

The Hippo pathway in colorectal cancer

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Abstract

Colorectal cancer (CRC) is one of the most frequently diagnosed neoplastic diseases worldwide. Currently, aside from traditional chemotherapy, advanced CRCs are treated with modern drugs targeting cellular components such as epithelial growth factor receptor (EGFR). Since up to 70% of metastasized CRCs are drug resistant, the description of recent progress in cellular homeostasis regulation may shed new light on the development of new molecular targets in cancer treatment. The Hippo pathway has recently become subject of intense investigations since it plays a crucial role in cell proliferation, differentiation, apoptosis and tumorigenesis. Components of the Hippo pathway are deregulated in various human malignancies, and expression levels of its major signal transducers were proposed as prognostic factors in colorectal cancer. In this review we focused on recent data regarding Hippo pathway, its up-stream signals and down-stream effectors. Hippo negatively regulates its major effectors, YAP1 and TAZ kinases, which act as transcriptional co-activators inducing expression of genes involved not only in tissue repair and proliferation but are also oncoproteins involved in tumour development and progression. The deregulation of Hippo pathway components was found in many malignancies.

The interactions between Hippo and Wnt/ β -catenin signalling, crucial in the maintenance of cell homeostasis, have been described in relation to the control of intestinal stem cell proliferation and CRC development. The recently discovered positive feedback loop between activated YAP1 and increased EGFR/KRAS signalling found in oesophageal, ovarian and hepatocellular cancer has been related to the CRC progression and resistance to EGFR inhibitors during CRC therapy. (*Folia Histochemica et Cytobiologica* 2015, Vol. 53, No. 2, 105–119)

Key words: CRC; Hippo pathway; YAP1/TAZ; Wnt signalling; EGFR/KRAS; intestinal stem cells

Abbreviations: AJ — adherent junction, AMOT — angiomin, APC — adenomatous polyposis coli, AREG — amphiregulin, Axl — Axl receptor tyrosine kinase, BMI1 — BMI1 polycomb ring finger proto-oncogene, β -TrCP — beta-transducin repeat containing E3 ubiquitin protein ligase, CK1 — casein kinase 1, CRC — colorectal cancer, CTGF — connective tissue growth factor, DSS — dextran sodium sulphate, DVL — Dishevelled protein, ECM — extracellular matrix, EGFR — epithelial growth factor receptor, EMT — epithelial-to-mesenchymal transition, FAT — FAT atypical cadherin, FZD — Frizzled protein, GPCR — G protein coupled receptor, GSK3 — glycogen synthase 3, ISC — intestinal stem cell, KO — knock out, KRAS — Kirsten rat sarcoma, LATS1/2 — large

tumor suppressor kinase 1/2, Lgr5 — leucine-rich repeat-containing GPCR5, LPR — Lipoprotein receptor-related protein, MOB1 — MOB kinase activator 1, MST1/2 — serine/threonine kinase 4/3, NF2 — neurofibromin 2, Oct-4 — octamer-binding transcription factor 4, RASSF — Ras association (RalGDS/AF-6) family member, SAV1 — Salvador family WW domain containing protein 1, STRIPAK — striatin interacting phosphate and kinase complex, TAZ — tafazzin, TCF4 — transcription factor 4, TEAD — TEA domain family member, TJ — tight junction, β -TrCP — β -transducin repeat-containing protein, VEGF — vascular endothelial growth factor, WB — Western blot technique, YAP1 — Yes-associated protein 1

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Colorectal cancer

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in males and the second in females, with an estimated 1.4 million cases and 693,900 deaths occurring in 2012 [1]. Five-year relative survival ranges

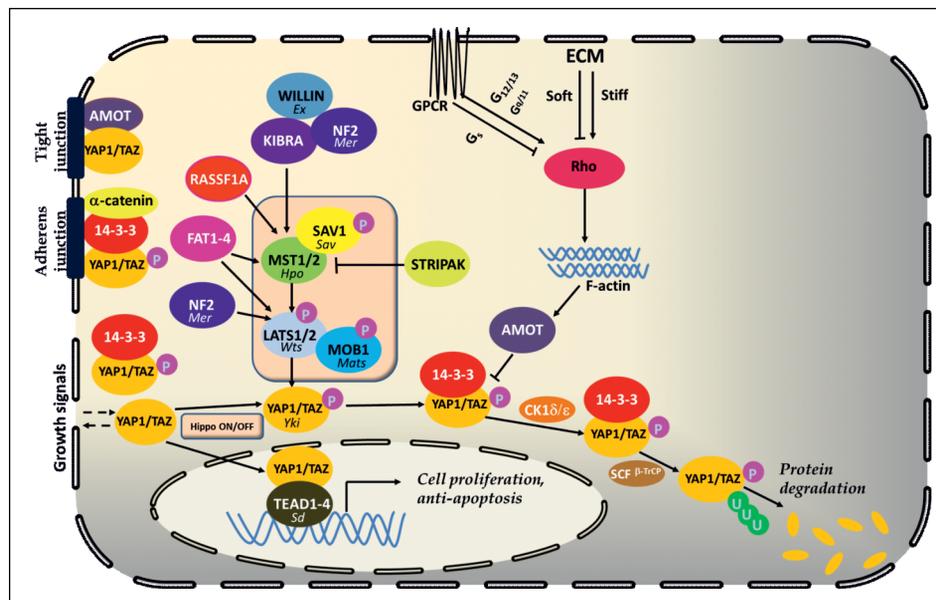


Figure 1. The mammalian Hippo pathway. *Drosophila* orthologue genes are shown below the names of the mammalian proteins. When the Hippo pathway is not active (e.g. during cell injury and repair or in cancer), the active effector proteins, YAP1 and TAZ, interact with TEAD1-4 transcription factors and promote transcription of genes involved in cell proliferation. During normal cell homeostasis, when the Hippo pathway is active, YAP1 or TAZ are inhibited due to their phosphorylation by core components of the Hippo pathway (SAV1, MST 1/2, LATS1/2 — shown in the central rectangle). In a phosphorylated form cytoplasmic YAP1/TAZ may interact with (i) the 14-3-3 protein, (ii) components of cell junctional complexes like AMOT or β -catenin, or (iii) may be degraded in proteasomes. YAP1/TAZ can be also regulated by other mechanisms such as cell polarity, GPCR signalling or ECM stiffness as described in the body text. Acronyms are explained on the first page

from more than 90% in patients with stage I disease to about 10% in patients with stage IV disease [2]. Treatment of CRC is one of the most expensive when diagnosed at later stages when prognosis is generally poor [3]. Although the knowledge of the background and the development of CRC has recently increased, there is a high need for more studies to find out well working prognostic, survival and diagnostic markers. Serum markers for routine CRC diagnostic such as CEA (carcinoembryonic antigen) and CA 19-9 showed good prognostic values and have been used as CRC tumour predictors [4]. The aim of this paper was to provide rationale to consider the expression of Hippo pathway genes as possible new prognostic genes in CRC.

