.[88] The critical role of ubiquitination as a PTM in facilitating the autophagy-dependent proteolysis of XIAP was also confirmed using E1 enzyme inhibitors.[88] In support of these last findings, prolonged autophagy induction with rottlerin caused apoptosis by downregulating the expression levels of XIAP, cIAP-1, Bcl-2 and Bcl-XL protein levels in pancreatic cancer stem cells[89] – Figure 6.

When considering the feedback loop (how XIAP/cIAP1/2 proteins impact on autophagy?), it is important to highlight that XIAP and cIAP1, through their E3 ubiquitin ligase activities, positively regulate autophagy: either by increasing the NF-kB capacity to promote the transcription of *BECN1* in HeLa, MEFs, MCF10A and DLBCL cell lines (SUDHL-5, SUDHL-8 and SUDHL-10),[73] or by assisting the efficient fusion of lysosomes with autophagosomes in HeLa, MEFs and dermal fibroblasts.[90] Distinct studies have shown the opposite effect of XIAP on autophagy by a distinct mechanism: phosphorylated active XIAP acts as an E3 ligase that mediates the rapid proteasomal degradation of Mdm2 (the major ubiquitin E3 ligase and inhibitor of p53), thus upregulating the cytosolic p53 levels which ultimately suppress autophagy.[91] This last effect was shown in a set of cell lines also used in the previous studies (MEFs and MCF10A cells) and additional ones (HCT116, HepG2, HEK293T, A549, MCF7cells), but using different time points of exposure and concentrations of the XIAP inhibitor, embelin: short periods (1-4 hours) and very low concentrations (50-200 nM)[91] *vs* longer time periods (16 hours) at higher concentrations of 10-20 µM in another study.[73] Additional studies have found that XIAP acts as an E3 ubiquitin ligase for p62 promoting its UPS-mediated degradation, thus suppressing the p62-selective autophagy in HCT116, HepG2, HEK293T, A549, and MDA-MB-cells.[92]

These different interactions between autophagy and diverse signalling pathways suggest that distinct initial conditions (intracellular networks and PTMs) may explain why various studies have obtain contrasting results when using different biological systems, or even the same systems but unrelated time points (or concentrations) of perturbations/ treatments.

Concluding remarks and open questions

We believe that multiple points of influence determined by PTMs (phosphorylation, ubiquitylation, acetylation, O-GlcNAcylation…), apart from the basal protein expression, modify the final outcomes in biological systems and define cell fate, acting as a switch on/off button. For example, both abnormal acetylation and deacetylation are associated with pathological conditions, particularly neurodegenerative diseases and cancer.[93-95] Our experimental observations, explained through a simple mathematical model, point out that a heterogenous biological system (same cell type, but with slightly different basal conditions or altered posttranslational processes) leads to different outcomes at the individual cell level and at different time points after perturbation. This supposition may explain how tumorigenic changes might start in only one or few cells in a dynamic process of alternating on-off steps, as the parameters involved might vary over time and space (e.g. transcriptional or PTMs caused by various environmental perturbations).

Our model for understanding the crosstalk between a signalling pathway and autophagy might provide insight into therapeutic opportunities in a variety of cancers (hepatocarcinoma, pancreatic adenocarcinoma, breast cancers, cancers of the head and neck etc..). Nevertheless, multiple parameters bring complexity to the system being considered, but, at the same time shed light on the importance of understanding tissue heterogeneity. This highlights the requirement for single cell analysis to assess transcriptional and post-translational modification levels in order to achieve the proper understanding of the biological processes that characterize the patient's cancer cells, by integrating single-cell fluorescence analysis of autophagy,[48] with data acquired from high-throughput omics platforms and machine-learning algorithms.[96,97] This may allow for the identification of intracellular changes that need to be repaired for reversing the cellular status to a benign state. Importantly, any biological process should be followed kinetically, as the same perturbation applied for different time periods might end up with contrasting outcomes.

Perturbing a metabolic process, either autophagy or any other catabolic/anabolic route, will inevitably impact on multiple transduction pathways. For instance, when common cancer hallmarks (increased cell proliferation, cell glycolysis) are investigated as readouts, one should be careful in designing the experiments and interpreting the data: the initial perturbation of one cellular process (e.g. autophagy) will disturb multiple signalling cascades with contrasting effects. For example, cell proliferation, differentiation and/or survival are promoted by YAP1, Wnt, and NF-kB signalling, but inhibited by TGFβ. Nevertheless, these signalling routes may react distinctively at various time points as different axes are switched on/off at early *vs*. late responses, or short *vs.* long perturbations: short periods of autophagy induction promotes resistance to cell death via NF-kB, while longer activation may induce autophagy-dependent cell death.[98-100] The system biology is further complicated and controlled, apart from the feedback loops, by the vast regulatory links (many still unknown) existing between the different axes affected by the initial perturbation (e.g. autophagy), and systems-level properties of the control networks can be integrated into relevant mathematical models.[26,30,83,100,101] Thus, the cellular biological complexity requires further in-depth study. However, the highest priority should be given to gathering and merging the existing research information to define the integrated relevant cellular networks using specialised bioinformatics platforms to facilitate the understanding and prediction of cell responses to external/internal stimuli with relevance to designing successful precision therapies.

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AUTHOR CONTRIBUTIONS

M.P. and R.T. created the computer simulations and prepared the related figures. All authors commented the results and wrote the manuscript together. D.C.R. supervised the study.

CONFLICT OF INTEREST

D.C.R is a consultant for Drishti Discoveries, Abbvie, PAQ Therapeutics, Aladdin Healthcare Technologies SE and Nido Biosciences. All other authors declare no competing interests.

Supporting information

Supporting Information is available from the Wiley Online Library or from the author.

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Figure legends

FIGURE 1. Schematic representation of the autophagy-YAP1 signalling control system. **(A)** The two contrasting effects of autophagy on cell survival and proliferation resulting from degrading either α-catenin (YAP1 inhibitor) or YAP1 are named as *positive* and *negative forward paths*, respectively. Various posttranslational modifications (PTMs) impact differently on the strengths of the two forward paths. The positive *feedback path* is represented by the transcriptional control exerted by YAP1 (in conjunction with TFEB or TEAD) on key autophagy genes. **(B)** Schematic diagram of the effect caused by the feedback path/loop in cells with either low or high basal levels of α-catenins, and normal or high initial autophagy flux/activity. The strength of the feedback loop is denoted by the parameter v_Y. Y_init indicates the initial YAP1 activity. YAP1 activity increases in low basal α-catenin conditions (YAP > Y_init), and decreases in high basal α-catenin cells (YAP1 < Y_init) upon autophagy inhibition.

FIGURE 2. Schematic representation of the effects exerted by the variation of c, r1 and r2 parameters upon autophagy inhibition.
(A) Variation of parameter c (the autophagy decay rate due to external perturbation): increase in the rate of autophagy decrease should cause YAP1 inhibition, and not activation at later time

points in cells with low basal α-catenin protein levels. **(B)** Variation of parameter r1 (controls the strength of the positive forward effect on YAP1 activity) and **(C)** r2 (controls the strength of the negative forward effect on YAP1 activity): increasing r1 or reducing r2 lowers the YAP1 activity, even causing YAP1 inhibition at later time points of autophagy inhibition in cells with low basal α-catenin protein levels.

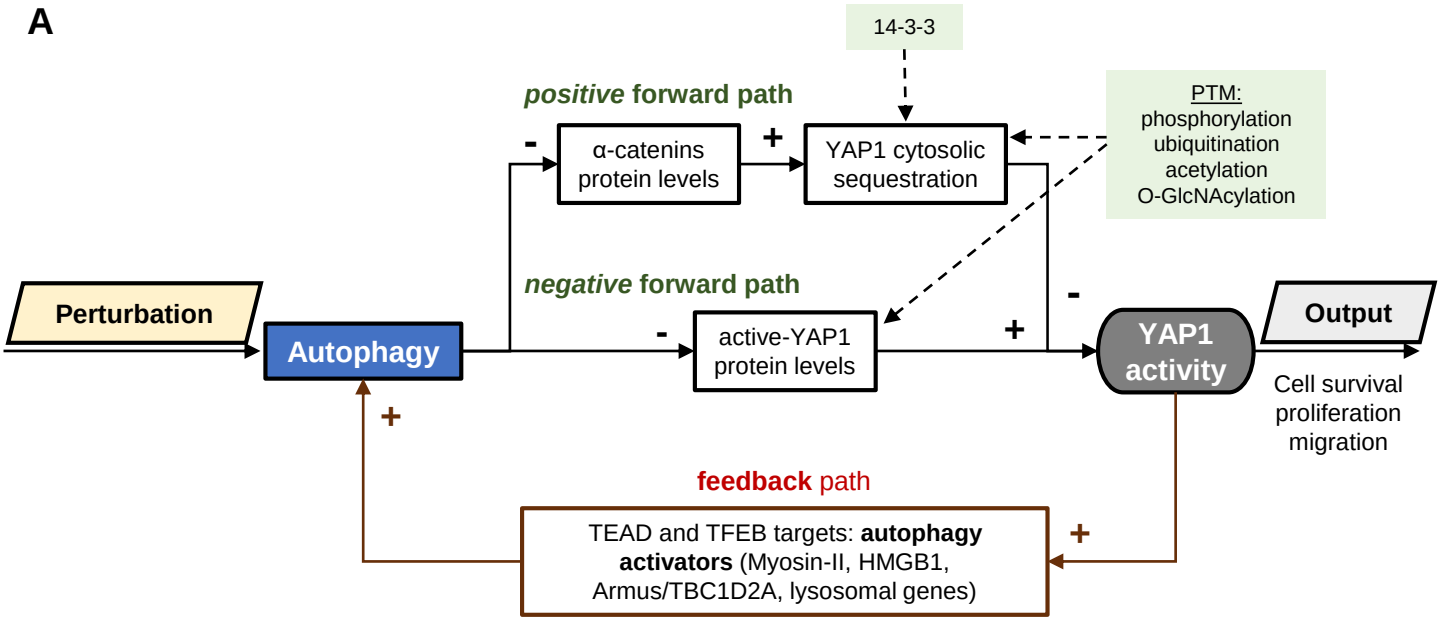
FIGURE 3. Schematic representation of YAP1 activity upon autophagy inhibition when one arm of the forward path is dominant. When the two arms (positive or negative) of the forward path are not balanced, the dominating arm dictates the YAP output irrespective of the initial α-catenin levels. For instance, large increase in negative arm (controlled by r2 parameter; over 5 times) or large decrease in the positive arm (controlled by parameter r1) would increase the YAP1 activity upon autophagy inhibition at early-time points irrespective of the initial α-catenin levels. Conversely, large reduction in the negative arm would cause the opposite effect: decreased YAP1 activity upon autophagy inhibition irrespective of the initial α-catenin levels.

