# UNIVERSITÀ DEGLI STUDI DI TRIESTE

## XXVII CICLO DEL DOTTORATO DI RICERCA IN BIOMEDICINA MOLECOLARE

# REGULATION OF YAP BY GLUCOCORTICOIDS

Settore scientifico-disciplinare: BIO/13


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## ABSTRACT

The Hippo signalling pathway is tumour suppressor cascade with a central role in the regulation of fundamental cellular biological processes, such as cell proliferation, apoptosis, organ size control and stem cell functions. The Hippo pathway transduces external signals that come to the cell into the nucleus, where it can control the expression of specific target genes, mainly involved in cell proliferation and differentiation. The Hippo pathway is an inhibitory pathway that control by phosphorylation and inhibition Yes-associated protein (YAP) coactivator, one of the two nuclear effectors of this signalling, involved in the regulation of proliferation and organ size.

As consequence, deregulation of Hippo tumor suppressor pathway or hyperactivation of its downstream effectors is often associated with formation, development and tumour dissemination.

Consistently, YAP is often over-expressed in a broad range of different tumours and it has aberrant activity in breast cancer as well as in several other human carcinomas. Up-regulation of YAP activity increases stem cell self-renewal in normal and cancer stem cells.

In this work we describe the identification of a new hormonal-dependent layer for YAP regulation in breast cancer by the glucocorticoids and we analyze the mechanisms through which this regulation occurs. We found that Glucocorticoid Receptor (GR) binds directly the YAP promoter and induces the transcription of YAP mRNA after GC stimulation in cancer cells. Moreover, GC lead to efficient YAP de-phosphorylation and transcriptional activation, in a transcription-independent manner, by inducing actin cytoskeleton reorganization.

Importantly, inhibition of the GR by means of RU486 (GR competitive antagonist) strongly blunted the expansion of the cancer stem cell pool in breast cancer cells by blunting the GR/YAP axis.

# INTRODUCTION

## 1.1 THE HIPPO PATHWAY

The Hippo signalling cascade is a highly evolutionally conserved tumour-suppressor pathway regulating tissue growth and cell fate (Harvey et al., 2013). It is important in the regulation of organ size by governing cell proliferation, stem cell properties, tissue regeneration and apoptosis (Hong et al., 2012). Following the first description in Drosophila melanogaster as an intrinsic mechanism that restricts organ size during development and that maintains tissue homeostasis throughout postnatal life, Hippo pathway has been found commonly deregulated in different type of cancers suggesting that altering Hippo signalling correlates with tumour initiation, progression and expansion (Cordenonsi et al., 2011).

The Hippo pathway integrates various upstream inputs from the plasma membrane into the nucleus, where it controls the transcription of several target genes that regulates cellular processes such as cell proliferation, differentiation and survival (Johnson and Halder, 2014).

## 1.2 THE HIPPO PATHWAY IN DROSOPHILA

In *Drosophila,* the first genes of the pathway isolated using the mosaic-based screens, were the tumour suppressor genes *warts* (wts), *hippo* (hpo) and *salvador* (sav). Loss of *wts* leads to robust cell-autonomous overgrowth in a variety of epithelial structures such as eyes, wings and legs (Pan, 2007). In 2002 Tapon et al. demonstrated that loss of *wts* or *sav* leads to increased cell proliferation and reduction in apoptosis showing the first evidence that these proteins regulate both processes. They additionally observed that loss of *wts* or *sav* is associated with increased levels of the cell cycle regulator Cyclin E (CycE) and the cell death inhibitor Diap1.

One year later the *Hpo* gene was identified, showing a similar loss-of-function overgrowth phenotype to that reported for *sav* or *wts* (Figure 1).

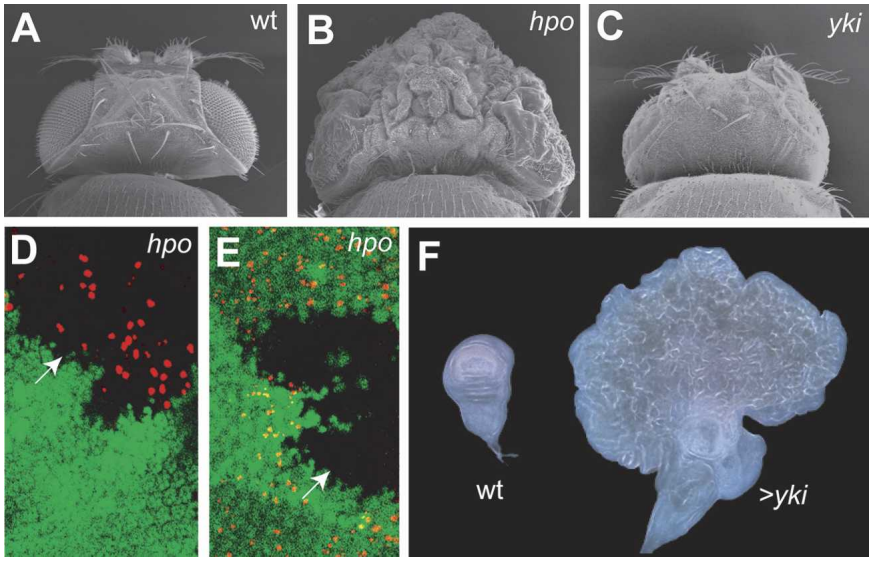



**Figure 1 The Hpo signaling pathway controls organ size in *Drosophila*.** Images from Huang et al. (2005). *A–C* show images of wild type (A) and flies in which *hpo* (B) or *yki* (C) function is specifically inactivated in the head. While inactivation of *hpo* leads to massive over- growth of the eye and head cuticles, inactivation of *yki* leads to the opposite phenotype. *D* and *E* show increased cell proliferation (D) and decreased cell death (E) in *hpo* mutant clones in the pupal eye. (D) While wild-type cells (green) had ceased cell proliferation (red), *hpo* mutant clones (black) continued to divide. (E) Conversely, normally occurring cell death can be detected in wild-type cells (green) but not in *hpo* mutant cells (black). *F* shows a wild-type wing imaginal disc (left) and a wing disc that overexpressed the *yki* gene (right). Yki overexpression leads to a dramatic increase in wing size (up to eight times the area of the wild-type wings) (Pan, 2007).

In 2005 Huang et al. identified the transcriptional coactivator Yorkie (Yki) as a critical substrate and downstream effector of Wts; this study demonstrates that Yki is the effector of the overgrowth phenotype observed in Hippo mutants. Yki is phosphorylated and inactivated by Wts. Overexpression of Yki recapitulates the loss-of-function *wts* phenotypes (increased *diap1* transcription and tissue overgrowth). Conversely, loss of *yki* leads to tissue atrophy and diminished *diap1* transcription, and genetic epistasis analysis placed *yki* downstream to *hpo, sav,* or *wts.*

## 1.3 THE HIPPO PATHWAY IN MAMMALS

Component of hippo signaling pathway are highly conserved during evolution (Pan, 2007) (Figure 2). In mammals the hippo core kinase is composed by the kinases Mst1 and Mst2 (mammalian STE 20-like protein kinase 1 and 2)(Harvey et al., 2003), Lats1 and Lats2 (the large tumour suppressor 1 and 2) (Hao et al., 2008; Oka et al., 2008; Zhang et al., 2008), Sav1 (the scaffolding protein Salvador homolog 1 which interacts with Mst1/2) and the adaptor proteins MOB kinase activator 1A (Mob1A) and

Mob1B (which interacts with Lats1 and Lats2, respectively).

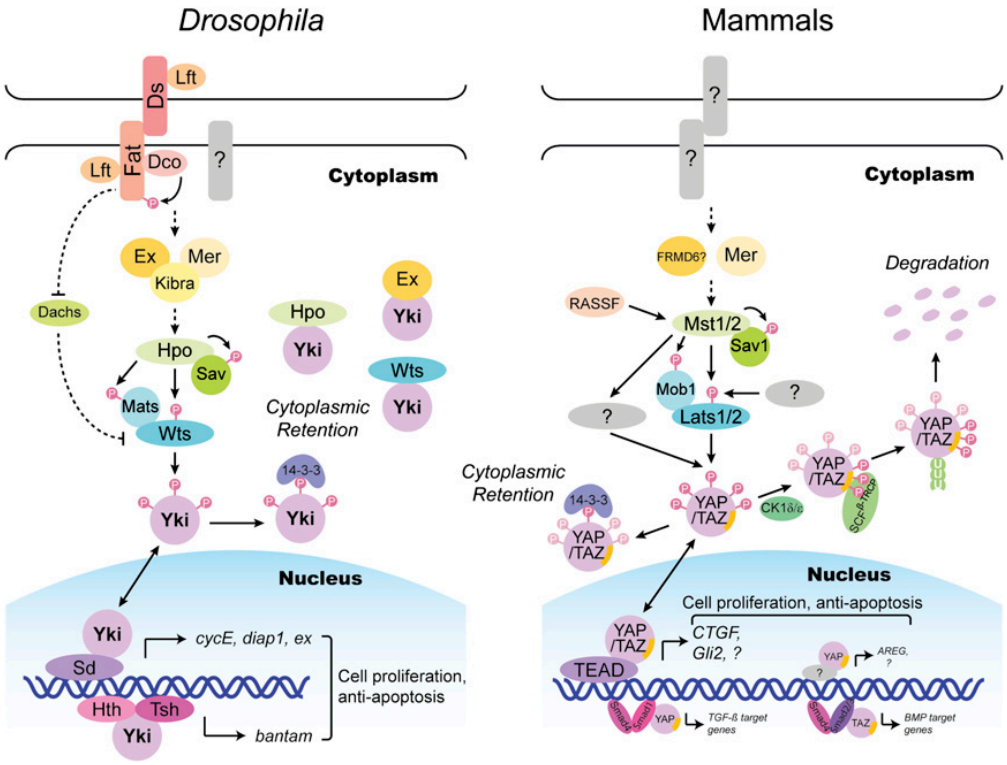



**Figure 2. Models of the Hippo pathway in Drosophila and mammals**. From Zhao et al., 2010.

When the Hippo pathway is active, MST kinases can bind to and phosphorylate Sav1, and this interaction enhances the kinase activity of Mst1/2. Together Mst1/2 and Sav1 phosphorylate and activate the two kinases Lats1 and Lats2 (Lats1/2) and Mob1; the latter, when phosphorylated, acts as a scaffolding protein and binds to Lats1/2, with an increase of their kinase activity. Last1/2 are regulated by a great number of proteins and among them, the most intensively studied is NF2 (Neurofibromin 2 or Merlin), which promotes Lats1/2 activation inducing their plasma membrane localization (Zhang et al., 2010). When activated, Lats1/2 and Mob1 cofactor, in turn, phosphorylate and inactivate their downstream targets: the transcriptional co-activator Yes-associated protein (YAP) and the transcriptional co-activator with PDZ-binding motif (TAZ) (Hong and Guan, 2012) (Figure 3).