The modern chemotherapy has adapted molecular findings in many cancers to focus on activation/inactivation of cancer-related intracellular pathways to eliminate or decrease expansion of tumour cells. Such drugs which target vascular endothelial growth factor-A (VEGF, *Bevacizumab*) or epidermal growth factor receptor (EGFR, *Cetuximab* and *Panitumumab*) have been introduced for the treatment of CRC [5]. However, it was noted that 50–70% of advanced CRC cases were resistant to both classical chemotherapeu-

tics and so called biological drugs [6]. In this review we also aimed to bring closer the possible associations between the Hippo pathway and drug resistance of CRC cells.

The Hippo pathway

The Hippo pathway is an important regulator of cell proliferation, growth and apoptosis [7, 8]. Moreover, it controls tissue homeostasis, organ size and stem cell functions. Its deregulation is frequently observed in many human cancers, suggesting that alterations of Hippo signalling are connected with tumour initiation and/or progression [9–11]. Hyperactivation of the Hippo pathway downstream effectors — YAP1 (Yes-associated protein 1) and TAZ (transcriptional co-activator with PDZ binding motif) may contribute to the development of cancer, however, their activation may also play a positive role in stimulating tissue repair and regeneration following injury [12, 13]. The general scheme of the Hippo pathway and interactions of mammalian Hippo components with upstream signals and effectors are presented in Figure 1 and listed in Table 1.

Table 1. Names and main functions of the most important Hippo pathway genes and proteins

Mammalian gene	<i>Drosophila</i> gene, protein	Protein name, aliases	Protein function
<i>NF2</i>	<i>Mer</i> , Merlin	Neurofibromin 2, Merlin, ACN, SCH, BANF	Signal transduction between cell-membrane and cytoskeletal proteins
<i>WWC1</i>	<i>Kibra</i> , ortholog	WW and C2 domain containing 1, KIBRA, HBEBP3, HBEBP36	Phosphoprotein involved in cell polarity, mitosis and cell migration [105]
<i>FRMD6</i>	<i>Ex</i> , Expanded	FERM domain containing 6, EX1, Willin	Protein linking cytoskeleton with plasma membrane [106]
<i>STK4</i>	<i>Hpo</i> , Hippo	Serine/threonine kinase 4, MST1, KRS2	Cytoplasmic kinase involved in stress-induced mitogen-activated protein kinase cascade
<i>STK3</i>		Serine/threonine kinase 3, MST2, KRS1	Kinase activated by proapoptotic molecules for the promotion of chromatin condensation during apoptosis, cardiomyocyte proliferation [107], regulation of osteoblast/osteoclast differentiation [108]
<i>RASSF1</i>		Ras association (RalGDS/AF-6) domain family member 1, RASSF1A, NORE2A	Signal transducer, microtubule stabilization, cell cycle arrest [109]
<i>SAVI</i>	<i>Sav</i> , Salvador	Salvador family WW domain containing protein 1, SAV, WW45	Regulation of protein degradation, RNA splicing and DNA transcription
<i>LATS1</i>	<i>Wts</i> , Warts	Large tumour suppressor kinase 1, WARTS	Serine/threonine kinase involved in mitosis: interacts with CDC2/cyclin A
<i>LATS2</i>		Large tumour suppressor kinase 2, KPM	Kinase which interacts with centrosomal proteins aurora-a and ajuba during mitosis [110]
<i>MOB1A</i>	<i>Mats</i>	MOB kinase activator 1A, MOB1, MATS,	Protein involved in mitotic exit network [111], microtubule stability control [112]
<i>YAPI</i>	<i>Yki</i>	Yes-associated protein 1, YAP, YKI	Downstream effector of Hippo pathway involved in development, repair and homeostasis
<i>TAZ</i>		Tafazzin, EFE, Taz1	Non-specific phospholipid-lysophospholipid transacylase involved in cardiolipin turnover [113]

Discovery and function of the Hippo pathway in *Drosophila*

The Hippo pathway was first described almost 20 year ago as a result of screening for mutant tumour suppressors in *Drosophila*. It was found that loss-of-function mutation of the Hippo pathway components revealed enormous overgrowth of fruit flies as a result of increased cell proliferation and decreased apoptosis [14]. The first identified elements of the pathway were its core components: *warts* (*wts*), *hippo* (*hpo*) and *salvador* (*sav*) (Figure 1) [14–17]. These genes belong to the hyperplastic group of *Drosophila* tumour suppressors, wherein mutations of these genes result in robust tissue overgrowth without alterations of cell differentiation status or cell polarity [18]. Further studies indicated that Hpo kinase in association with its adaptor protein Sav, phosphorylates and activates complex formed

by Wts kinase, which is associated with an activating subunit Mats [19]. The kinase activity of Hpo could be antagonised by dSTRIPAK — phosphatase complex [20]. The core kinase cassette of the Hippo pathway acts as a suppressor of a downstream element — Yorkie (Yki) [21]. Subsequent biochemical studies showed that Wts directly phosphorylates and inhibits Yki. Yki is a transcriptional co-activator that lacks DNA binding domain. It cooperates with nuclear transcription factors, like Scalloped, and enables transcription of genes which promote cell proliferation (*e.g.*, *Myc*) and inhibits apoptosis (*e.g.*, *diap1*) [22, 23]. When the cell receives growth inhibiting signals, *i.e.* due to contact inhibition, it activates core components of the pathway that phosphorylate Yki. This leads to the cytoplasmic sequestration of Yki and its binding to the 14-3-3 protein, which finally results in Yki protein degradation [21, 24].