FIGURE 4. Schematic representation of various autophagy-transduction pathways control systems. (A) Autophagy-Wnt/β-catenin signalling crosstalk. Both forward and feedback paths present two arms: positive and negative. **(B)** Autophagy-TGFβ/Smads signalling crosstalk. The forward path present two arms: positive and negative, while the feedback is only positive.

FIGURE 5. Schematic representation of the autophagy-NF-kB signalling control system. Both forward and feedback paths present two arms: positive and negative.

FIGURE 6. Schematic representation of the autophagy-XIAP/cIAP signalling control system. Both forward and feedback paths present two arms: positive and negative, each of them with multiple mechanisms.

FIGURE 1


A
14-3-3
positive forward path
α-catenins protein levels
YAP1 cytosolic sequestration
PTM: phosphorylation ubiquitination acetylation O-GlcNAcylation
negative forward path
Perturbation
Autophagy
active-YAP1 protein levels
YAP1 activity
Output
Cell survival proliferation migration
feedback path
TEAD and TFEB targets: autophagy activators (Myosin-II, HMGB1, Armus/TBC1D2A, lysosomal genes)

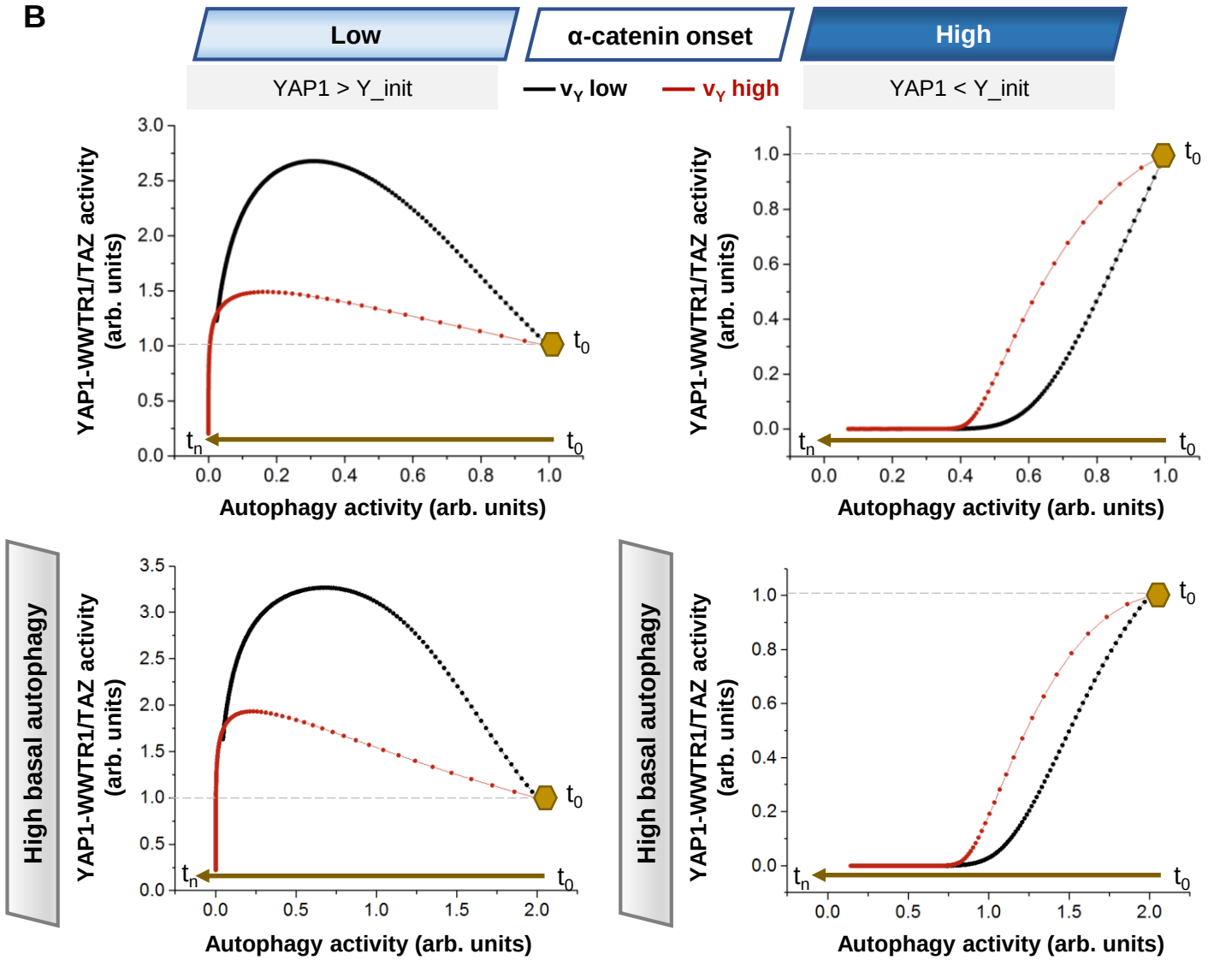

B
Low
α-catenin onset
High
YAP1 > Y_init
v_Y low
v_Y high
YAP1 < Y_init
YAP1-WWTR1/TAZ activity (arb. units)
Autophagy activity (arb. units)
t_0
t_n
High basal autophagy