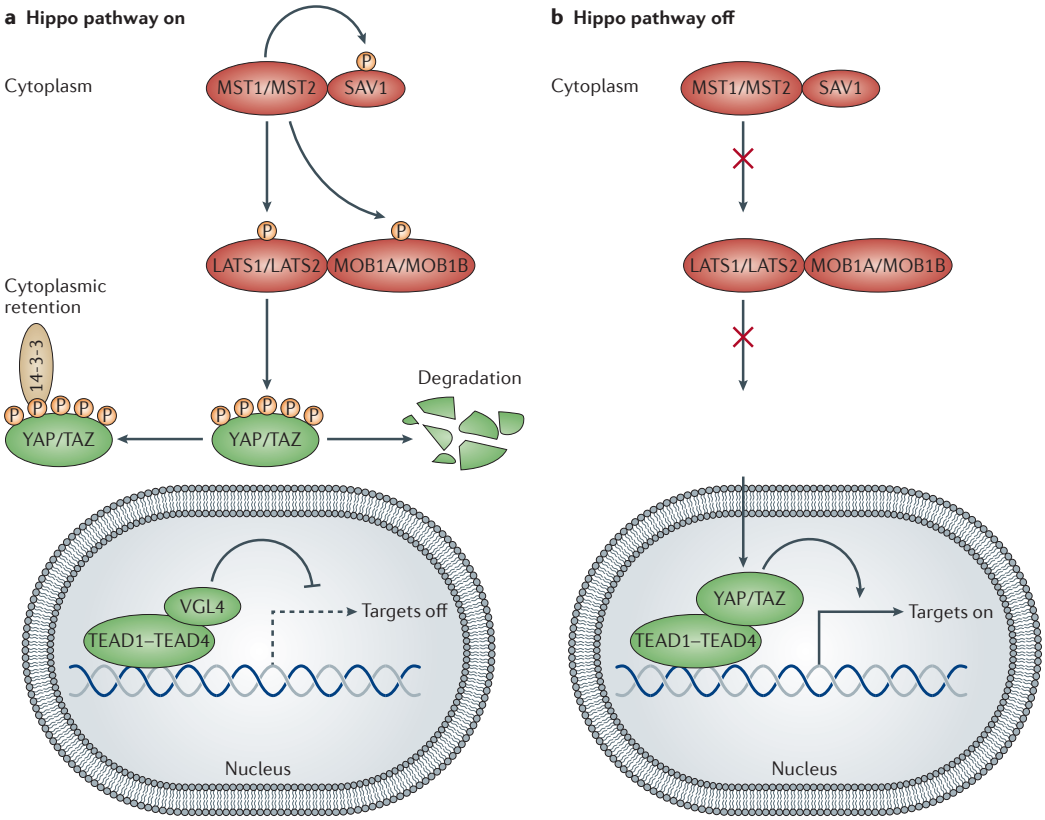



**Figure 3 The core of the Hippo signalling pathway and its mode of action**. Schematics of the core pathway components and how they interact are depicted. (a) When the Hippo pathway is on, mammalian STE20-like protein kinase 1 (MST1) or MST2 phosphorylate Salvador homolog 1 (SAV1), and together they phosphorylate and activate MOB kinase activator 1A (MOB1A), MOB1B, large tumour suppressor homolog 1 (LATS1) kinase and LATS2 kinase, which then phosphorylate Yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ). Phosphorylated YAP and TAZ are sequestered in the cytoplasm by the 14-3-3 protein and shunted for proteasomal degradation. As a result, the TEA domain-containing sequence-specific transcription factors (TEADs) associate with the transcription cofactor vestigial-like protein 4 (VGL4) and suppress target gene expression. (b) When the Hippo pathway is off, the kinases MST1, MST2, LATS1 and LATS2 are inactive, so YAP and TAZ are not phosphorylated and instead accumulate in the nucleus where they displace VGL4 and form a complex with TEADs, which promotes the expression of target genes. From Johnson, R. & Halder, G. The two faces of Hippo: targeting the Hippo pathway for regenerative medicine and cancer treatment. Nat. Rev. Drug Discov. 13, 63–79 (2014).

LATS1/2-mediated phosphorylation occurs on multiple residues (S61, S109, S127, S164, S381 in human YAP, and S66, S89, S117, S311 in human TAZ) inhibiting YAP/TAZ activity trough different mechanisms. Phosphorylated YAP on S127 or TAZ on S89 are retained in the cytoplasm by interaction with 14-3-3-proteins (Zhao et al., 2010). Moreover, phosphorylation of YAP on S381 or TAZ on S311 serves as priming event for successive phosphorylation by Casein Kinase (CK) 1 δ/ε and creation of a phosphodegron motif, that tags the proteins to E3 ubiquitin ligase $SCF^{\beta\text{-}TrCP}$ (β-transduction repeat-containing E3 ubiquitin protein ligase) recognition and

subsequent ubiquitin-mediated proteasomal degradation (Liu et al., 2010; Zhao et al., 2010). On the other hand, when the Hippo pathway is not active, YAP and TAZ are de-phosphorylated and accumulate in the nucleus where they drive target genes expression. However, since they lack any DNA-binding domain, YAP and TAZ control transcription by interacting with a range of DNA-binding transcription factors, such as the TEAD/TEF family transcription factors (TEAD1/2/3/4) and activate their target genes such as the connective tissue growth factor (CTGF) and the Cysteine-rich angiogenic inducer 61 (CYR61).

When TEADs proteins don't interact with YAP and TAZ, they form complexes with the Transcription cofactor vestigial-like protein 4 (VGL4), which represses target gene expression (Guo et al., 2013; Koontz et al., 2013).

Other transcription factors interacting with YAP and TAZ are the p53-family member p73, the Runt family members Runx1 and Runx2, Pax3, Pax8, the thyroid transcription factor-1 (TTF1), TBX5, the peroxisome proliferator-activated receptor γ (PPARγ), and SMAD1/2/3/4 (Piccolo et al., 2014).

## 1.4 MECHANISMS OF REGULATION OF THE HIPPO PATHWAY

In the last years the number of signals and mechanisms able to regulate the Hippo pathway is rising progressively. MSTs are regulated by a number of proteins including: TAO (thousand and one aminoacid protein) and MARK1 (MAP/microtubule affinity regulating kinase 1) which directly phosphorylates and activates MSTs (Johnson and Halder, 2014). KIBRA (kidney and brain protein) and Expanded are instead adaptor proteins for MSTs activity (Genevet et al., 2010). LATSs kinases are regulated by NF2, the tumour suppressor protein Merlin, which promotes LATSs activation by inducing their plasma membrane localization (Hamaratoglu et al., 2006).

YAP and TAZ are regulated by different signalling: extracellular factors, cell–cell adhesions, cell polarity and mechano-transduction. The Crumbs homolog complex (CRB) localizes to apical junction and regulates cell polarity (Johnson and Halder, 2014). Together with AMOT (angiomotin adaptor proteins) CRB inhibits YAP by promoting its cytoplasmic retention. Another regulator of cell polarity is Scribble,

which is required for the recruitment of MST and/or LATS to TAZ (Cordenonsi et al., 2011).

E-cadherin localization at adherens junctions suppresses the nuclear localization and activity of YAP by regulating MST kinase activity (Bhat et al., 2011). Moreover, the E-cadherin-associated protein $\alpha$-catenin regulates YAP by sequestering the YAP-14-3-3 protein complex in the cytoplasm (Schlegelmilch et al., 2011).

YAP and TAZ are also directly regulated by the extracellular matrix (ECM) and stiffness: cells that are grown on stiff ECM show high YAP/TAZ nuclear localization, whereas cells cultured on low stiffness display inactivated YAP and TAZ in the cytoplasm. This layer of regulation requires RHO GTPase proteins activity and tension of the actomyosin cytoskeleton. Although it is unclear how actin regulates YAP and TAZ, the small GTPase RHO-A protein is the prime regulator of this actin dependency (Dupont et al., 2011; Sorrentino et al., 2014).