The components and function of the Hippo pathway in mammals

The core kinases cassette and downstream effectors of the *Drosophila* Hippo pathway are highly conserved in mammals (Figure 1): MST 1/2 (homologues of Hpo), SAV1 (Sav homologue), LATS 1/2 (Wts homologues), MOB1 (homologues of Mats) and YAP1 and its paralogue TAZ (homologues of Yki) [25]. It was shown that expression of human genes can rescue the phenotypes of corresponding *Drosophila* mutants *in vivo* [15, 19, 21]. The first kinase MST1/2 can be activated in two different ways: (1) by caspase-dependent cleavage under apoptotic stress [26], or (2) by binding to one of Ras association domain family (RASSF) proteins, RASSF1A [27]. Activated MST1/2 interacts with SAV1 through the SARAH domains presented on both proteins what leads to the phosphorylation and activation of their direct substrates LATS1/2 [28]. MOB1 protein is also phosphorylated by MST1/2, which results in its enhanced interaction with LATS1/2 and formation of a complex that phosphorylates and inhibits activity of YAP1 and/or TAZ (Figure 1) [28, 29].

LATS1/2-dependent phosphorylation of Ser127 of YAP1 and Ser89 of TAZ in sequences HXRXXS (H-histidine; R-arginine; S-serine; X-any amino acid) are the most important reactions within this mechanism since this results in binding of 14-3-3 sites and causes segregation of YAP1 and TAZ in the cytoplasm [30–32]. When LATS1/2 phosphorylates Ser397 of YAP1 and Ser311 of TAZ, casein kinase1 ϵ/δ subsequently phosphorylates Ser400 and Ser403 of YAP1 and Ser314 of TAZ thereby leading to ubiquitination and degradation of YAP1 and TAZ proteins [31]. However, it has to be noted that many other kinases, not involved in the Hippo pathway, can phosphorylate YAP1 in a specific way, depending on the signals received by the cell. Akt kinase, a pro-survival kinase, was shown to negatively regulate the YAP1-dependent transcription of pro-apoptotic genes [31]. It was also found that after DNA damage, c-Abl kinase phosphorylates YAP1 at Y407 (tyrosine phosphorylation) which enhances the YAP1-p73 interaction, prevents Itch-mediated ubiquitination of p73 and activates transcription of pro-apoptotic genes [33, 34].

Regulation of the YAP1/TAZ activity

Upstream regulators of YAP1/TAZ activity can act either through Hippo pathway core components or independently of Hippo kinases. For instance, in the epithelial cells apical-basal polarity and planar cell polarity regulate the Hippo pathway. In *Drosophila* epithelium apically localized Merlin (Mer), Expanded (Ex) and Kibra proteins form a complex (Figure 1) which acts by binding to Sav, Hpo and Wts to activate

the Hippo pathway in a cooperative manner or *via* direct binding of Ex and Yki to restrain Yki level in cytoplasm and inhibit its transcriptional activity [35, 36].

Function of this apical protein complex is conserved also in mammals. The complex of Nf2 (neurofibromin2, a Mer ortholog), KIBRA and FRMD6 (also known as WILLIN, as potential Ex ortholog) inhibits YAP1 activity (Figure 1) [37]. *Nf2*, a tumour suppressor gene is currently the only one known Hippo pathway gene that is mutated in cancer (Table 2) [10], especially in cancers of central nervous system [38].

Planar cell polarity not only regulates the position of a cell in the epithelial layer, but also plays a crucial role in the control of development. In *Drosophila* the protocadherins Fat (Ft) and Dachshous (Ds) modulate Wts activity; however, the role of their mammalian orthologues in the Hippo pathway is not so well understood and requires further studies [39].

Tight junctions (TJ) and adherens junctions (AJ) play an important role in intercellular contacts being responsible for the permeability barriers. Structural proteins of TJ and AJ were shown to interact with YAP1 (Figure 1). Cytoplasmic angiominin (AMOT) proteins can inhibit YAP1/TAZ *via* physical interactions and transfer of the YAP1/AMOT complexes from cytoplasm to tight junctions or actin cytoskeleton (Figure 1). Moreover, AMOT proteins also activate LATS1/2 to phosphorylate YAP [40]. Another TJ protein, ZO-2, was reported to increase nuclear localization of YAP and tight junction localization of TAZ [41, 42]. It was demonstrated that α -catenin, a component of adherens junction, together with 14-3-3 protein and phosphorylated form of YAP1 form a complex that inhibits YAP1 activity [43].

It has been shown that YAP1/TAZ activity can also depend on environmental cues such as extracellular matrix stiffness, cell tension, cell attachment/detachment [38, 44]. It was observed, that the nuclear localization of YAP/TAZ resulting in TEAD genes' activation was found during proliferation of endothelial and epithelial cells as well as during osteoblast differentiation [38]. In breast cancer (MCF10A) and mouse fibroblast NIH-3T3 cell cultures it was found that YAP1 overexpression and phosphorylation can overcome cell contact inhibition [29]. Recent reports appraise the role of G protein-coupled receptors (GPCRs) and their cooperation with Rho GTPase and actin cytoskeleton in negative or positive modifications of YAP1 or TAZ activity. Depending on the class of G proteins, but also on the type of their ligands, the effects on YAP1/TAZ activity regulation might be completely different. For instance, lysophosphatidic acid, sphingosine 1-phosphate and peptide agonists of thrombin receptors induce, whereas