FIGURE 2

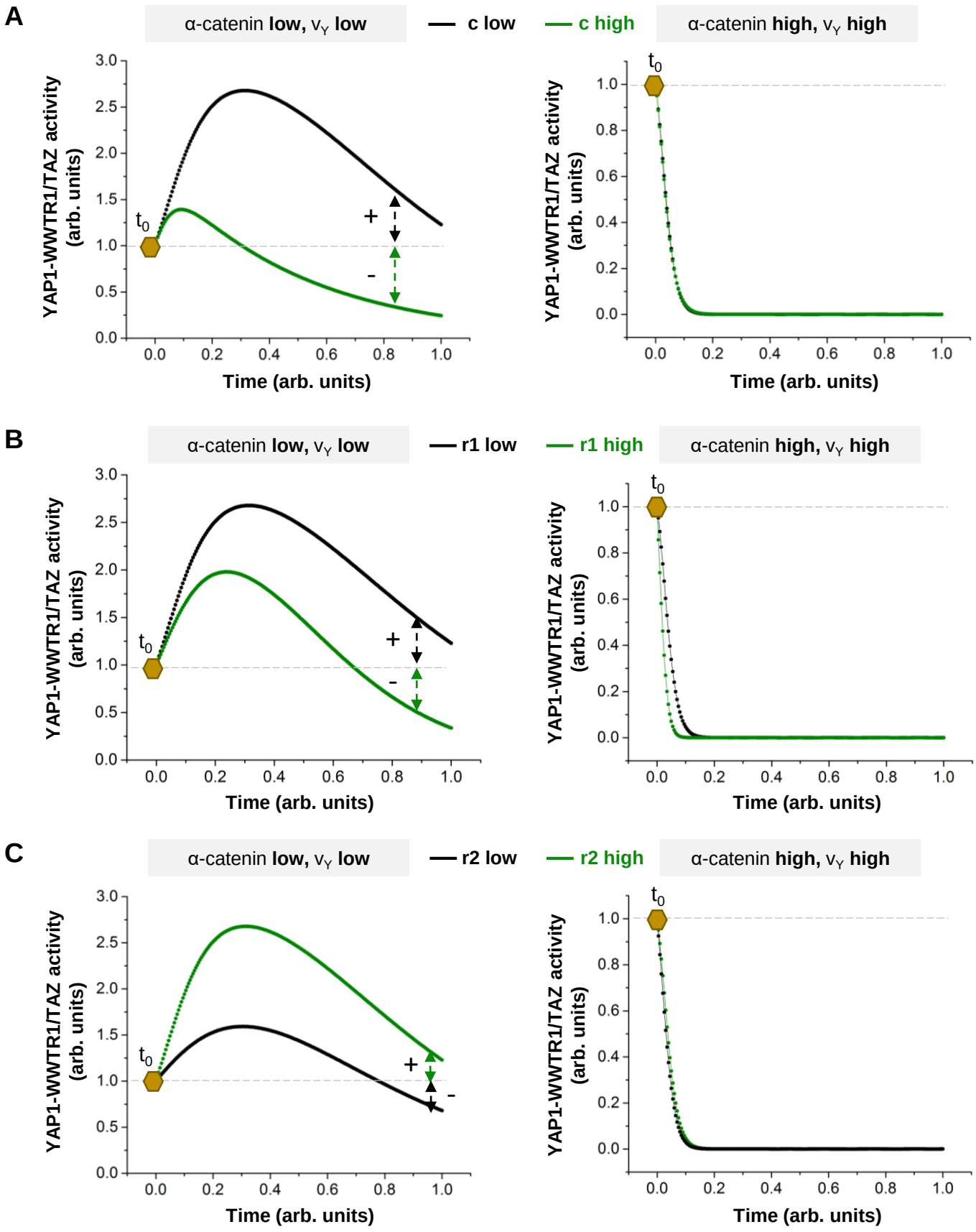

A
α-catenin low, v_Y low
c low
c high
α-catenin high, v_Y high
YAP1-WWTR1/TAZ activity (arb. units)
Time (arb. units)
t_0
+
-
B
α-catenin low, v_Y low
r1 low
r1 high
α-catenin high, v_Y high
YAP1-WWTR1/TAZ activity (arb. units)
Time (arb. units)
t_0
+
-
C
α-catenin low, v_Y low
r2 low
r2 high
α-catenin high, v_Y high
YAP1-WWTR1/TAZ activity (arb. units)
Time (arb. units)
t_0
+
-

FIGURE 3

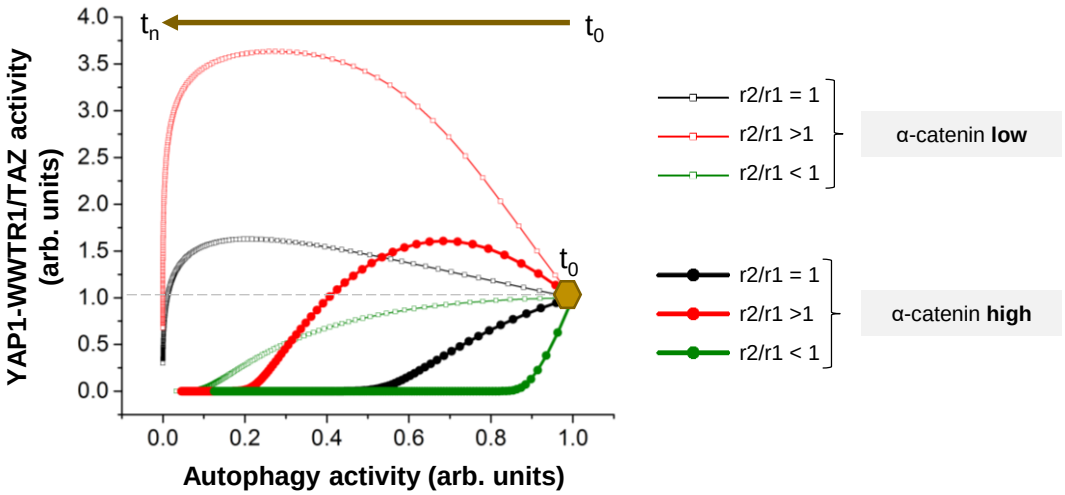

t_n
t_0
t_0
YAP1-WWTR1/TAZ activity (arb. units)
Autophagy activity (arb. units)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
0.0
0.2
0.4
0.6
0.8
1.0
r2/r1 = 1
r2/r1 >1
r2/r1 < 1
α-catenin low
r2/r1 = 1
r2/r1 >1
r2/r1 < 1
α-catenin high

FIGURE 4

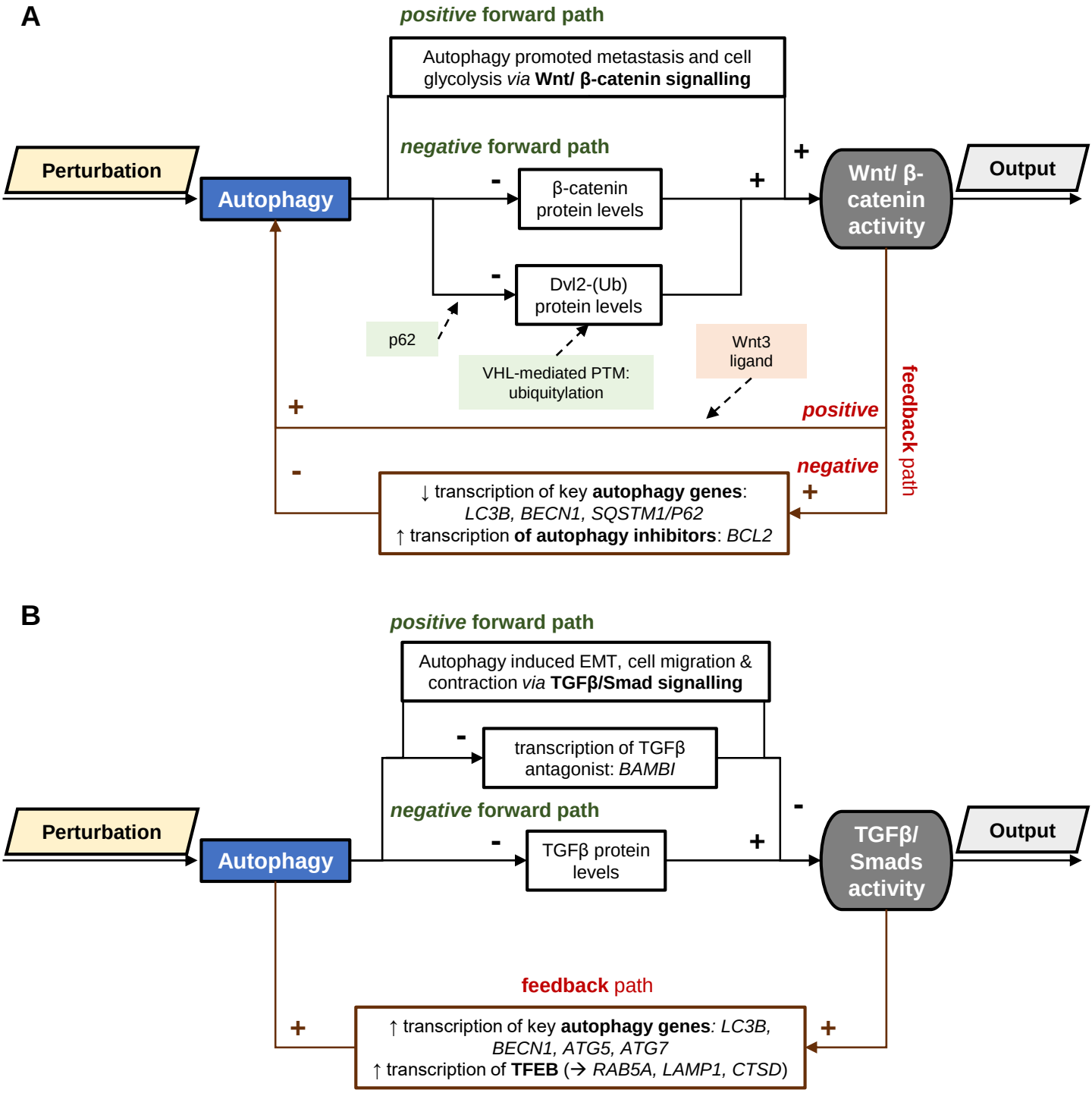

A
positive forward path
Autophagy promoted metastasis and cell glycolysis via Wnt/ β-catenin signalling
negative forward path
Perturbation
Autophagy
β-catenin protein levels
Dvl2-(Ub) protein levels
Wnt/ β-catenin activity
Output
p62
VHL-mediated PTM: ubiquitylation
Wnt3 ligand
positive
negative
feedback path
↓ transcription of key autophagy genes: LC3B, BECN1, SQSTM1/P62
↑ transcription of autophagy inhibitors: BCL2
B
positive forward path
Autophagy induced EMT, cell migration & contraction via TGFβ/Smad signalling
transcription of TGFβ antagonist: BAMBI
negative forward path
Perturbation
Autophagy
TGFβ protein levels
TGFβ/ Smads activity
Output
feedback path
↑ transcription of key autophagy genes: LC3B, BECN1, ATG5, ATG7
↑ transcription of TFEB (→ RAB5A, LAMP1, CTSD)