The Hippo pathway is also regulated by GPCRs (G-protein coupled receptors) proteins; they transduce extracellular signals to the interior of the cell by using heterotrimeric G proteins that consist of α-, β- and γ-subunits. Among the Gα proteins, Gα11, Gα12, Gα13, Gαi, Gαo and Gαq can activate YAP and TAZ, whereas Gαs-coupled signals repress them. This regulation is mediated by LATS kinases (Yu et al., 2012). In addition to GPCRs, the cytokine receptor leukaemia inhibitory factor receptor (LIFR) is able to regulate YAP; in fact it has been shown that loss of LIFR in non-metastatic breast cancer cells induces migration, invasion and metastatic colonization through activation of YAP while restoring LIFR expression in highly malignant tumour cells suppresses metastasis by activating the Hippo kinase cascade that leads to phosphorylation, cytoplasmic retention and functional inactivation of YAP (Chen et al., 2012) (Figure 4).

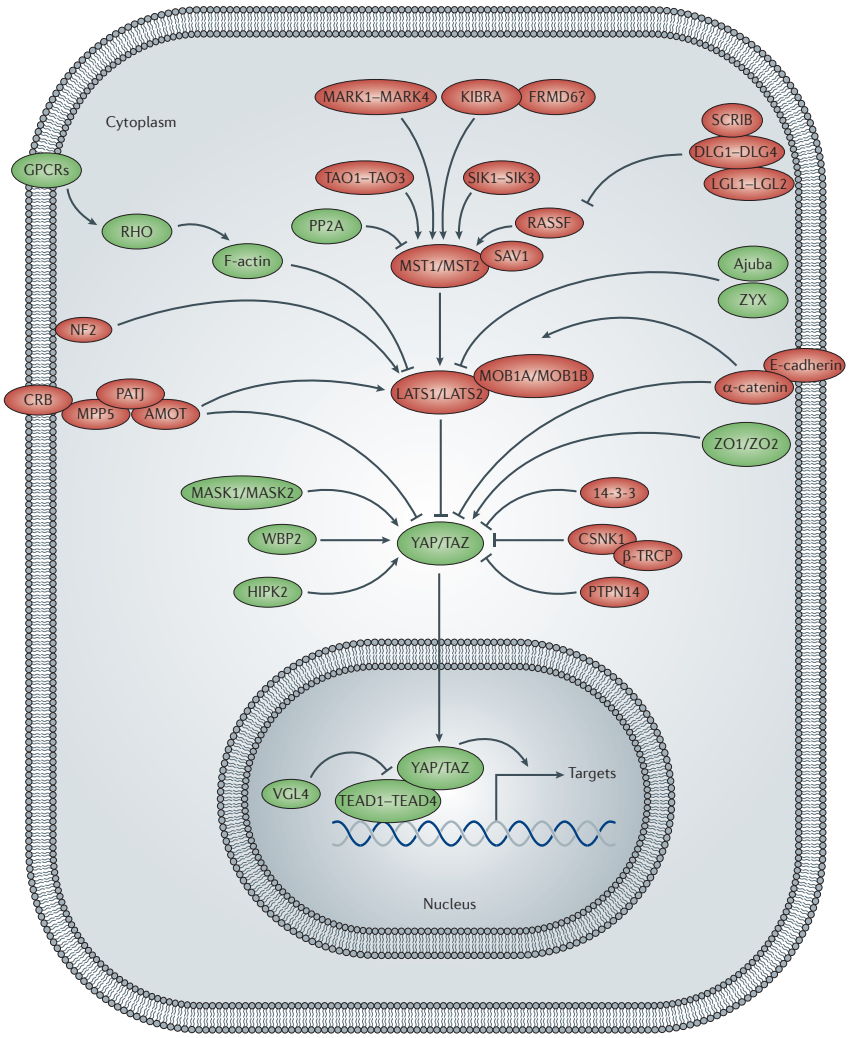



**Figure 4. The Hippo pathway network.** Mammalian Hippo pathway components that promote the activity of Yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ) are shown in green, whereas those that inhibit YAP and TAZ activity are shown in red. AMOT, angiomotin; β-TRCP, β-transducin repeat-containing E3 ubiquitin protein ligase; CSNK1, casein kinase 1; CRB, Crumbs homolog; DLG, discs large homolog; FRMD6, FERM domain-containing protein 6; GPCR, G protein-coupled receptor; HIPK, homeodomain-interacting protein kinase; KIBRA, kidney and brain protein; LATS, large tumour suppressor homolog; MARK, MAP/microtubule affinity-regulating kinase; MASK, multiple ankyrin repeats single KH domain-containing protein; MOB1A, MOB kinase activator 1A; MST, mammalian STE20-like protein kinase; NF2, neurofibromin 2 (also known as Merlin); PP2A, protein phosphatase 2A; PTPN14, protein tyrosine phosphatase, non-receptor type 14; RASSF, RAS association domain-containing family protein; SAV1, Salvador homolog 1; SCRIB, Scribble homolog; SIK, salt-inducible kinase; TAO, thousand and one amino acid protein kinase; TEAD, TEA domain-containing sequence-specific transcription factor; VGL4, vestigial-like protein 4; WBP2, WW domain-binding protein 2; ZO, zona occludens protein; ZYX, Zyxin protein. From Johnson et al., 2014.

In addition to these upstream regulators, there are several other proteins that modulate the activity of YAP/TAZ. Among them, Homeodomain-interacting protein kinase 2 (HIPK2), which promotes YAP abundance; 14-3-3 proteins, which mediate cytoplasmic YAP/TAZ retention after Hippo pathway activation; casein kinase 1 and β- TRCP, which mediate YAP/TAZ protein degradation; and finally protein tyrosine

phosphatase non-receptor type 14 (PTPN14), which promotes the nucleus-to-cytoplasm translocation of YAP during contact inhibition (Johnson and Halder, 2014). YAP and TAZ regulate also tumour cell niches by modulating cell–cell and cell–matrix interactions through the production of secretory proteins, such as cysteine-rich angiogenic inducer 61 (CYR61), amphiregulin (AREG; an epidermal growth factor family member) and connective tissue growth factor (CTGF).

## 2.1 YAP AND TAZ PROTEIN STRUCTURE

The Yes-associated protein (YAP) transcription co-activator is a key regulator of organ size and development (Dong et al., 2007). The human YAP gene, located at 11q22, can be transcribed into at least two major isoforms of that are derived by differential splicing (Sudol et al., 2012). These are YAP1, containing one WW domain and YAP2, containing two WW domains. Molecular structure of YAP is composed by an N-terminal proline-rich domain, a TEAD-binding region, WW domains, an SH3-binding motif, a coiled-coil domain, a transcription activation domain and a C-terminal PDZ-binding motif (figure 5).

YAP mRNA is expressed in a wide range of tissues, except peripheral blood leukocytes (Zhao et al., 2010). Since it lacks of a DNA-binding domain, YAP controls gene expression modulating the activity of some transcription factors. The WW domains reflect the sequence motif containing two conserved tryptophan (W) residues and they have been shown to bind PPXY motif.

Two proteins binding to the WW domain of YAP have been identified as interacting partners: WBP-1 and WBP-2 (WW domain Binding Protein). Moreover, several transcription factors such RUNX, ErbB4 cytoplasmic domain, and Smad1 contain the PPXY motif and could be potential targets for YAP, although none of them has been shown to mediate the growth-promoting function of YAP.

Phosphorylation of YAP by the Hippo pathway leads to its sequestration in the cytoplasm and/or to its proteasomal degradation. Therefore, the Hippo pathway regulates YAP by both spatial (nuclear-cytoplasmic translocation) and temporal (degradation) mechanisms. Mechanistically, tumour suppressor kinases LATS1/2 directly phosphorylate YAP at five serine/threonine residues (defined by the

consensus HxRxxS). Mutation in these serine residues makes YAP insensitive to inhibition by the Hippo pathway; in fact the most common used mutant-YAP protein expresses serine to alanine mutations in all these LATS phosphorylation sites (Piccolo et al., 2014).

TAZ, also known as WWTR1 (WW-domain containing transcriptional regulator 1, WWTR1), was first identified as a 14-3-3 binding protein. Sequence analysis revealed that TAZ shares homology with Yes associated protein (YAP), previously identified as a binding partner of the SH3 domain of the Src-family kinase Yes. Both TAZ and YAP contain WW domain, a 14-3-3 binding motif, a coiled-coiled motif in the transactivation domain and a PDZ-binding motif in the C-terminal (Liu et al., 2011) (Figure 4). Those motifs and domains are critical for regulating TAZ function.

TAZ has been shown to interact with and regulate multiple transcription factors, such as Runx2 (runt-related transcription factor 2) (Cui et al., 2003), PPAR perioxisome proliferator-activated receptor PPAR) (Hong et al., 2005), TBX5 (T-box transcription factor 5, TBX5) (Murakami et al., 2005), TEADs (TEA domain family members, TEAD) (Chan et al., 2009), TTF-1 (thyroid TF1, TTF1), PAX3 (paired box homeotic gene 3, PAX3).

TAZ phosphorylation at Ser89 mediated by LATS generates a 14-3-3 binding site and promotes TAZ its sequestration in the cytoplasm (Lei et al., 2008).

TAZ contains four consensus HxRxxS motifs. Besides Ser89, LATS kinase also phosphorylates TAZ at Ser 66, Ser117 and Ser311. TAZ is a very unstable protein with a half-life of 2 hours, indicating that protein degradation is the main route for TAZ inhibition (Piccolo et al., 2014).