Table 2. The involvement of Hippo pathway proteins in human diseases

Gene's name	Role in human diseases	Role in CRC development
<i>NF2, Merlin</i>	Mutation leads to neurofibromatosis type II; lesions in the CNS and eyes and skin [114, 115] TSG in some cancers due to mutation and underexpression [10]	Barely studied; point mutations were not critical [49]
<i>WWC1, KIBRA</i>	Polymorphisms associated with Alzheimer's disease [116]	Not studied
<i>FRMD6, human Expanded</i>	Polymorphisms associated with Alzheimer's disease [117], CCL: downregulation leads to resistance to Taxol I MC10FA cells [118]	Not studied
<i>STK4, MST1 and STK3, MST2</i>	Phosphorylation loss of function in prostate cancer [119], CCL: overexpression inhibits cell proliferation and promote apoptosis in HepG2 cells [87] Mouse double KO: cholangiocarcinoma [46, 101], HCC [120]	Decreased mRNA level in tumour associated with node metastasis [121], mouse double KO: crypt dysplasia, colon adenoma [46, 78, 120]
<i>RASSF1, RASSF1A</i>	TSG: promoter hypermethylation and decrease expression in cancer: thyroid [122], oesophageal [123], prostate [124], colorectal, breast [125]	TSG; promoter hypermethylation and decrease expression in CRC [126, 127]
<i>SAVI, Salvador</i>	TSG: LOH and downregulation in renal cell carcinoma [128], no gene mutation in stomach, liver and lung cancer [129] CCL: overexpression induces apoptosis in MCF-7 cells [130]	No gene mutation in CRC [129]
<i>LATS1</i>	TSG: decrease expression in cancer: glioma [131], NSLC [132], sarcoma [133] and astrocytoma [134]. CCL: LATS1 degradation inhibits apoptosis in MCF10A cells [135]	TSG: promoter hypermethylation and mRNA decrease in CRC [86]
<i>LATS2</i>	TSG: decrease expression in: malignant mesothelioma (and LOH) [136], NSLC (no mutation found) [137] and astrocytoma [134] OG: overexpression in AML [138], nosopharyngeal carcinoma [139]	TSG: downregulation in CRC [87]
<i>MOB1, Mob1</i>	Targeted by NS5A protein of hepatitis C virus [140]. Mouse KO: various cancer types developed [141]	TSG: downregulation in CRC [142]
<i>TAZ</i>	Mutations in Barth syndrome [143] OG: overexpression in breast cancer [144], HCC [145]	OG: overexpression in CRC [85]
<i>YAP1</i>	OG: overexpression in NSCLC [88], prostate [90], breast [91], gallbladder cancer [146] and glioma [89] TSG: decreased expression in breast cancer [92]	OG: overexpression in CRC [83], in CRC cases resistant to cetuximab [102] TSG: underexpression in CRC [79]

AML — acute myeloid leukaemia; CCL — cancer cell line(s); HCC — hepatocellular carcinoma; KO — knock out; LOH — loss of heterozygosity; NSCLC — non-small cell lung cancer; OG — oncogene; TSG — tumour suppressor gene

epinephrine or glucagon repress YAP1/TAZ activity [45, 46]. YAP1 could also be regulated by matrix stiffness independently from LATS1/2 phosphorylation. In cells cultured in soft matrix YAP and TAZ become phosphorylated even if all known LATS1/2 phosphorylation-dependent sites in YAP protein are inactivated by mutations [47, 48].

Hippo pathway cross talk with Wnt/ β -catenin and EGFR/KRAS signalling

The above presented ways of YAP1/TAZ activity regulation show the complexity of molecular inter-

actions within the Hippo pathway. Moreover, cross talks of the Hippo pathway with other signalling pathways like Notch, EGF or Sonic hedgehog have also been described [49]. Due to the space constraints they will not be discussed. However, in this review we focus on the relationships between Hippo and Wnt- β catenin signalling pathways because of their crucial role for the maintenance of intestinal epithelium homeostasis (Figure 2A). Additionally, we will also discuss cross talk between Hippo and EGFR/KRAS signalling pathways since their interactions may be involved in drug resistance during CRC chemotherapy (Figure 3) [50, 51].

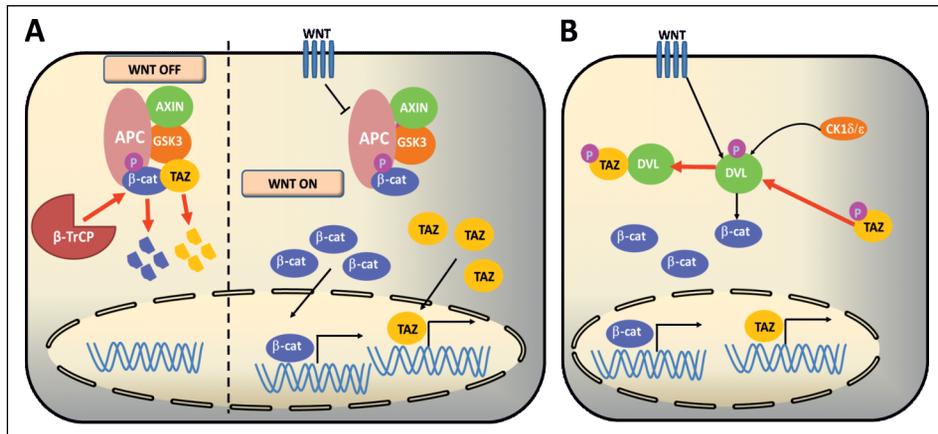


Figure 2. Interactions between Hippo pathway effectors TAZ and YAP and Wnt/ β -catenin pathway. **A.** When Wnt pathway is not triggered (WNT OFF), β -catenin and TAZ are inactivated by degradation *via* destruction complex. When Wnt is triggered (WNT ON), *e.g.* during tissue regeneration, the destruction complex is bound to Wnt/Frizzled and LRP5/6 resulting in the inhibition of either β -catenin or TAZ degradation. This leads to the accumulation of β -catenin and/or TAZ in the nucleus [38, 57–59]; **B.** Wnt/ β -catenin pathway is restricted by TAZ inhibition. On the contrary, YAP1 gene expression is enhanced by β -catenin/TCF4 complex [60]