FIGURE 5

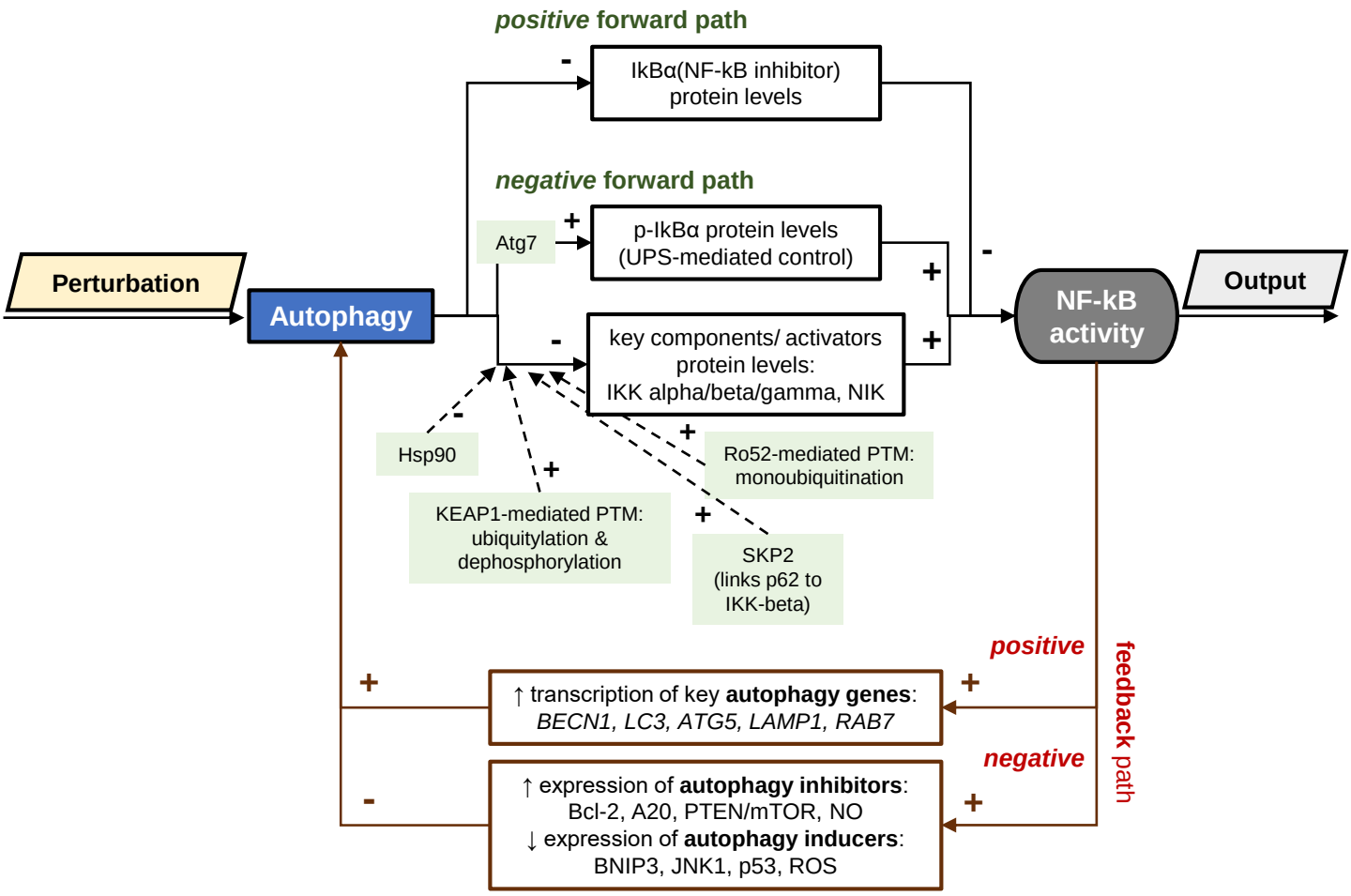
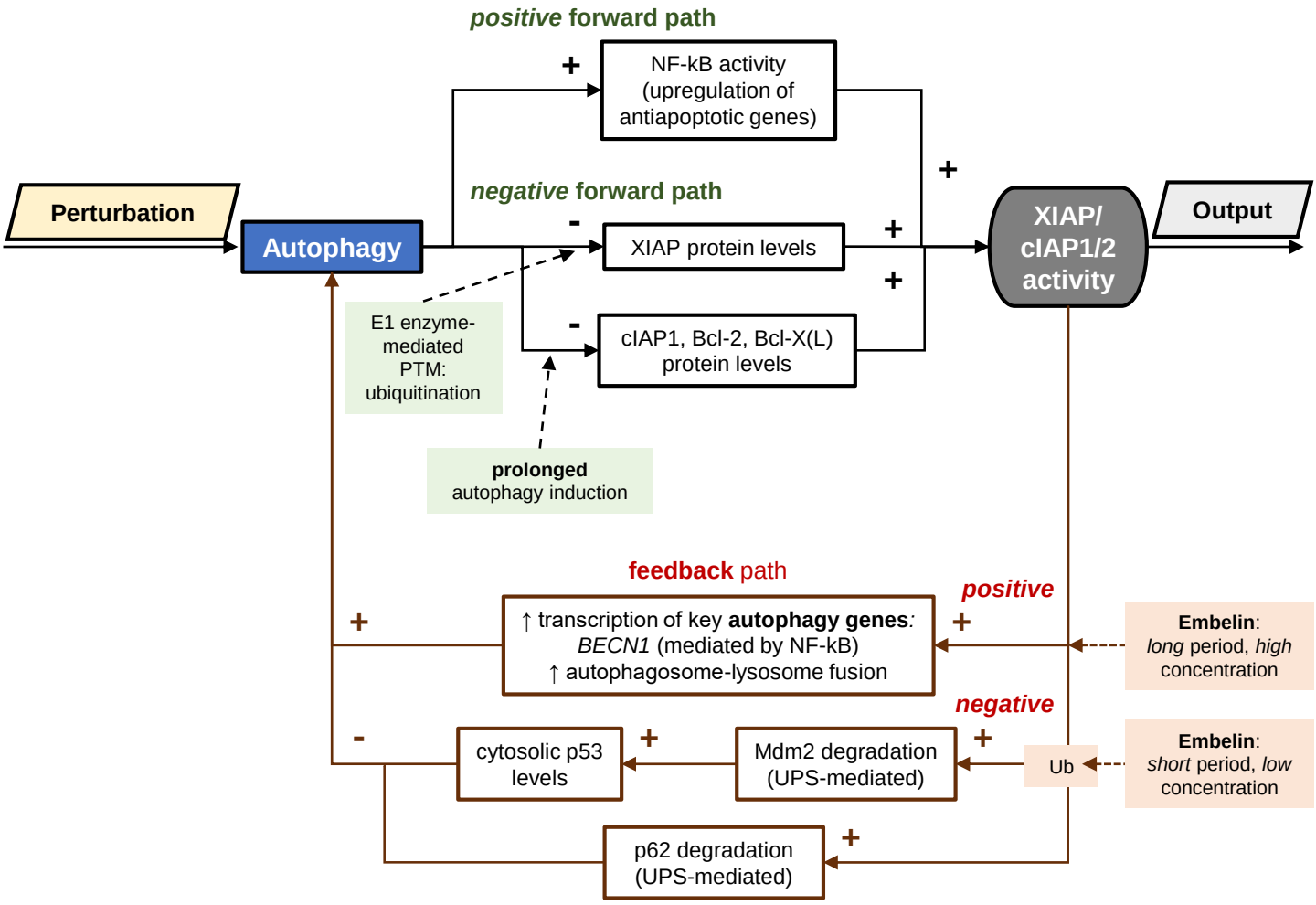

positive forward path
IkBα(NF-kB inhibitor) protein levels
negative forward path
Atg7
p-IkBα protein levels (UPS-mediated control)
Perturbation
Autophagy
NF-kB activity
Output
key components/ activators protein levels: IKK alpha/beta/gamma, NIK
Hsp90
Ro52-mediated PTM: monoubiquitination
KEAP1-mediated PTM: ubiquitylation & dephosphorylation
SKP2 (links p62 to IKK-beta)
positive
feedback path
↑ transcription of key autophagy genes: BECN1, LC3, ATG5, LAMP1, RAB7
negative
↑ expression of autophagy inhibitors: Bcl-2, A20, PTEN/mTOR, NO
↓ expression of autophagy inducers: BNIP3, JNK1, p53, ROS

FIGURE 6


positive forward path
NF-kB activity (upregulation of antiapoptotic genes)
negative forward path
Perturbation
Autophagy
XIAP protein levels
cIAP1, Bcl-2, Bcl-X(L) protein levels
XIAP/ cIAP1/2 activity
Output
E1 enzyme-mediated PTM: ubiquitination
prolonged autophagy induction
feedback path
positive
↑ transcription of key autophagy genes: BECN1 (mediated by NF-kB)
↑ autophagosome-lysosome fusion
Embelin: long period, high concentration
negative
cytosolic p53 levels
Mdm2 degradation (UPS-mediated)
Ub
Embelin: short period, low concentration
p62 degradation (UPS-mediated)