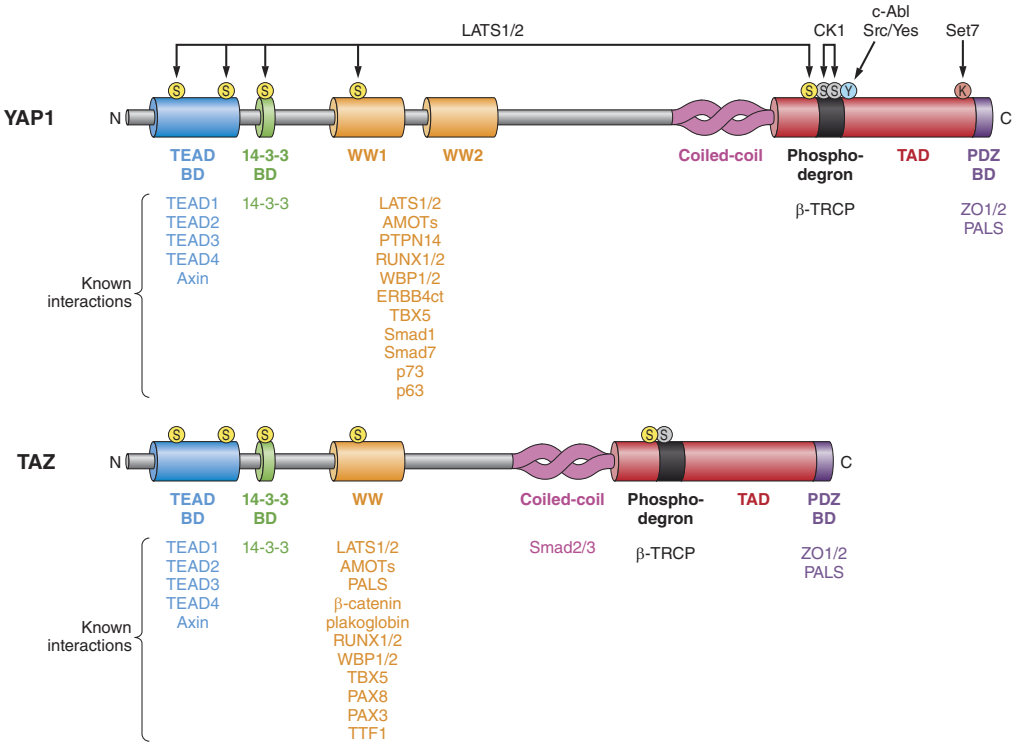



**Figure 5. Schematic representation depicting the multiple domains of YAP and TAZ, the mapped interactions with other proteins, and the residues targeted by post-translational modifications**. The five serines of YAP and the corresponding four serines of TAZ that are targeted by LATS1/2 phosphorylation are shown in yellow, the CK1 phosphorylation sites on both proteins are shown in gray, and the c-Abl phosphorylation site on YAP is shown in cyan. The lysine residue of YAP targeted for methylation by Set7 is also shown. TEAD BD is the TEAD binding domain. 14–3-3 BD is the domain that binds 14–3-3 proteins upon phosphorylation by LATS1/2. TAD is the transcriptional activation domain. PDZ BD is the small COOH-terminal domain able to interact with proteins bearing PDZ domains. From Piccolo et al., 2014.

## 2.2 YAP FUNCTION IN ORGANS AND TISSUES

YAP and TAZ have very important biological functions in several tissues. One of the most important role of YAP and TAZ is the control of the organ size during development. In particular YAP overexpression in mice liver is sufficient to induce a four-fold increase in liver mass due to proliferation of mature hepatocytes; this also leads to the acquisition of biliary duct/liver progenitor cell traits by the hepatocytes (Piccolo et al., 2014). This phenotype is dependent on TEAD-mediated gene responses, in fact crossing TRE-TEAD2-DN (TEAD2- dominant-negative under the control of a tetracycline-responsive element) transgenic mouse model with mouse model in which human YAP protein is overexpressed in a liver-specific manner lead to hepatomegaly and tumorigenesis suppression driven by YAP overexpression as shown in figure 6 (Liu-Chittenden et al., 2012). Moreover Liu-Chittenden and

collegues identified a small-molecule inhibitor, verteporfin, that was able to disrupt YAP/TEAD interaction open the possibility that inhibiting TEAD–YAP interaction is a pharmacologically viable strategy against the YAP oncoprotein function.

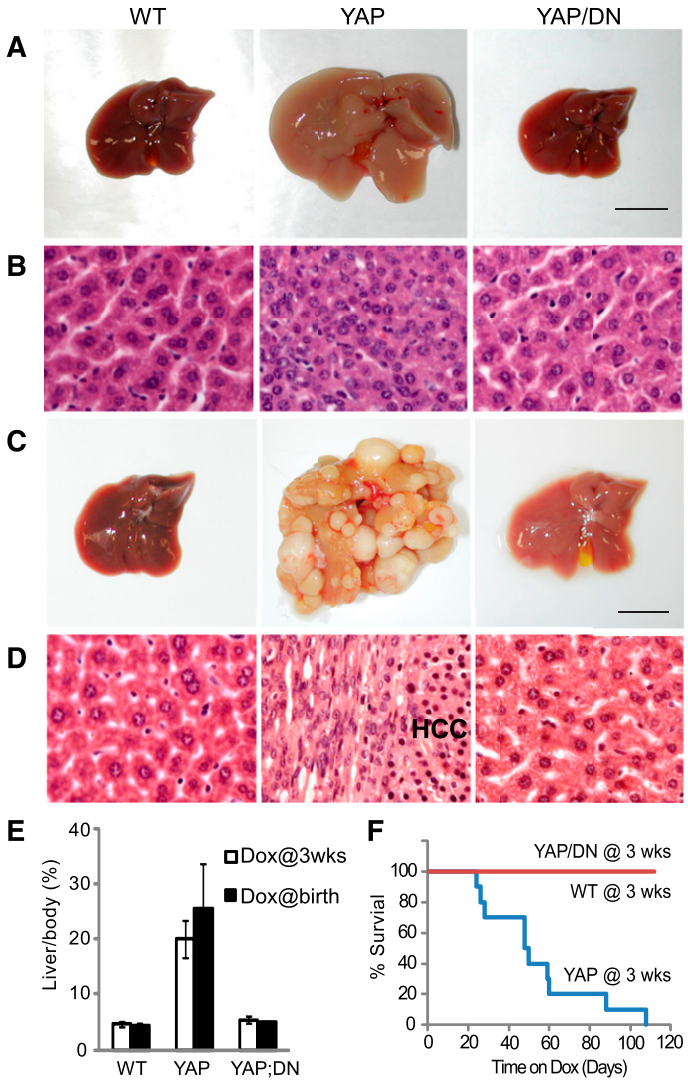



**Figure 6. TEAD2-DN suppressed hepatomegaly and tumorigenesis driven by YAP overexpression.** (A,B) Whole amount (A) and hema- toxylin/eosin (H&E) staining (B) of livers from wild-type (WT), YAP, and YAP/TEAD2-DN mice treated with 0.2 g/L Dox for 2 wk starting at 3 wk of age. Bar, 1 cm. (C,D) similar to A and B except that mice were treated with 1 g/L Dox for 8 wk starting at birth. (E) Quantification of liver-to-body weight ratio for animals analyzed in A and C. Values are mean 6 SEM; n $ 3 for each data point. (F) Survival curves of wild-type, YAP, and YAP/TEAD2-DN mice subjected to 0.2 g/L Dox treatment starting at 3 wk of age. From Liu-Chittenden et al., 2012).

On the contrary YAP liver specific inactivation leads to reduction in hepatocyte proliferation, defective bile duct morphogenesis and increase in apoptosis. As a consequence, mice display a mild liver enlargement, steatosis, and progressive fibrosis (Zhang et al., 2010). *YAP* liver conditional knockout mice display decreased duct cell proliferation and enhanced parenchymal damage, suggesting a positive role

for YAP in liver regeneration (Bai et al., 2012).

These studies reveal an important role for Hippo/YAP signaling in liver biology.

Recently it was demonstrated that YAP plays an important role in cardiomyocytes proliferation; in fact its deletion in embryonic cardiomyocytes leads to a reduction in cell proliferation and in heart hypoplasia while YAP overexpression increases heart size (Xin et al., 2013). Deletion of *YAP* postnatally was associated with progressive dilated cardiomyopathy: in this case it has been demonstrated a specific role of YAP and TAZ in heart disorders because the inactivation of both *TAZ* and *YAP* accelerated the beginning of the cardiac disease, and the complete *YAP/TAZ-null* hearts became unable to sustain postnatal life. Moreover, it was shown that in adult heart overexpression of activated YAP induced the regenerative response to myocardial infarction (Del Re et al., 2013).

These evidences open a new window for the development of new therapeutic approaches that, by inhibiting Hippo kinases, could facilitate heart regeneration in patients suffering cardiac damage.

Recently a key role of YAP in tissue regeneration of intestinal epithelial regrowth following injury has emerged. Elevated YAP expression is seen in mice treated with dextran sodium sulphate (DSS), a chemical that results in injury and inflammation of the large intestine and initiation of regenerative response. Mice depleted for YAP in the colonic epithelium do not show defects in intestinal homeostasis but are unable to efficiently undergo a regenerative response following DSS treatment, indicating a role for Hippo signalling in repressing regenerative responses that involve stem cell activation (Cai et al., 2010).

Barry and colleagues demonstrated that upon whole-body irradiation, YAP-deficient mice showed crypt hyperplasia and overgrowth in both small intestine and colon.

The authors confirmed the growth-suppressive function of YAP by stimulation with the Wnt agonist R-spondin1 (Rspo1), a potent growth factor for intestinal crypts. Loss of YAP increased Wnt/Rspo1 hypersensitivity and induced massive hyperplasia, which was accompanied by upregulation of Wnt targets and intestinal stem cell markers. Together, these data suggest an opposite roles of YAP in the intestine: on one hand YAP serves as an oncoprotein, promoting growth upon DSS-induced intestinal damage, on the other YAP acts as a growth-repressive protein that restricts

Wnt/Rspo1-induced intestinal stem-cell expansion and regeneration after irradiation-induced injury.