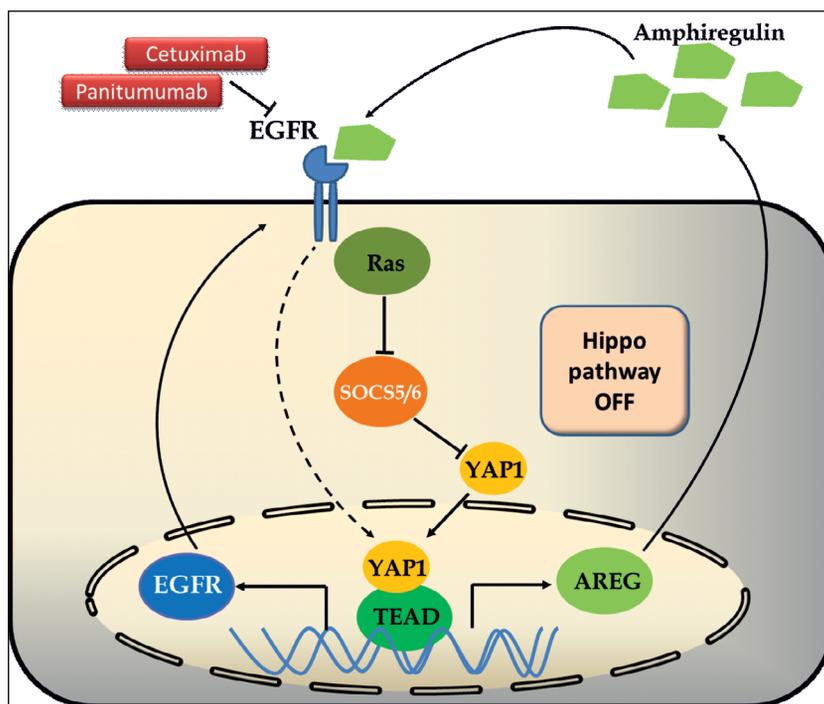


Figure 3. Indirect relationship between EGFR/KRAS and YAP. A positive autocrine regulation between EGFR and YAP1 is established *via* amphiregulin expression [50, 61]. Cetuximab and Panitumumab are humanized monoclonal antibodies against EGFR used in CRC therapy [61]

The interactions of the Hippo and Wnt signalling pathways

Organs grow up to pre-defined size and shape, which are controlled by different signalling pathways that receive external signals and translate them into respective cellular processes. Cell-cell junctions, cell

adhesion, cell polarity, growth factors or other small signalling molecules, like hormones and cytokines, control whether a cell should enter process of proliferation, apoptosis or differentiation. Several studies have emphasized the critical role of the Wnt/ β -catenin pathway for gastrointestinal (GI) tract homeostasis. Uncovering its interactions with Hippo pathway

increased our understanding of the mechanisms of intestinal epithelium homeostasis in physiology and pathology [38].

The effector of the Wnt signalling (WNTS) pathway is the transcriptional co-activator β -catenin [52]. WNTS ligands include many types of proteins that act as morphogens, controlling cell differentiation and proliferation. These molecules bind to cell membrane receptor complexes composed of Frizzled (FZD) and Lipoprotein receptor-related (LPR) proteins and thereby regulate the activity of β -catenin [38, 53, 54]. β -catenin plays a central role in several developmental processes, such as regulation of gene transcription [53], stem cell renewal [55] and epithelial-to-mesenchymal transition (EMT) [56]. When a cell does not receive WNT signals, β -catenin is maintained at low level in the cytoplasm through its degradation in AXIN destruction complex. This complex is composed of the scaffolding protein AXIN, the tumour suppressor *adenomatous polyposis coli* gene product (APC), casein kinase 1 (CK1) and glycogen synthase 3 (GSK3) [38, 53, 57, 58]. Due to sequential CK1 and GSK3 phosphorylation of β -catenin N-terminal region, the phosphorylated β -catenin could be recognized by β -TrCP, an E3 ubiquitin ligase subunit so that subsequent β -catenin ubiquitination leads to its proteasomal degradation (Figure 1 and 2A). Continuous elimination of β -catenin prevents it from nuclear translocation, and thereby Wnt target genes are repressed by the DNA-bound T cell factor/lymphoid enhancer factor, belonging to the TCF/LEF family of transcriptional factors [54].

Binding of Wnt ligand induces the formation of a complex composed of FZD receptor, LRP5/6 co-receptor and WNT ligand together with the recruitment of the scaffolding protein Dishevelled (DVL) [54]. In effect, since β -catenin is not degraded, it accumulates in the cytoplasm and migrates to nucleus, where it forms a complex with TCF/LEF what enables transcription of Wnt target genes [38, 53, 59].

In 2010 Varelas et al. found that activation of the Hippo pathway restricts Wnt/ β -catenin signalling by interaction of TAZ and DVL in the cytoplasm (Figure 2B) [60]. TAZ interaction with DVL inhibits CK1 binding and Wnt3A-induced DVL phosphorylation, thereby inhibiting Wnt3A-induced transcriptional response. Loss of TAZ in a cell culture and in the kidneys of *Taz*-null mice results in increased DVL2 phosphorylation, enhanced cytoplasmic and nuclear β -catenin accumulation and Wnt-target genes' induction [60]. Furthermore, the inhibition of the Hippo pathway activity leads to increased nuclear TAZ level so that reduced TAZ-DVL binding results in increased target gene expression in both pathways (Figure 2B) [60].

Hippo upregulates EGFR/KRAS pathway

Recent reports [50, 61–63] revealed that the Hippo pathway effector, YAP1 protein, is involved in the regulation of EGFR/KRAS signalling (Figure 3). During homeostasis, degradation of YAP1 is triggered by ubiquitin ligase complex substrate recognition factors SOCS5/6. It was found that during cancerogenesis EGFR-activated Ras, a small GTP-binding protein, downregulates SOCS5/6 expression, which increases level and half time of YAP1 in the cytoplasm [50]. Non-phosphorylated YAP1 may induce transcription of *EGFR* as well as *AREG* (amphiregulin) genes. Expression of the *EGFR* gene leads to up-regulation of the above-described positive feedback loop (Figure 3). Moreover, amphiregulin proteins (secretory products of *AREG* gene) bind to EGFR and act as autocrine growth factors. Such relationship between YAP1 and the EGFR pathway was found to be important in the progression of oesophageal cancer in patients treated with EGFR inhibitors [61]. Recently published results of knockdown of YAP1 and knockdown of ERBB3 study suggested presence of an autocrine loop between YAP1 and EGFR which may control ovarian cell tumourigenesis and cancer progression [63].