Supporting information

The complexity of biological control systems: an autophagy case study

Mariana Pavel†, Radu Tanasa† So Jung Park, and David C. Rubinsztein*

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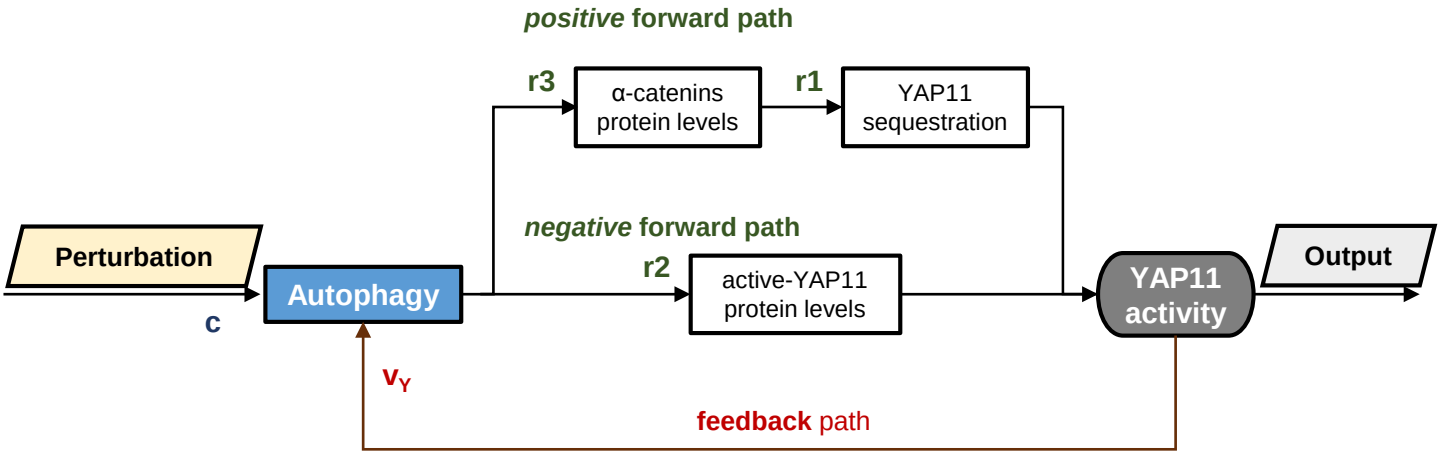
*Address correspondence to: dcr1000@cam.ac.uk

Mathematical model



$$(1)\quad \frac{dA}{dt} = -cA - v_Y\left(1 - e^{-\delta_A Y}\right)A$$

$$(2)\quad \frac{dY}{dt} = -r_1 YC + r_2\left(1 - e^{-\delta_Y A}\right)\left(1 - \frac{Y}{Y_{\max}}\right)Y$$

$$(3)\quad \frac{dC}{dt} = r_3 v_C v_Y\left(1 - e^{-\delta_C A}\right)\left(1 - \frac{C}{C_{\max}}\right)C$$

Figure S1 | The mathematical model proposed for the crosstalk between autophagy and YAP1 signalling. The three differential equations are those detailed in our previous publication: Pavel, Park et al. Nature Communications 2021. The parameters varied in the present study are: c, r1, r2, and r3.

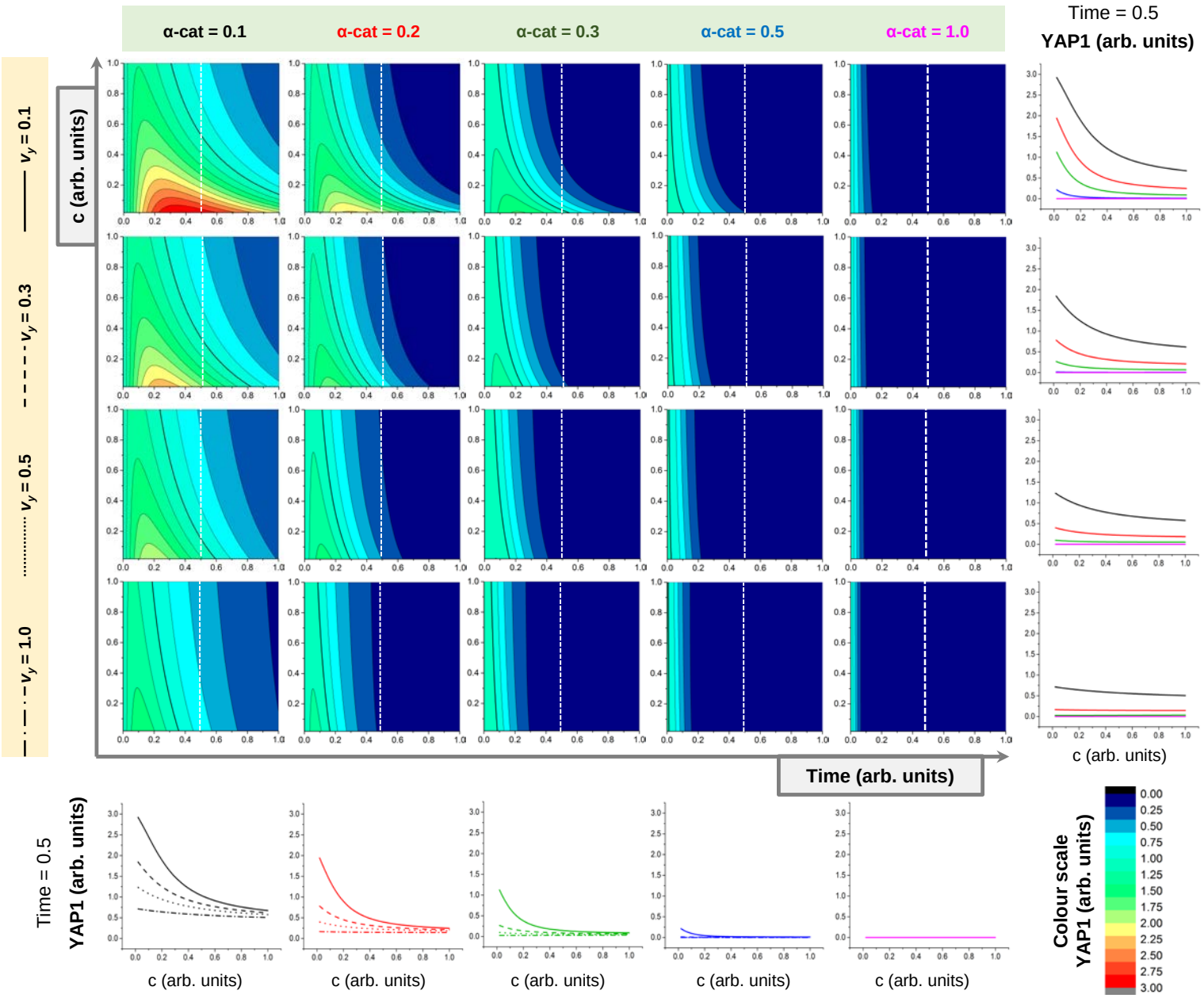


Figure S2 | The effect of parameter *c* on the dynamics of YAP1 activity when different initial α-cat and v_Y values are considered upon autophagy down-regulation.

Predicted time variation of absolute YAP1 activity (arbitrary units) when various initial α-cat and v_Y values (the strength of YAP1 influence on autophagy activity (Pavel et al. 2018)) are considered. Time parameter is shown on the horizontal axis, while the parameter *c* is extended on the vertical axis.

The colour scale on the bottom right indicates the estimated arbitrary values of YAP1 activity for the contour plots. The black highlighted contour line corresponds to YAP1 = 1.0 (values below 1.0 denote reduction in YAP1 activity, while values above 1.0 denote increase in YAP1 activity). The profiles indicated by vertical white dotted lines (corresponding to the time step = 0.5) are pictured in the line graphs plotted on the bottom (v_Y is varied; X axis – c variation, Y axis – YAP1 activity) or on the right (initial α-cat is varied; X axis – c variation, Y axis – YAP1 activity). For all plots: A_init = 1.0, Y_init = 1.0.