Overexpression of activated YAP can specifically expand the epidermal stem cell compartment in the basal layer of the epidermis while *YAP* deletion from the basal layer of the embryonic epidermis, shows reduced stratification caused by reduction in keratinocyte proliferation and reduced stem cells self-renewal in mice (Schlegelmilch et al., 2011). Cottini et al. in 2014 demonstrated a role for YAP1 as a tumor-suppressor gene in hematological cancers. In fact, they observed that although nuclear ABL1 triggers cell death through its interaction with the Hippo pathway coactivator YAP1 in normal cells, this type of tumours escape apoptosis preventing nuclear ABL1-induced apoptosis as a result of genetic inactivation or reduced expression of the Hippo transcriptional cofactor *YAP1;* in this study they proposed a new synthetic-lethal approach in which inhibition of the kinase STK4, that normally reduce YAP levels, reactivates YAP1 and triggers ABL1-dependent apoptosis, providing the rationale for developing STK4 inhibitors for clinical evaluation in haematological malignancies (Cottini et al., 2014).

## 2.3 THE HIPPO PATHWAY IN HUMAN CANCERS

Consistent with the critical role of the Hippo signalling in growth control, widespread dysregulation or mutations in the Hippo pathway are associated in a variety of human carcinomas. However, the mechanisms that lead to Hippo pathway deregulation and YAP/TAZ activation in human cancer are still not well understood.

In general, components of the Hippo pathway can be divided in tumour suppressors and tumour promoters: while the core module members Mst1/2, Sav1, Lats1/2 and Mob1 and upstream regulators are mostly involved in tumour suppressive functions, YAP and TAZ and their activators have mainly been described to have oncogenic roles. Abnormally elevated levels and nuclear localization of YAP and TAZ, indeed, have been reported in many human cancers, including breast, liver, lung, skin, colon and ovarian cancers (Harvey et al., 2013). In line with this, preclinical studies have already described similar phenomena in murine tissue: transgenic expression of YAP in mouse liver results in a dramatic increase of liver mass in a reversible manner,

hyperplasia, and eventually leads to tumour formation (Camargo et al., 2007; Dong et al., 2007), confirming the important role of YAP in organ size regulation and tumorigenesis (figure 7). Moreover, in mouse models, YAP activation is able to drive expansion of multipotent undifferentiated progenitor cells and to induce severe dysplasia along the entire intestinal epithelium (Camargo et al., 2007).

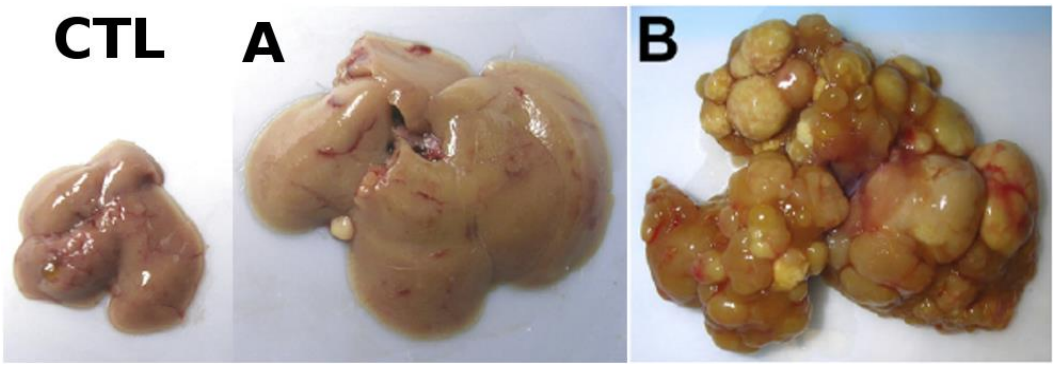



**Figure 7. YAP induces organ growth and tumour formation in vivo**. A normal mouse liver (control = CTL) and transgenic YAP over-expressing mouse livers after 4 weeks (A) or for 3 months (B) from birth. Note the increased in liver size (after 4 weeks - A) and the widespread development of hepatocellular carcinoma throughout the liver (after 3 months - B). Modified from Pan, D. The hippo signaling pathway in development and cancer. Dev. Cell 19, 491–505 (2010) and from Dong, J. et al. 2007).

Despite the fact that Hippo activity seems to be frequently deregulated in different human cancers, most of the Hippo pathway genes are not commonly mutated (Harvey et al., 2013), with the exception of NF2/Merlin gene. NF2 is a tumour-suppressor protein that controls cell growth and contact- dependent inhibition of proliferation and that has been characterized as a Hippo regulator (Zhang et al., 2010). NF2 gene is mutated with high frequency in neurofibromatosis type 2, a familiar cancer syndrome characterized by the development of malignant peripheral nerve sheath tumours (Asthagiri et al., 2009). Moreover, inactivation of NF2 gene is also frequently observed in malignant pleural mesothelioma (Bianchi et al., 1995). Although mutations of other Hippo pathway components in human cancer are less common, evidence supports a role of this pathway in human tumorigenesis: down-regulation of Mst1/2, Sav1, Lats1/2 and Mob1 (tumour suppressor components of the pathway) has been reported in various human cancers by many authors (Pan, 2010; Zhao et al., 2010).

However, the fact that the core components of the Hippo pathway are essentially

unaffected by mutations, suggests that mechanisms other than direct mutations lead to the aberrant activation of YAP and TAZ in cancer, such as a metabolism-related mechanism, as recently reported (Sorrentino et al., 2014) (Wang et al., 2015; Mo et al., 2015), or hormonal mechanisms. Since YAP is the major downstream effector of the Hippo pathway, it is not surprising that it functions as a tumour promoter: over-expression of YAP in human non-transformed mammary epithelial cells results in phenotypic alterations that are hallmarks of cancer transformation, including growth factor-independent proliferation, suppression of apoptosis and EMT (Overholtzer et al., 2006; Zhao et al., 2008). Amplification of YAP gene locus has been observed in several human cancers, such as intracranial ependymomas, medulloblastomas, oral and oesophageal squamous cell carcinomas, non-small cell lung cancer and ovarian cancer (Hong and Guan, 2012). Consistently, elevation of YAP protein levels has been identified in many human cancer cell lines and primary tumours (Harvey et al., 2013). Moreover, it has also been reported that high expression of YAP as well as deregulation of the Hippo pathway often correlate with poor patient prognosis (Harvey et al., 2013); for instance, YAP is an independent prognostic marker for overall survival and disease-free survival for patients with hepatocellular carcinoma (Xu M. Z. et al. 2009).

Importantly, emerging evidence suggests that the Hippo pathway can modulate its effects on tissue size by the direct regulation of stem cell proliferation and maintenance (Mo et al., 2014; Ramos and Camargo, 2012). In particular, recent studies have reported that YAP and TAZ are implicated in stem cell and progenitor cell self-renewal and expansion, as well as in embryonic development (Camargo et al., 2007; Cao et al., 2008; Lian et al., 2010).

## 2.4 THE HIPPO SIGNALLING AND STEM CELL PROPERTIES

The association of the Hippo signalling with stem cell properties has been recently extended to include cancer stem cells (CSCs). According to the model that described CSC characteristics, tumour has a phenotypic heterogeneity in its cell population: it is composed by a small pool of cancer cells, the cancer stem cells, that exhibit the ability of self-renewal and unlimited growth, and from which all the other mature

neoplastic cells, with only limited capacity to divide and survive, originate (Clarke et al., 2006; Nguyen et al., 2012; Schulenburg et al., 2010). Indeed, a cancer stem cell is able to divide both in a symmetric and asymmetric way: through the former division it can expand the cancer stem cell pool that remains undifferentiated, while through the latter division it creates a daughter cell (multipotent progenitor) that exits the stem cell state and differentiates into a specialized end cell that makes up the bulk of the tumour and that has limited proliferative capacity and thus limited survival. Although CSCs are only a minority of the whole tumour, they are thought to be the real driving force of the disease. Indeed, conventional chemotherapy and radiotherapy are effective against bulk population of tumour cells, while they have a relatively poor effect on CSCs (Dean et al., 2005; Diehn et al., 2009). As a consequence, even if the bulk of the tumour is eliminated, few CSCs that survive can regenerate the tumour and lead to tumour recurrence.

In this regard, TAZ has recently been shown to be a stimulator of CSC traits and to be a central mediator of metastatic ability and chemo-resistance of breast cancer stem cells (Cordenonsi et al., 2011) (Bartucci et al., 2015). Indeed, over-expression of TAZ can lead to the acquisition of the mesenchymal phenotype in mammary epithelial cells, a feature called epithelial to mesenchymal transition (EMT) that is commonly observed in high-grade tumours and that correlates with metastatic spreading (Hanahan and Weinberg, 2011).

Moreover, hyper-activation of TAZ contributes to chemotherapy resistance (Lai et al., 2011) which, as said, is a peculiarity of CSCs. Finally TAZ resulted to be over-expressed in about 85% of poor- differentiated breast cancer (G3) and to correlate with poor prognosis in patients (Cordenonsi et al., 2011). Other studies have also shown that nuclear TAZ is highly expressed in high-grade glioblastomas (Bhat et al., 2011). Ectopic expression of TAZ leads to increased invasion, self-renewal, and tumour initiating capacity to generate properties similar to mesenchymal-like stem cells. Collectively, it is clear that TAZ enhances the self-renewal capacity and tumorigenic potential contributing to both the initiation and the progression of breast cancer and glioma.