The role of the Hippo pathway in intestinal epithelial cells

Hippo pathway in intestinal epithelium homeostasis

The inner surface of the intestinal tube is lined with a single layer of epithelial cells that play both absorbing and secreting functions. Major differentiated cell types are enterocytes (which are involved in the uptake of nutrients), goblet cells (which produce mucus), enteroendocrine cells (which produce hormones) and Paneth cells. Except for Paneth cells, all cell types migrate as clonal lineages to the tip of the villus within 4–5 days, where they are shed into the lumen [64]. Post-mitotic Paneth cells are relatively long-lived (5–6 weeks) [65] and intermingle with intestinal stem cells (ISC) at crypt bottoms to secrete ISC niche factors and function in innate immunity. Colon has a simple columnar epithelium that lacks Paneth cells; instead, so called 'deep secretory cells' are thought to be responsible for the ISC niche function [64, 66]. Regardless of the differences in morphology and functions, epithelial cell types in intestinal crypts are organized into three compartments. All epithelial lining cells are derived from intestinal stem cells that occupy the base of crypts (1st compartment). ISC divide, migrate and start to differentiate into transient-amplifying cells in the middle of crypts height (2nd compartment).

Transient-amplifying cells finally differentiate into absorptive cells — enterocytes and secretory cells (goblet cells and enteroendocrine cells), which expand from the top third part of the crypt and surface epithelium to the tip of the villus or colon surface epithelium (3rd compartment) [67].

The base of intestinal crypts makes a niche for ISCs, where two types of stem cells can be found: cycling crypt base columnar cells (that cycle asymmetrically) and quiescent (+4) cells [68]. Leucine-rich repeat-containing GPCR5 (*Lgr5*) is a marker present on the crypt base columnar cells, that are intestinal stem cells or long-lived multipotent progenitor cells [68, 69]. It has been shown that *Lgr5* gene is a downstream target of Wnt pathway [69]. Single *Lgr5*+ stem cell can rebuilt an entire crypt-villus-like structure, but knock-out (KO) of *Lgr5* gene has no detectable effect on murine intestinal development, probably since other related markers may attain function of the *Lgr* protein [68, 70]. For (+4) quiescent cells there are currently no commonly-accepted receptors, however, *Bmi1* and *Musashi-1* have recently been proposed [71, 72]. *Bmi1* expression has been found in distinct cells located near the bottom of crypts, which comprise rarely occurring, slowly cycling cells located predominantly in the +4 position [71]. It was shown that *Bmi1*+ cells in the situation of conditional *Lgr5*+ cell deletion, can give rise to *Lgr*+ cells and support crypt-villus regeneration [71, 72]. On the basis of these and other findings a model in which *Lgr5*+ cells mediate homeostatic self-renewal, whereas *Bmi1*+ cells mediate injury-induced regeneration has been proposed [73].

Physiological level of Wnt signalling controls the rate of a crypt proliferation. The elevated level of Wnt signalling is a characteristic feature of ISC in intestinal crypts [64] whereas deregulated Wnt signalling can lead to colorectal cancer development [64]. R-spondin binds to LGR4/LGR5 receptors and enhances WNT signals in ISC, but tumour suppressor RNF43 activity negatively controls WNT signals in ISC by ubiquitinating FZD receptors [74, 75]. Furthermore, Paneth cells constitutively secrete Wnt3 ligand, but the additional source of Wnt proteins also exists in the surrounding stromal cells. Notch signalling in the intestinal epithelium is involved in inhibition of the secretory phenotype of the differentiating ISC [76].

The relationships between Hippo pathway and Wnt signalling suggest a role of the Hippo pathway in the maintenance of the intestinal epithelium homeostasis. The first reports concerning the Hippo pathway in intestinal epithelium suggested oncogenic role of YAP1. It was demonstrated that endogenous YAP1 is expressed in the base of crypts where the ISCs reside [77]. In the mouse model, when *YAP1*

was activated in a doxycycline-inducible manner, in contrast to control mice, cell proliferation upon *YAP1* activation was observed not only in the base of crypts but extended through the whole length of crypt's epithelium [77]. The experimental inhibition of YAP1's activity resumed differentiation of crypts' epithelial cells. Interestingly, YAP1 activation induced intestinal dysplasia and was associated with high level of nuclear β -catenin expression, characteristic to active Wnt pathway [77].

The role of Hippo pathway in the regeneration of intestinal epithelium

The role of YAP1 in tissue regeneration was first described in dextran sodium sulphate (DSS)-induced colon regeneration model. In normal tissue, YAP1 protein is present along the entire crypt in both types of cells: proliferating and post-mitotic [13]. Moreover, conditional *YAP1* KO in the intestinal epithelium revealed no visible defects in cell differentiation, cell death, cell proliferation or cell migration along the crypt-villus axis [13]. Five days of DSS treatment of wild-type mice resulted in intestinal inflammation and slightly decreased YAP1 protein level. However, two days after DSS withdrawal, YAP1 protein level dramatically increased. Increased YAP1 protein level was observed (by immunohistochemistry and WB) in regenerating crypts [13]. This study revealed the possibility that Hippo signalling may be constitutively activated during cell and tissue regeneration, however, during normal cell homeostasis YAP1 becomes inactivated. Moreover, the inactivation of tumour suppressors that normally restrict YAP1 function may lead to tumour formation and/or overgrowth of intestinal epithelium [13]. *E.g.*, conditional *Sav1* knock-out mice exhibited enlargement of crypts in both colon and small intestine with increased number of proliferating cells. Interestingly this dysplasia was completely diminished by loss of YAP1 [13].

The role of MST1 and MST2 kinases in the intestinal epithelium was also investigated [13, 78]. Ablation of MST1/2 kinases in mouse intestinal epithelium caused marked expansion of stem cell compartment and loss of secretory cells throughout small and large intestine. Decreased phosphorylation, enhanced abundance and nuclear localization of YAP1 were observed as a result of MST1/2 deletion in intestinal epithelium with simultaneous activation of Wnt and Notch signalling [78]. These results correlate with other data obtained upon activation of the nuclear form of YAP1 with mutated LATS1 phosphorylation site [13], because both of

these conditions lead to the Hippo pathway-independent activation of YAP1.