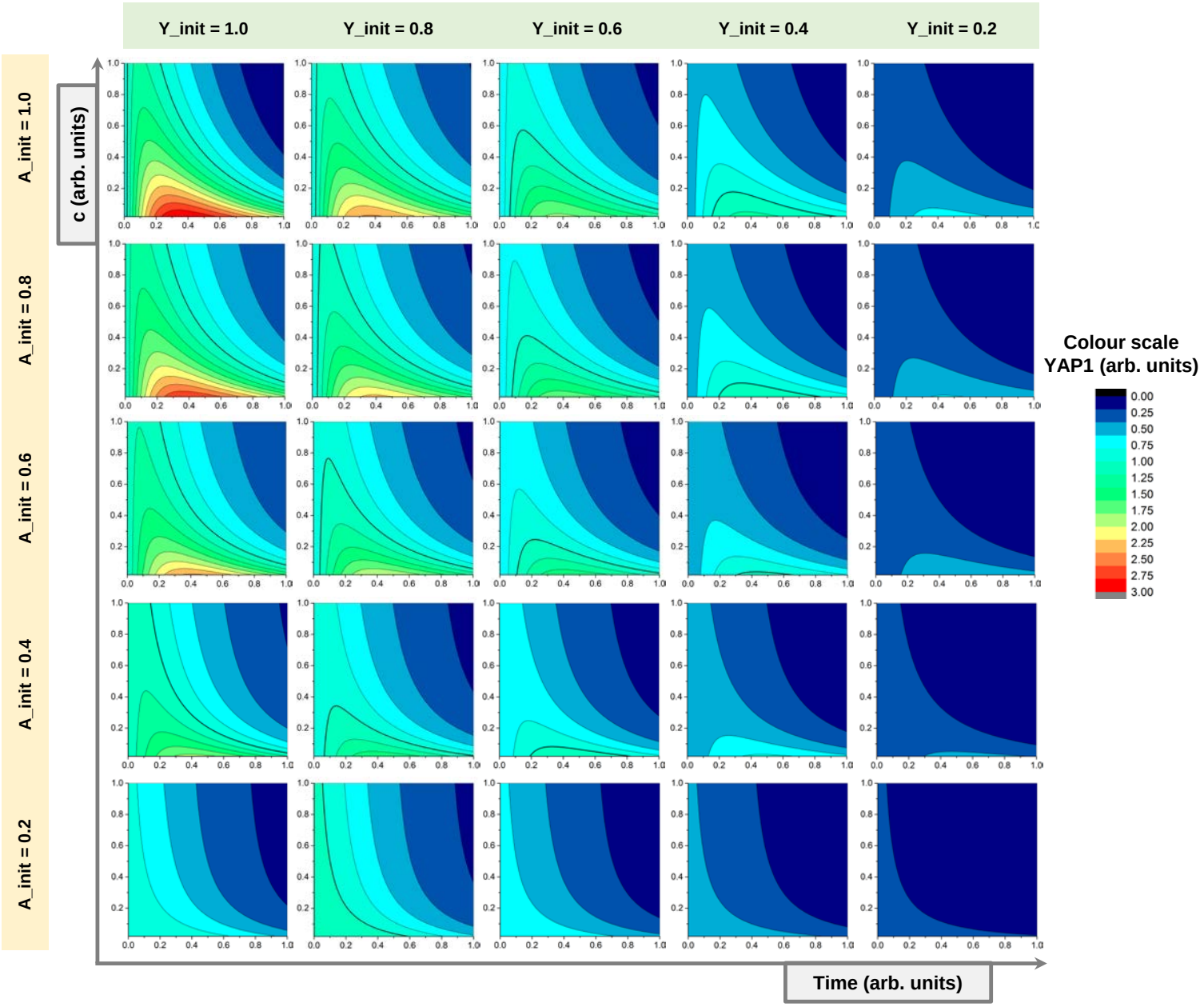


Figure S3 | The effect of parameter *c* on the dynamics of YAP1 activity when different initial autophagy (A_init) and YAP1 activity (Y_init) values are considered upon autophagy down-regulation.
Predicted time variation of absolute YAP1 activity (arbitrary units) when various initial autophagy (A_init) and YAP1 activity (Y_init) values are considered, and α-cat = 0.1, v_Y = 0.1. Time parameter is shown on the horizontal axis, while the parameter c is extended on the vertical axis.
The colour scale on the right indicates the estimated arbitrary values of YAP1 activity for the contour plots. The black highlighted contour line corresponds to YAP1 = 1.0.

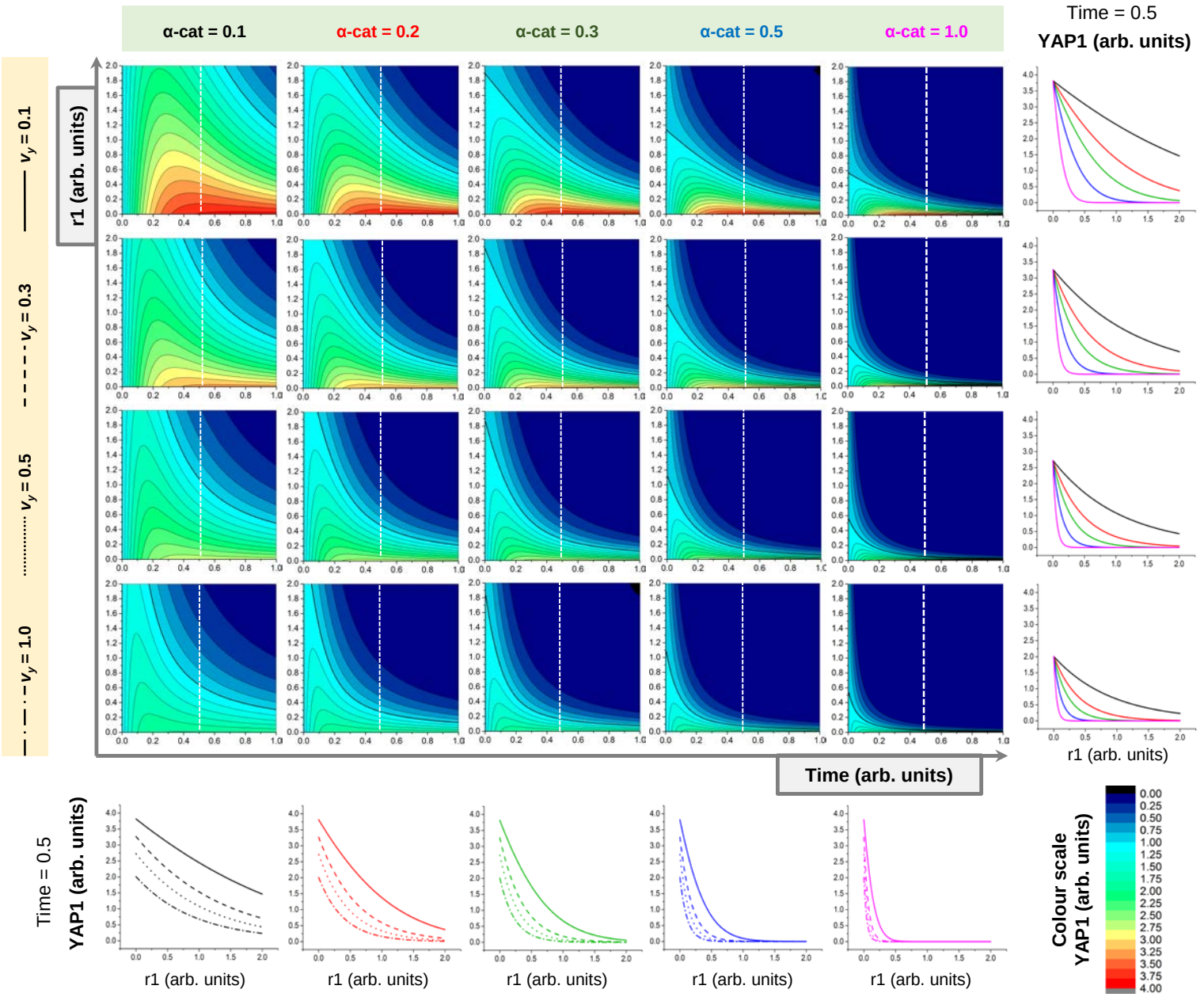


Figure S4 | The effect of parameter *r1* on the dynamics of YAP1 activity when different initial α-cat and v_Y values are considered upon autophagy down-regulation.

Predicted time variation of absolute YAP1 activity (arbitrary units) when various initial α-cat and v_Y values (the strength of YAP1 influence on autophagy activity (Pavel et al. 2018)) are considered. Time parameter is shown on the horizontal axis, while the parameter r1 is extended on the vertical axis.

The colour scale on the bottom right indicates the estimated arbitrary values of YAP1 activity for the contour plots. The black highlighted contour line corresponds to YAP1 = 1.0 (values below 1.0 denote reduction in YAP1 activity, while values above 1.0 denote increase in YAP1 activity). The profiles indicated by vertical white dotted lines (corresponding to the time step = 0.5) are pictured in the line graphs plotted on the bottom (v_Y is varied; X axis – r1 variation, Y axis – YAP1 activity) or on the right (initial α-cat is varied; X axis – r1 variation, Y axis – YAP1 activity). For all plots: A_init = 1.0, Y_init = 1.0.