Instead, poor data about YAP contribution in promoting CSC characteristics have been reported until now. YAP has been reported implicated in stem cell features of

neuroprogenitor cells and embryonic stem cells and activation of YAP expands multipotent progenitor cells leading to loss of differentiation in the pancreas and in the stem cell compartment of the intestine. Recent studies have provided additional evidence for the role of YAP in stem cell traits. According to these data, YAP acts as an essential downstream effector of the oncogenic KRAS signalling and it promotes resistance development and tumour recurrence in pancreatic adenocarcinoma (Kapoor et al., 2014) and lung adenocarcinoma (Shao et al., 2014). In these KRAS-dependent cancer models, indeed, YAP has emerged to be able to bypass loss of oncogenic KRAS signalling through regulation of the EMT-like transcriptional program. Moreover, a study has shown that YAP confers stem cell phenotypes to airway epithelial cells, thus regulating airway epithelial size and architecture (Zhao et al., 2014). Indeed, up-regulation of YAP increases stem cell self-renewal and blocks terminal differentiation and, consistently, YAP overexpression in differentiated secretory cells causes them to partially reprogram and adopt a stem cell-like identity. Furthermore, another study has highlighted that YAP activation is able to de-differentiate hepatocytes into ductal cells bearing characteristics of hepatic progenitors, such as self-renewal capability (Yimlamai et al., 2014), confirming a positive role of YAP in stemness. Only recently, however, YAP has been described as a major determinant of CSC properties onto a wide variety of non-transformed cell types of gastrointestinal origin, including oesophageal epithelium cells, immortalized embryonic liver cells, as well as in oesophageal cancer cells (Song et al., 2014). All together, these observations suggest that the ability of YAP to maintain stem cell property and to confer CSC traits might be a relevant part of the mechanism responsible for its oncogenic behaviour.

In summary, the Hippo pathway is strongly linked to tumorigenesis and provides several novel potentials targets for emerging therapeutic strategies. Deregulation and/or hyper-activation of YAP and TAZ functions have been described in a broad range of different human carcinomas and their activity promotes multiple cancer cell phenotypes. Thus, it is reasonable to consider YAP and TAZ as direct or indirect potential targets for new anticancer therapies. Several preclinical data support this idea: experiments performed with human cancer cell lines have proved that reduction of YAP and TAZ levels leads to decreased cell proliferation *in vitro* and *in*

*vivo* in xenograft assay (Diep et al., 2012). Consistently, it seems possible that targeting the main down-stream effectors of the Hippo pathway, YAP and TAZ, might inhibit characteristics of cancer cells at many levels.

## 3.1 GLUCOCORTICOIDS SIGNALLING

Glucocorticoids (GCs) are a class of Corticosteroid hormones that are either secreted from the *zona fasciculata* of the adrenal gland during exposure to acute and chronic stress or administered pharmacologically mainly in clinical setting of inflammation. Natural Glucocorticoids (Cortisol in human) are cholesterol-derived hormones whose synthesis and relapse are under circadian and stress-associated regulation by the hypothalamic-pituitary-adrenal (HPA) axis (Biddie et al., 2012). Furthermore, the availability of natural GCs in tissues is regulated by corticosteroid-binding globulin in serum and by locally expressed 11β-hydroxysteroid dehydrogenase (11β -HSD) enzymes. Two iso-enzymes are responsible for regulating local cortisol levels highly expressed in key metabolic tissues including liver, adipose tissue, and the central nervous system: 11β-HSD type I, which converts inactive Cortisone to active Cortisol, and 11β-HSD type II, which is responsible for the reverse reaction that inactivates cortisol (Draper and Stewart, 2005).

GCs play a central role in critical biological processes that are necessary for life, such as control of intermediary metabolism, cell growth, apoptosis and differentiation, immune and inflammatory reactions, as well as central nervous system and cardiovascular functions (figure 8). Imbalance in glucocorticoid levels such as chronic elevation or deficiency can result in pathological conditions, such as Cushing's disease and Addison's disease, respectively (Kadmiel and Cidlowski, 2013) (Vegiopoulos and Herzig, 2007). Synthetic Glucocorticoids resemble natural Glucocorticoids but differ from the latter by their potency and metabolic clearance and also because synthetic GCs do not bind corticosteroid-binding globulin and are thereby not susceptible to their regulation.

Prednisone/Prednisolone, Betamethasone, Dexamethasone, Fluticasone and Budesonide are among the most commonly prescribed GCs and due to their potent anti-inflammatory effects, recommended for a variety of medical conditions such as:

chronic inflammatory disorders including asthma, rheumatic disease and skin infections as well as for immunosuppression in patients undergoing organ transplantation. In addition to their anti-inflammatory properties, Corticosteroids have been exploited for their anti-proliferative, pro-apoptotic and anti-angiogenic actions for the treatment of haematological cancers (Frankfurt and Rosen, 2004; Vilasco et al., 2011).

Both natural and synthetic GCs mediate their effects on target cells by binding to their intracellular receptor, the human Glucocorticoid Receptor.

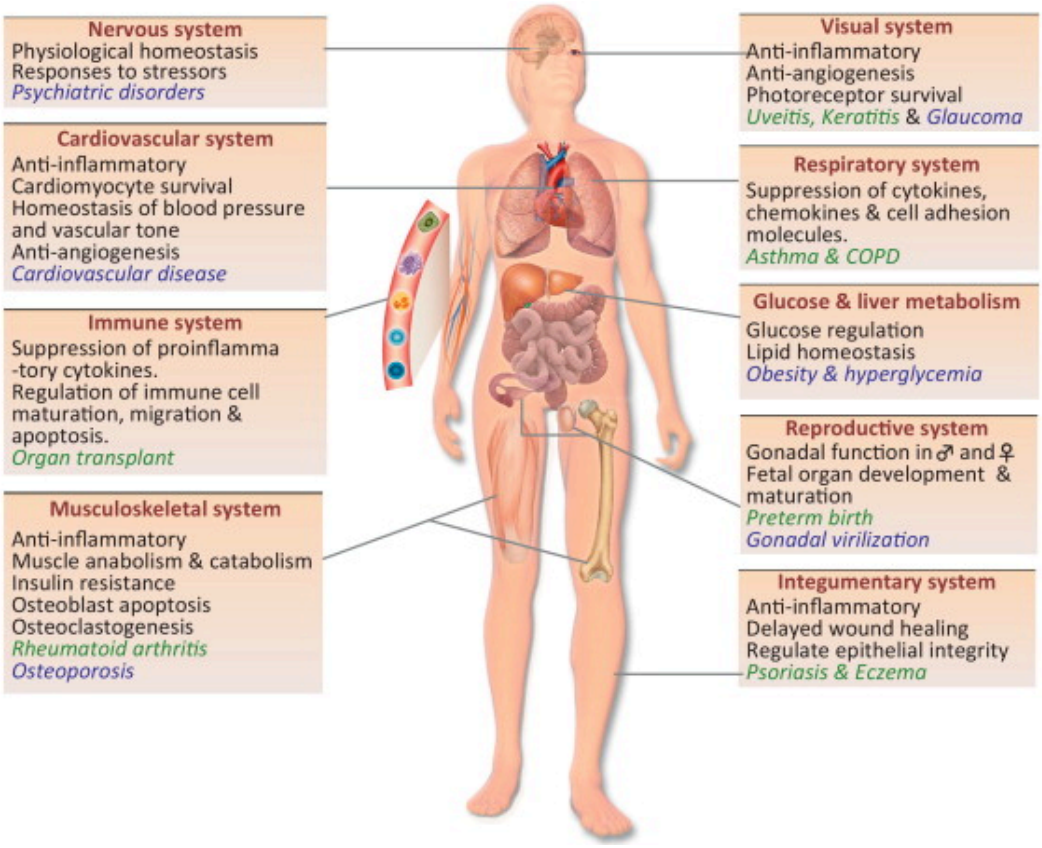



**Figure 8. Scheme of glucocorticoids function in the body.**

## 3.2 GLUCOCORTICOID RECEPTOR ACTIVITY

Glucocorticoid Receptor, hereafter called GR, is expressed in nearly all tissue types and belongs to the nuclear receptor superfamily of transcription factor proteins acting as a ligand-dependent transcription factor that positively or negatively regulates the expression of glucocorticoid-responsive genes (Nicolaides et al., 2010;

Zhou and Cidlowski, 2005).

In the absence of Glucocorticoids, the GR resides in an inactive state in the cytoplasm forming a complex with heat shock proteins (HSPs) 90, 70, 50, 20 and other chaperone proteins. When cells are exposed to GCs, the GR undergoes a conformational change that triggers its translocation to the nucleus, where it can exert its actions mainly as a transcription factors (Nixon et al., 2012) exerting its action as trans-activator or trans-repressor, but also independently from the ligand as described in figure 9 (Nixon et al., 2012).