Divergent views on the role of YAP1 in intestinal tissue regeneration have emerged recently. It has been found that in strong cooperation with Wnt signalling the Hippo pathway controls the regeneration stage after injury. However, cytoplasmic YAP1 restricted elevated Wnt signalling independently of AXIN-APC-GSK complex, and in some cases acted synergistically with the destruction complex to control the subcellular localization of β -catenin [79]. In mouse overexpression of *YAP1* gene inhibited the Wnt-mediated intestinal regeneration and *vice versa* a loss of *YAP1* led to hyperactive Wnt signalling and expansion of stem cell niche during regeneration. It was found that YAP1 nuclear localization correlated with active Wnt signalling whereas its cytoplasmic localization inhibited Wnt pathway [79].

Control of injury-induced proliferation of cells is crucial for maintaining the proper number of cells in the regenerating organ. The insufficient proliferation may lead to atrophy, whereas the dysregulated proliferation may lead to tumourigenesis [80]. For this reason focusing on mechanisms involved in regeneration of such organs like intestine, that is permanently exposed to potentially injuring factors, may provide important clues to the pathomechanisms of uncontrolled cellular proliferation that characterizes neoplasms, including colorectal cancer (Figure 4).

The role of Hippo pathway in colorectal cancer

Although the most common mutations in CRC involve APC gene and dysregulated β -catenin signalling, YAP/TAZ can contribute to these mutations [81].

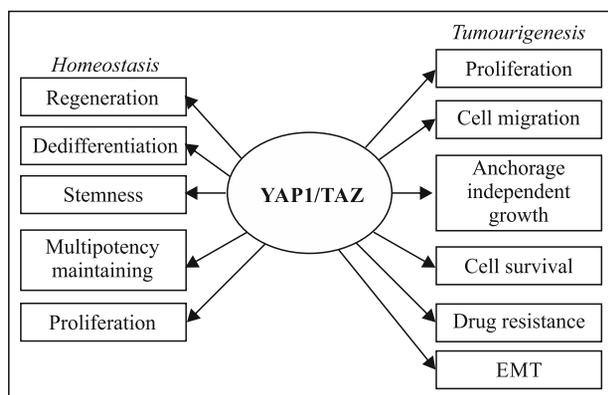


Figure 4. Summary of cellular functions of Hippo pathway effector proteins YAP1/TAZ in intestinal epithelium homeostasis and tumourigenesis

Therefore, expression studies of Hippo pathway components in CRC have been reported by some groups [78, 82–86], however, only Liang et al. presented complex data regarding collaborative expression analysis of the most important Hippo genes [87]. They found decreased mRNA ratios of *LATS1* and *MST1/2* as well as increased mRNA levels of *YAP*, *TAZ*, *TEAD* and *OCT4* in CRC in comparison to healthy colon [87]. Most of the genes coding for the Hippo pathway proteins were shown to function in many types of cancer either as tumour suppressors or as oncogenes (Table 2). The most commonly focused gene, *YAP1*, was found to be involved in tumour development and progression in different malignancies, including CRC. Although increased level of the YAP1 protein (assessed by WB) was found in most studies [83, 88–91] suggesting its oncogenic role, the under-expression was also observed in breast [92] and colorectal cancer [79]. The study of Barry et al. showed decreased expression of YAP1 in high grade tumours and stage IV [79]. The authors suggested that *YAP1* is a Wnt target gene which plays a role in a negative feedback loop to limit Wnt-initiated signals in CRC development [79].

This hypothesis sheds a new light on the possible role of the Hippo pathway in the Wnt-related pathomechanisms of the CRC development. As previously mentioned, AXIN destruction complex that contains APC protein tightly regulates β -catenin level in Wnt pathway (Figure 2A). Common mutations in *APC* gene are found in most CRC cases [81, 93, 94]. These mutations lead to an elevated β -catenin level in the cell nucleus where it acts as co-activator of transcription factors of the TCF/LEF family. The expression of the TCF4 transcription factor and the activation of Wnt target genes’ transcription by the β -catenin/TCF4 transcription complex were documented in colonocytes [52, 95]. It was proved that β -catenin is required for the *YAP1* gene expression in HCT116, an advanced CRC cell line [95]. *YAP1* gene silencing in SW620 (colon adenocarcinoma) and HCT116 (metastatic CRC) cell lines resulted in positive regulation of anchorage-independent cell growth, because decreased YAP1 level reduced growth of colonies in soft agar, especially of SW620 cells. YAP1 was detected in the cytoplasm of HCT116, SW620, SW480, RKO, LS174, and HT29 CRC cell lines, which suggests the properly functioning of Hippo pathway kinase cassette [95]. Moreover, WB protein analysis of nuclear and cytoplasmic fractions from cells grown in high confluence revealed that cell density did not affect YAP1 nuclear localization [95]. The analysis of YAP1/ β -catenin expression in primary and metastatic colorectal tumours revealed that out of the 36 primary tumours examined, 86% scored positively

for nuclear localization of β -catenin and YAP1, while only 3% lacked nuclear expression of either protein [95]. The distribution was comparable in metastatic CRC tumours, which suggested that Wnt/ β -catenin signalling pathway and Hippo/YAP1 pathway converge to promote colon cancer [95].

Expression of Hippo pathway components as prognostic factors in colorectal cancer

Increased expression of YAP1 in CRC was found by many groups [13, 46, 78, 96]. *E.g.*, Wang et al. showed that YAP was overexpressed in 52.5% (73/139) of CRC tumours with a predominant localization in the nucleus [97]. Furthermore, YAP1 protein expression in CRC patients correlated with cyclin D levels, nodal status and pTNM stage. The authors proposed that YAP1 may be a prognostic factor in CRC because of the correlation between its high expression and shorter overall survival [97].

Wang et al. also found that YAP1 and TAZ expression levels were significantly associated with the lymph node status in CRC [82]. It is known from other clinical and histopathological studies that have been performed for 25 years in a large surgical centre that the cut-off values of lymph node ratio are strong independent prognostic factors for CRC patients [98]. Therefore, high prognostic impact of lymph node metastases is a background of the statement regarding the role of YAP1 and TAZ in the prognosis of CRC patients [82].