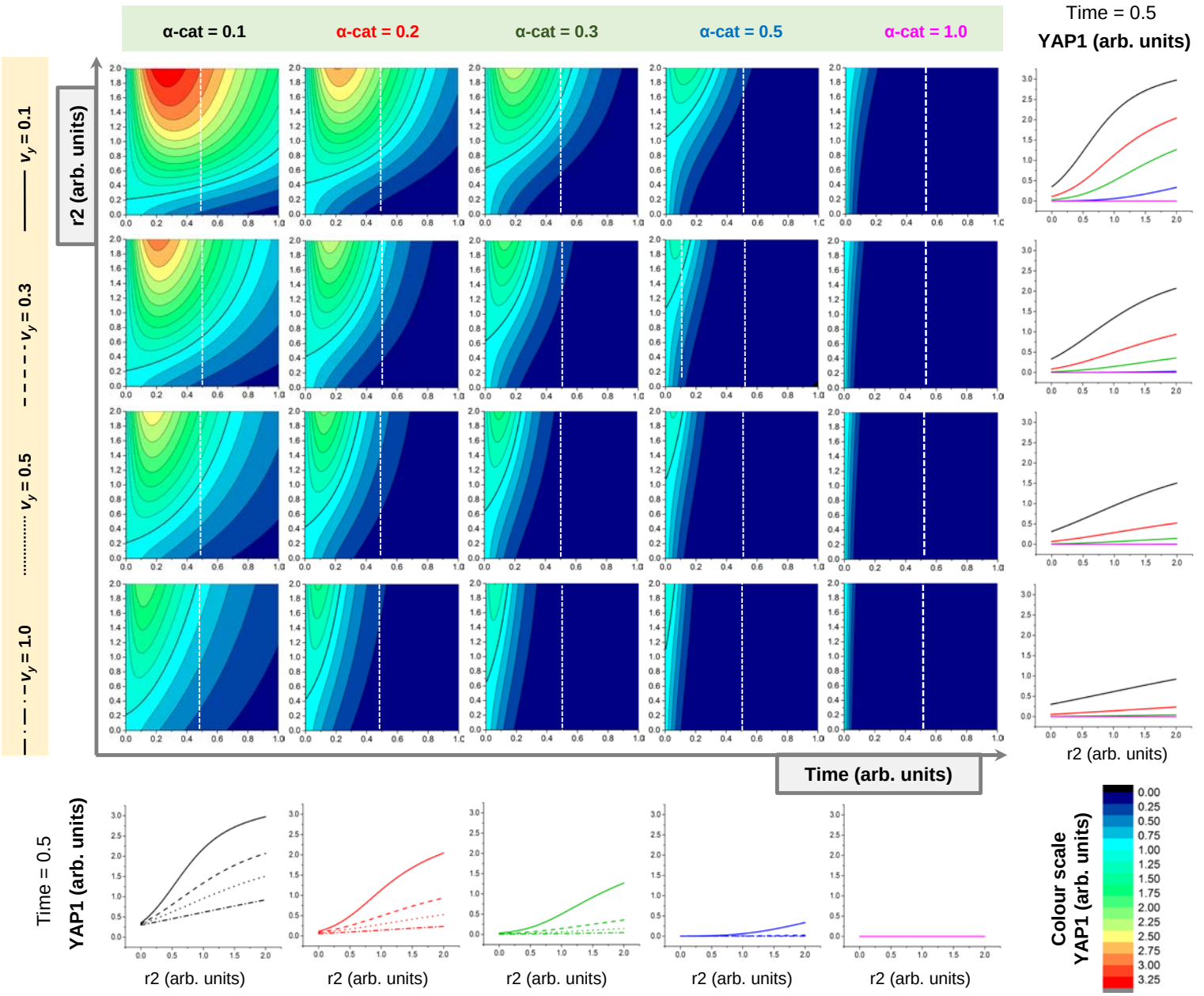


Figure S5 | The effect of variable *r2* on the dynamics of YAP1 activity when different initial α-cat and v_Y values are considered upon autophagy down-regulation.

Predicted time variation of absolute YAP1 activity (arbitrary units) when various initial α-cat and v_Y values (the strength of YAP1 influence on autophagy activity (Pavel et al. 2018)) are considered. Time parameter is shown on the horizontal axis, while the parameter r2 is extended on the vertical axis.

The colour scale on the bottom right indicates the estimated arbitrary values of YAP1 activity for the contour plots. The black highlighted contour line corresponds to YAP1 = 1.0 (values below 1.0 denote reduction in YAP1 activity, while values above 1.0 denote increase in YAP1 activity). The profiles indicated by vertical white dotted lines (corresponding to the time step = 0.5) are pictured in the line graphs plotted on the bottom (v_Y is varied; X axis – r2 variation, Y axis – YAP1 activity) or on the right (initial α-cat is varied; X axis – r2 variation, Y axis – YAP1 activity). For all plots: A_init = 1.0, Y_init = 1.0.

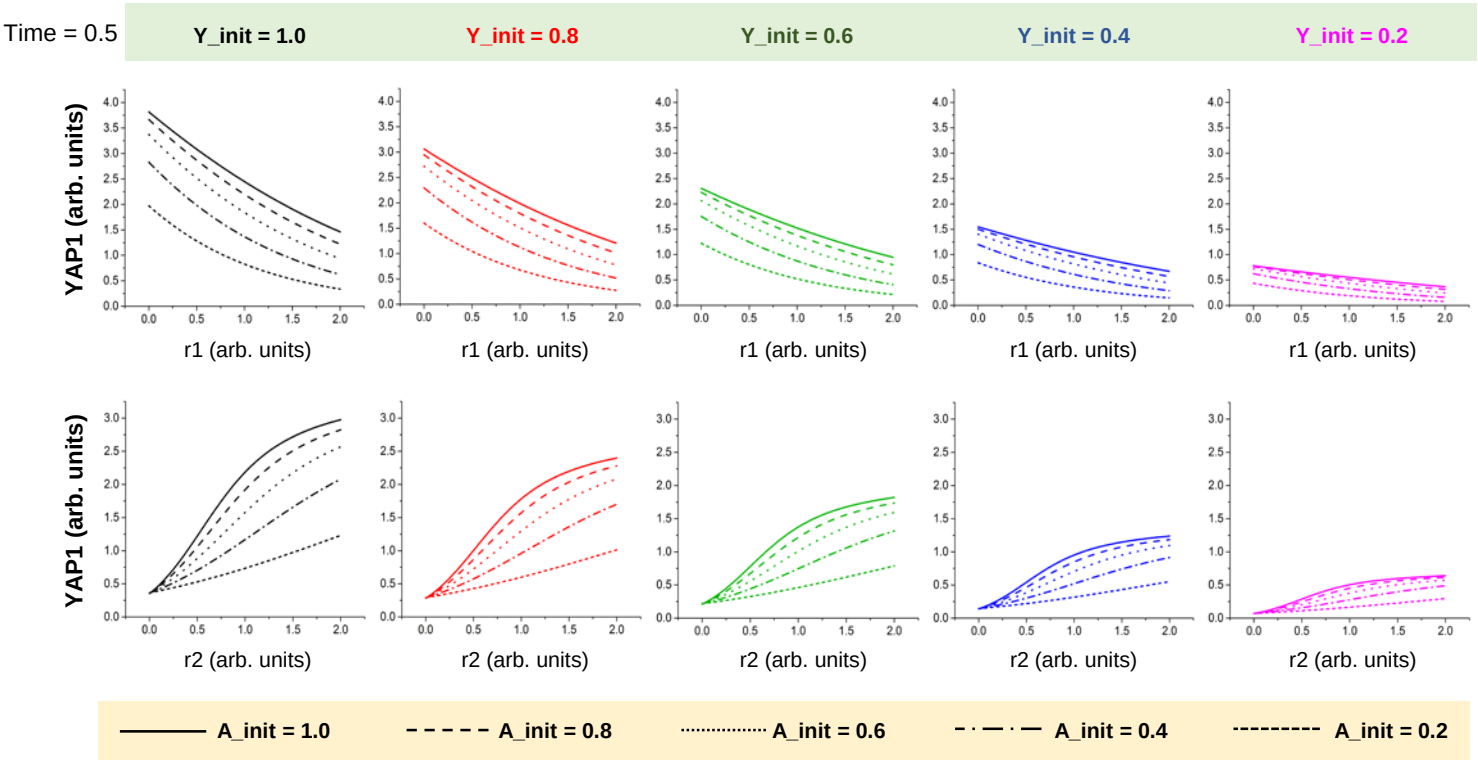


Figure S6 | The effect of variables *r1* and *r2* on the dynamics of YAP1 activity when different initial autophagy (A_init) and YAP1 (Y_init) values are considered upon autophagy down-regulation.
Predicted dynamics of YAP1 activity (arbitrary units) when various initial autophagy (A_init) and YAP1 activity (Y_init) values are considered at a fixed time after initiation of autophagy downregulation (time = 0.5), and α-cat = 0.1, v_Y = 0.1. The profiles at the indicated time point are pictured as line graphs: Y-axis – YAP1 activity; X-axis – r1 (on top) and r2 (on bottom).The colour legend for initial YAP1 variation is shown on the top of the graph, while the initial autophagy values are listed on the bottom (different line styles).