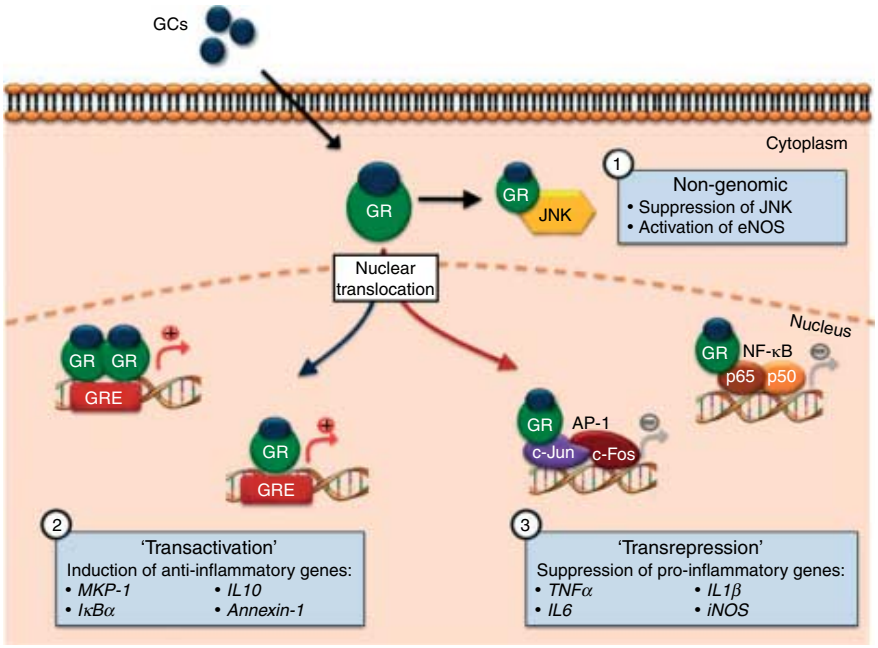



**Figure 9. Glucocorticoid (GC) action**. GCs act through several mechanisms to exert anti-inflammatory effects: 1) non-genomic pathways involve GC receptor (GR)- mediated direct interactions with second messenger proteins, including the MAPK protein JNK, inhibiting the activation of this signalling pathway. 2) GR-mediated transactivation of key anti- inflammatory genes involves direct DNA binding of both GR dimers and monomers/multimers to GC-response elements (GRE) in the promoter region of target gene. 3) Transrepression of pro- inflammatory genes does not require direct DNA binding of GR, but rather 'tethering' of GR monomers to DNA-bound pro-inflammatory transcription factors. From Nixon et al., 2012.

GR is the product of a single gene, NR3C1, located on chromosome 5q31–32 in humans; alternative splicing of GR generates two main isoforms: hGR-α and hGR-β. Furthermore, alternative translation initiation sites and post-translational modifications of the GR result in many other GR isoforms and a complex array of receptor molecules (Duma et al., 2006; Kadmiel and Cidlowski, 2013). The hGR-α isoform contains three distinct functional domains – the C-terminal ligand binding domain (LBD), the DNA-binding domain (DBD) and the N-terminal transactivation

domain (NTD). The NTD has a strong transcriptional activation function (AF-1), which allows for the recruitment of co-regulators and transcription machinery; the LBD also contains a transactivation domain (AF-2) involved in transcriptional activation of target genes; the two zinc-finger motifs present in the DBD recognize and bind specific DNA sequences, that is, the glucocorticoid response elements (GREs) on the promoter of target genes (Gruver-Yates and Cidlowski, 2013; Nicolaides et al., 2010) (figure 10). Thus, the hGR-α isoform is able to bind to GCs, undergo conformational changes, dissociate from the HSPs, homodimerize and translocate into the nucleus, where it can recruit co-regulators and interact directly with GREs to exert transcriptional effects. The GR-α/GRE complex results in stimulation or inhibition of the GRE- mediated gene transcription (trans-activation effect).

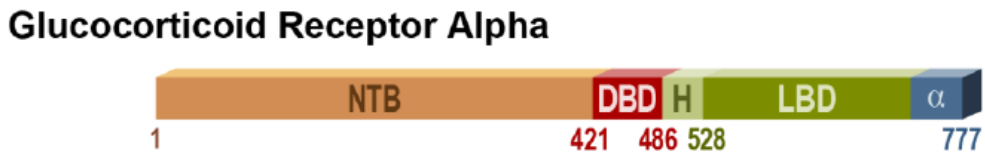



**Figure 10. Glucocorticoid Receptor Alpha (α-GR) structure**. Modified from Gruver-Yates, A. L. & Cidlowski, J. a. Tissue-specific actions of glucocorticoids on apoptosis: a double-edged sword. Cells 2, 202–23 (2013).

Alternatively, the ligand-activated hGR-α can modulate gene expression independently of binding to GREs, by interacting with other transcription factors, such as activator protein 1 (AP-1), nuclear factor-κB (NF-κB) and signal transducers and activators of transcription 5 (STAT5), which are involved in cellular proliferation, survival and inactivation of apoptosis, invasion, angiogenesis and carcinogenesis (Moutsatsou and Papavassiliou, 2008) (Figure 9).

Conversely, the hGR-β isoform resides constitutively in the nucleus and acts as a natural dominant-negative inhibitor of the hGR-α isoform (Charmandari et al., 2004).

GCs regulate the expression of a wide array of target genes by both positive and negative regulatory mechanisms. The genes encoding glucocorticoid-induced leucine zipper (GILZ), serum/glucocorticoid- regulated kinase 1 (SGK1), tristetraproline (TTP), and mitogen-activated protein kinase phosphatase-1 (MKP-1) are examples of genes up-regulated by activated GR. Examples of genes negatively regulated by GR are β-

arrestin 2, osteocalcin, and the GR gene, NR3C1, itself (Kadmiel and Cidlowski, 2013).

## 3.3 GLUCOCORTICOIDS SIGNALLING IN ORGANS

Glucocorticoids produce marked effects on energy metabolism by inhibiting glucose utilization by peripheral tissues such as muscles and stimulating glucose breakdown to maintain the necessary circulating levels to mount a stress response (Negi et al., 2009).

Glucocorticoids are the main hormones involved in the adaptation of the body to chronic stress. In fact, a genome-wide analysis of GC-regulated target gene networks in liver revealed that the GR controls many aspects of hepatic energy metabolism (Vegiopoulos and Herzig, 2007). The best-known GC function is the ability to stimulate gluconeogenesis. In fact GC lead to a marked increase in the levels of enzymes involved in the conversion of amino acids in glucose and cause the mobilization of amino acids from extra hepatic tissue. As consequence more amino acids are released in the plasma and become available to enter in the gluconeogenesis process that leads to the increase in glycogen storage in the liver cells. On the other hand glucocorticoids are also involved in a moderate decrease in the rate of glucose utilization from different tissues in the body leading to increased glucose concentration in the blood that stimulates insulin secretion.

Glucocorticoids play also an important role in the function and homeostasis of the central nervous system (CNS). Chronic exposure to supra-physiologic levels of glucocorticoids (GCs) in Cushing's syndrome (CS) is associated with an increased prevalence of sleep disturbances, mood alterations, psychiatric diseases, cognitive impairment, and anatomical brain changes (Bourdeau et al., 2005). Different studies demonstrated the presence of brain atrophy and psychiatric disturbances such as depression in patients affected by Cushing's syndrome.

Glucocorticoids regulate bone physiology decreasing the number and the function of osteoblasts. These effects lead to a suppression of bone formation, a central feature in the pathogenesis of Glucocorticoid-induced osteoporosis. Glucocorticoids decrease the replication of cells of the osteoblastic lineage, reducing the pool of cells that may differentiate into mature osteoblasts (Canalis et al., 2007). In addition to

inhibiting the differentiation of osteoblasts, glucocorticoids inhibit the function of the differentiated mature cells. Glucocorticoids inhibit osteoblast-driven synthesis of type I collagen, the major component of the bone extracellular matrix, with a consequent decrease in bone matrix available for mineralization. Moreover glucocorticoids induce the apoptosis of osteocytes. As a result, the normal maintenance of bone through this mechanism is impaired and the biomechanical properties of bone are compromised.

In addition to profound changes in the physiology and function of multiple tissues, stress and elevated glucocorticoids can also inhibit reproduction (Whirledge and Cidlowski, 2010). The stress-induced rise in glucocorticoids represses GnRH (gonadotropin-releasing hormone) secretion, which can result in hypogonadism. Stress induces an elevation in glucocorticoid concentration, which precedes a decline in testosterone concentration in the male as reported in patients with Cushing Syndrome not only at the hypothalamic and pituitary level, but also directly in the testes inducing Leydig cell apoptosis, reducing the number of Leydig cells per testis (Whirledge and Cidlowski, 2010). Glucocorticoids display also an important role in adipose tissue and excess of GC levels have been associated to the Metabolic syndrome, a clustering of conditions which include abdominal obesity, elevated triglycerides, low high-density lipoprotein, high fasting blood glucose, and high blood pressure (Wang et al., 2005). In Cushing's syndrome infact increased secretion of GCs leads to obesity, hypertension, hyperlipidemia and glucose intolerance (Arnaldi et al., 2003).

Consistently, treatment of obese rats with the GR antagonist RU486 or adrenalectomy, oppose the obese phenotype in these animals (Langley and York, 1990; Livingstone et al., 2000).

During mammalian fetal growth, the late gestation rise in fetal glucocorticoid levels is essential for the maturation of tissues and organs in preparation for birth. Mice lacking GR (GR-/- mice) die neonatally because they are unable to inflate their lungs due to severe pulmonary immaturity (Rog-Zielinska et al., 2013).

A direct effect of glucocorticoids on the cardiomyocyte structure and function has been demonstrated revealing a direct and dynamic role for glucocorticoids and GR signaling in the modulation of cardiomyocyte function (Ren et al., 2012).