Other reports showed the prognostic value of YAP1 and TAZ transcription levels for CRC patients. A positive correlation between TAZ and YAP1 mRNA expression ratios and their downstream target genes *AXL* and *CTGF* was found in tumour tissues of 522 CRC patients [85]. In the same study it was demonstrated that TAZ, but not YAP mRNA level can be used to predict survival of CRC patients [85]. Increased TAZ mRNA level may not necessarily correlate with the increase of its transcriptional activity since post-translational modifications like phosphorylation may cause its cytoplasmic sequestration. Therefore Yuen et al. proposed to associate TAZ expression with the expression of its two downstream genes, *AXL* and *CTGF*. Patients whose tumours overexpressed one, two or all of the three genes had an increasing risk for disease progression [85]. Based on microarray technique, it was also shown that target genes involved in EMT, migration and invasion, colon cancer progression, cancer signalling, angiogenesis and others factors engaged in tumourigenesis, presented differences in their expression level between high and low grade CRC patients which correlated

with *TAZ-AXL-CTGF* expression [85]. Two potential therapeutic targets, *ANO1* and *SQLE*, were also identified: in patients with upregulated *TAZ-AXL-CTGF* expression, a decrease in expression of either *ANO1* and *SQLE* or both genes was associated with the survival of *TAZ-AXL-CTGF* high grade colon cancer patients [85]. Although the identification of EMT markers can serve as potential prognostic CRC factors, the most recent data suggest caution when interpreting large scale transcriptome data from cancer due to contamination of tumour samples with stromal cells [99].

In a quantitative PCR study which focused on the expression of all Hippo elements in CRC, the mRNA levels of *MST1* and *LATS2* were decreased to higher extent in colorectal cancer tissues than in colorectal adenomas or adjacent non-tumour tissues (non-tumour tissues > adenomas > cancer) [87]. The mRNA expression levels of *YAP*, *TAZ* and *TEAD1* increased in colorectal cancer compared to colorectal adenomas and non-tumour tissues (cancer > adenomas > non-tumour tissues). Similar results were obtained on protein level by WB analysis. The clinicopathologic analysis revealed that expression levels of *MST1*, *LATS2* and *CDX2* (Caudal type homeobox transcription factor 2, marker of throphoectoderm which plays important roles in endoderm and intestinal development) in patients with lymph node metastasis were significantly lower than those in patients without lymph node metastasis. Finally the mRNA levels of *MST1*, *LATS2* and *CDX2* gradually decreased with the TNM stage (I > II > III > IV) and the mRNA levels of *YAP1*, *TAZ* and *TEAD1* gradually increased with TNM (IV > III > II > I) [87].

Associations of Hippo pathway with therapeutic strategies in CRC

Personalized therapy of advanced cancer was applied as a result of molecular progress in cancer cell biology. Another important reason for personalized therapy in CRC was the occurrence of chemoresistance of tumour cells in some patients when the traditional drug, 5-fluorouracil (5FU) was introduced in metastatic CRC therapy [100]. Recent reports on HT29 CRC cell line revealed high level of YAP in 5FU resistant cells. It was found that after exposing the cells to 5FU YAP became localized in cytoplasmic compartment of cells and phosphorylated causing the cells to enter quiescence. Increased YAP protein levels were also seen in human CRC liver metastases and were correlated with CRC relapse [43, 101].

The recently introduced biological drugs act directly on specific cellular targets, mainly growth

factor receptors. Chemotherapy based on modern molecular-targeting drugs like *Cetuximab*, *Regorafenib* or *Panitumumab* (humanized monoclonal antibodies against EGFR, CEGFR2-TIE2 and EGFR, respectively) showed some efficacy in advanced CRC, but there is only scarce data regarding targeted CRC therapy in respect to the Hippo pathway.

Lee et al. assessed the possible connection between cetuximab-treated CRC patients and *YAP1* mRNA expression; patients with CRC were divided into two groups: one group with activated *YAP1* in CRC (AYCC) and the second one with inactivated *YAP1* in colorectal cancer (IYCC). Statistical analysis revealed that patients with AYCC had slightly more advanced disease than had patients with IYCC (but patients with stage IV were excluded from this analysis). In the group of patients with stage I–III, the AYCC patients demonstrated much shorter survival rates than IYCC patients [102]. During analysis of all tumour stages patients (n = 80) who received cetuximab monotherapy, it was found that only in IYCC patients tumour shrinkage (complete or partial remission) occurred. *YAP1* activation was significantly associated with poor response to cetuximab therapy in colorectal cancer [102]. Although more studies are necessary, it appears that similarly as in head and neck cancer in which *YAP1* is a potential marker for cetuximab resistance [103], *YAP1* expression in colorectal cancer could be regarded as a potential marker of cetuximab therapy effectiveness.

Recently, the association of the EGFR/KRAS pathway with *YAP1* expression has been observed in drug-resistant oesophageal cancer [61]. Since the presence of drug-resistant CRC emerged as an important clinical problem [6, 104], we propose that a possible association of the *YAP1* expression with the EGFR signalling pathway in CRC should be also studied.

Conclusions

The Hippo pathway is an evolutionary conserved regulator of cell proliferation, death and differentiation. It controls organ size and tissue homeostasis and acts mainly through inhibition of the activity of *YAP1* and *TAZ* kinases, its major nuclear effectors. The activity of the Hippo pathway can be modulated by cross talk with other signalling pathways such as Wnt or EGFR/*KRAS* which are important in the control of intestinal epithelium's homeostasis. In the intestine, inhibition of the Hippo pathway activity results in stem cell expansion and neoplastic growth. Although core components and their influence on key nuclear effectors are well established, mechanisms that de-regulate the

Hippo pathway, are poorly understood. The available experimental and clinical data strongly suggest that the role of the Hippo pathway should be further explored under different pathological conditions such as cell/tissue injury or neoplastic transformation of intestinal epithelium. Moreover, the possible involvement of the Hippo pathway components in drug resistance of advanced CRC should also be investigated.

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