r2/r1 ratio - variation

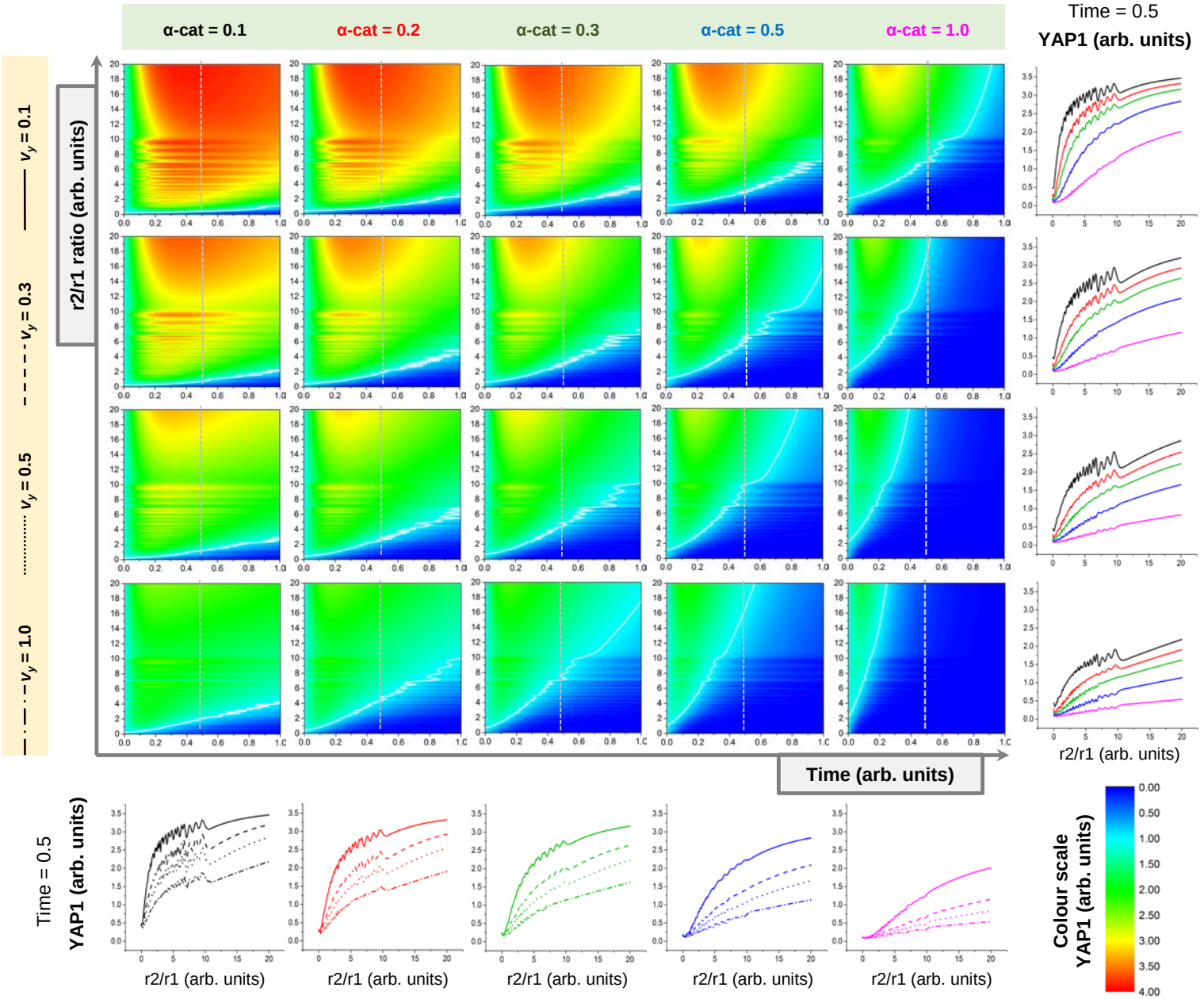


Figure S7 | The effect of *r2/r1* ratio on the dynamics of YAP1 activity when different initial α-cat and v_Y values are considered upon autophagy down-regulation.

Predicted time variation of absolute YAP1 activity (arbitrary units) when various initial α-cat and v_Y values (the strength of YAP1 influence on autophagy activity (Pavel et al. 2018)) are considered. Time parameter is shown on the horizontal axis, while the variation of r2/r1 ratio is extended on the vertical axis.

The colour scale on the bottom right indicates the estimated arbitrary values of YAP1 activity for the contour plots. The black highlighted contour line corresponds to YAP1 = 1.0 (values below 1.0 denote reduction in YAP1 activity, while values above 1.0 denote increase in YAP1 activity). The profiles indicated by vertical white dotted lines (corresponding to the time step = 0.5) are pictured in the line graphs plotted on the bottom (v_Y is varied; X axis – r2/r1 ratio, Y axis – YAP1 activity) or on the right (initial α-cat is varied; X axis – r2/r1 ratio, Y axis – YAP1 activity). For all plots: A_init = 1.0, Y_init = 1.0.

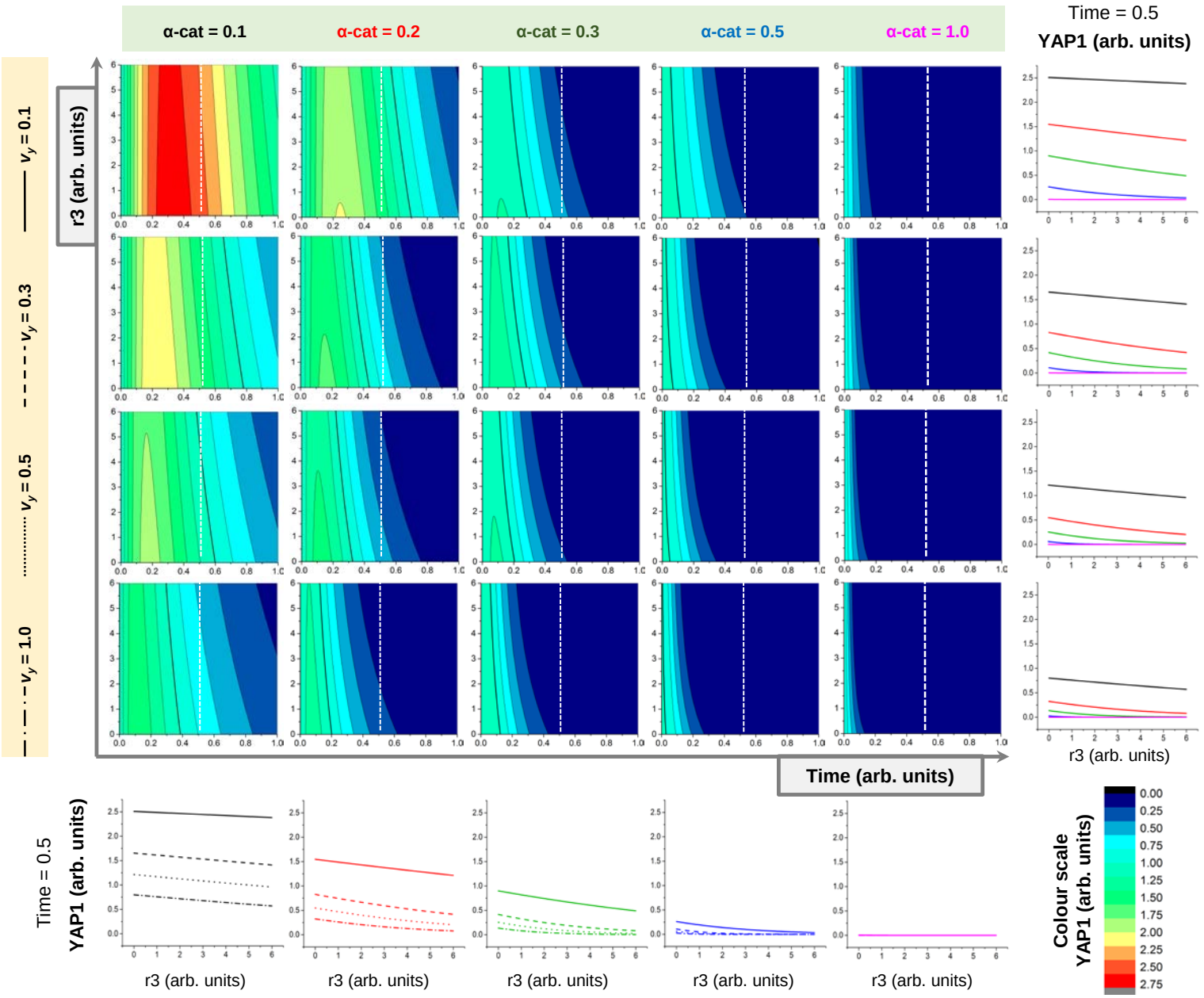


Figure S8 | The effect of variable *r3* on the dynamics of YAP1 activity when different initial α-cat and v_Y values are considered upon autophagy down-regulation.

Predicted time variation of absolute YAP1 activity (arbitrary units) when various initial α-cat and v_Y values (the strength of YAP1 influence on autophagy activity (Pavel et al. 2018)) are considered. Time parameter is shown on the horizontal axis, while the parameter *r3* is extended on the vertical axis.

The colour scale on the bottom right indicates the estimated arbitrary values of YAP1 activity for the contour plots. The black highlighted contour line corresponds to YAP1 = 1.0 (values below 1.0 denote reduction in YAP1 activity, while values above 1.0 denote increase in YAP1 activity). The profiles indicated by vertical white dotted lines (corresponding to the time step = 0.5) are pictured in the line graphs plotted on the bottom (v_Y is varied; X axis – r3 variation, Y axis – YAP1 activity) or on the right (initial α-cat is varied; X axis – r3 variation, Y axis – YAP1 activity). For all plots: A_init = 1.0, Y_init = 1.0.