## 3.4 GLUCOCORTICOIDS SIGNALLING IN CANCER

As previously said, glucocorticoid signalling is almost ubiquitously diffuse in various organs. From a clinical point of view, steroid therapy has been remarkably effective in treating various diseases, due to its anti-inflammatory, anti-proliferative, pro-apoptotic, and anti-angiogenic roles. Among these, pro-apoptotic action is the most-well-characterized feature that has been exploited for cancer treatment. Indeed, GCs are potent inducers of apoptosis in many cell types and tissues, such as skeletal and muscular system, circulatory system, nervous system, endocrine system, reproductive system, and the immune system. But it is owing to their ability to induce apoptosis in lymphocytes that synthetic GCs have been widely used in the treatment of haematological malignancies (Frankfurt and Rosen, 2004; Smith and Cidlowski, 2010). Physiological GC-induced apoptosis plays an important role in the development and in the functions of the immune system: GCs are important for T cell selection, immune system homeostasis, and resolution of the immune response following clearance of infection. High doses of Glucocorticoids are known to induce apoptosis in thymocytes, T cells, B cells, macrophages, mature but not immature dendritic cells, eosinophils, and natural killer cells. Indeed, according to their ability to efficiently kill lymphoid cells, Glucocorticoids have been included in essentially all chemotherapy protocols for hematopoietic malignancies: they are prescribed to treat acute lymphoblastic leukemia (ALL), chronic lymphoblastic leukemia (CLL), multiple myeloma (MM), Hodgkin Lymphoma (HL) and Non-Hodgkin's Lymphoma (NHL) (Smith and Cidlowski, 2010). Glucocorticoid chemotherapy is limited, however, by the emergence of GC-induced apoptosis resistance in lymphoid malignancies that is increasingly reported and studied (Kofler et al., 2003; Schlossmacher et al., 2011). Besides their use as powerful cytotoxic agents in an oncological setting for the treatment of lymphoid malignancies, Glucocorticoids are also used as a co-medication in the therapy of solid tumors to support chemotherapy, either because of their effectiveness in treating the malignancy, or for decreasing oedema, pain, electrolyte imbalance, nausea and emesis or to reduce cytotoxic reactions caused by other treatment regimens (Grote et al., 2006; Rutz and Herr, 2004). Indeed, GCs are often associated with a substantial, but temporary, improvement in symptoms

caused by advanced disease. However, the divergent GR activity in different cell types is striking when comparing GCs effects on lymphocytic malignancies versus epithelial cell-derived cancers. In fact, while synthetic GCs are routinely used to induce apoptotic cell death in malignant lymphoid cells, in epithelial (i.e. “solid”) tumours GCs have been reported to play opposite effects: GCs stimulate anti-apoptotic gene expression and antagonize the ability of chemotherapy treatment to effectively induce cell death (Herr et al., 2009; Zhang et al., 2006). Moreover several retrospective analysis have suggested that GC administration induces chemotherapy resistance in cancers of the breast and lung, and enhances the risk of skin cancer and perhaps lymphoma (Herr and Pfitzenmaier, 2006). Pre-clinical data suggest that GCs show diverse end even contradictory effects on chemo-sensitivity in many non-haematological tumour cells. Dexamethasone showed an anti-apoptotic effect in a variety of cell lines derived from malignant solid tumours, including breast cancer, brain cancer, cervical cancer, bone cell cancer, melanoma, and neuroblastoma. Data also suggest that Glucocorticoids diminish chemotherapy effectiveness and induce treatment resistance in solid tumors, such as prostate cancer, pancreatic cancer and ovarian carcinomas (Zhang et al., 2006). Thus, the therapeutic outcome of many solid tumours may be negatively impacted by glucocorticoids treatment depending on the type of cancer. In this context it is important to point out how glucocorticoids treatment may be particularly detrimental in breast cancer.

In epithelial breast cells GCs are generally cytoprotective also in normal cell types (Moran et al., 2000) and there is an anti-apoptotic effect of GCs in cancers derived from this tissue. In fact, in vitro data suggest that Dexamethasone could protect breast cancer cells from chemotherapy-induced apoptosis (Moran et al., 2000). Moreover, two breast cancer and ovarian xenograft studies have reported that pre-treatment with Dexamethasone could selectively inhibit response to Taxol (Paclitaxel)-induced apoptosis (Pang et al., 2006; Sui et al., 2006). Interestingly, a recent study has shown that pre-treatment with Mifepristone (RU486), a GR antagonist, could potentiate the efficacy of chemotherapy in GR positive TNBCs inhibiting the anti-apoptotic signalling pathways of GR and increasing the cytotoxic efficiency of Paclitaxel (Skor et al., 2013). Finally, in a recent meta-analysis of primary

breast tumour gene expression from 1378 early stage breast cancer patients with long-term clinical follow-up, high expression of the gene encoding the GR (NR3C1) has been found to significantly correlate with shorter relapse-free survival in patients with oestrogen receptor negative (ER-) breast cancer either treated or untreated with adjuvant chemotherapy (Pan et al., 2011). Conversely, in patients with ER+ breast cancer, a high level of primary tumour GR expression has been associated with a better outcome relative to low GR expression. From a clinical point of view, high GR expression correlates with a relatively poor prognosis in patients with ER- breast cancer and with a significantly increased risk of early relapse compared to patients with ER- but low GR expression tumours, regardless of adjuvant chemotherapy treatment.

All together, these data provide evidence suggesting that exogenous GCs, and subsequent GR activation in tumour cell, inhibit cancer cell death pathways promoting cell survival in breast cancer.

## 4.1 BREAST CANCER

Breast cancer is the most common malignancy diagnosed in women around the world, both in developed and in developing countries (Benson and Jatoi, 2012; Boyle and Howell, 2010). Breast tumours are annually responsible for almost 1.6 million new cases of cancer worldwide and approximately 520,000 deaths were recorded in 2012 (data from IARC, GLOBOCAN project 2012). Although the incidence of breast cancer is increasing almost everywhere throughout the world, the mortality rate is declining in many high-income countries (Autier et al., 2010) but a significant percentage of these patients still dies, mainly due to the treatment failures and the inability to prevent metastatic spreading of the disease.

Management of breast cancer relies on the availability of clinical and pathological prognostic and predictive factors to stratify individual patients for appropriate therapy. The Nottingham Histological Score (modification of the Scarff-Bloom-Richardson grading system) (Elston and Ellis, 1991) is the most used grading system and is recommended by the World Health Organization (WHO), the American Joint Committee on Cancer (AJCC) and the Royal College of Pathologists (UK RCPath). In

this score the nuclear grade, the tubular formation and the mitotic index are considered to distinguish invasive carcinomas into three different groups with prognostic relevance: grade 1 (G1), grade 2 (G2) and grade 3 (G3) tumours. These grades are representative of the degree of loss of differentiation and of the of acquisition of various mutations and provide a predictor of outcome in patients with invasive breast cancer; in fact G3 tumors (46%) are associated with malignancy and high risk of metastasis occurrence and the majority of deaths in these patients occurs within 10 years. However, since the biology of breast cancer varies a lot from one tumour to another, histological grade is not a sufficient guide to characterize all these biological differences. In fact, breast cancer presents an intrinsic inter-tumoral and intra-tumoral heterogeneity and this is one of the main factors that determine risk of disease progression and therapeutic resistance (Polyak, 2011).

In the last years the biology of this tumour became more clear: there are ways now to identify sub-types of breast cancer: at least five main molecular classes have been distinguished by gene-expression profiling and histological staging:

- luminal-A characterized by the expression of oestrogen receptor (ER) and its target genes, low rate of proliferation and good prognosis;
- luminal-B characterized by high proliferation rate and incomplete sensitivity to endocrine therapy (Prat et al., 2012).
- HER2-positive breast cancers showing amplification or high expression of the ERBB2 oncogene. These tumours are poorly differentiated and have an aggressive natural history: they display high proliferation rate, high frequency of cerebral metastases and worse prognosis.
- Normal-like breast cancer that is a small group of usually well differentiated, ER-positive and HER2- negative cancers. They are characterized by the expression of normal tissue genes.
- Basal-like breast cancer subtype represents approximately 15% of all breast cancers, it is defined by the expression of markers usually present in normal breast myoepithelial cells (CK5/6 e CK17). Among them a large percentage are TNBC (Triple-Negative breast cancer) characterized by large tumour size, high histological grade, high frequency of lymph node affection and metastases.

In the last years our knowledge of the clinical behaviour of breast cancer has increased substantially. Identification of several prognostic subgroups and prediction of hormone- responsive and hormone-resistant disease has led to more rational use of endocrine and cytotoxic treatments. Breast cancer is sensitive to multiple cytotoxic compounds like anthracyclines, taxanes, topoisomerase inhibitors, nucleoside analogues, commonly used as chemotherapeutics, both in breast cancer and in other cancers (Dobbelstein and Moll, 2014). However, the side effects of these treatments are severe and sometimes can be as lethal as the cancer itself. In fact, due to their lack of specificity, these compounds affect all rapidly dividing cells indiscriminately and thus also interest tissue with a high rate of turnover, i.e. bone marrow, skin and epithelium of gastro-intestinal tract. Importantly, these chemotherapeutics can also give rise to secondary malignancies (Dobbelstein and Moll, 2014).

As in other solid tumours, development of breast cancer is a multi-factorial process represented by a chain of cellular and molecular events that reflect accumulation of genetic and epigenetic alterations. The acquisition of new mutations in a subpopulation of tumour cells is often responsible for drug-resistance. Thus, the pathways required for cancer growth and drug-resistance need to be further investigated for the clinical advancement of targeted therapies; so the molecular characterization of signalling pathways that drive tumour formation and progression represents a key step in the development of new treatment modalities and targeted cancer therapy.

## AIM OF THE THESIS

The Hippo signalling pathway has emerged to play a central role in the regulation of cellular biological properties, which are fundamental in both organ size control and tumorigenesis. The YAP oncogene is the main nuclear effector of the Hippo tumour-suppressor cascade. It is regulated by a wide number of upstream signals and proteins that influence its activity in mediating cellular biological processes, such as cell proliferation, apoptosis and stemness. Deregulation of these signals can lead to cancer formation, maintenance and expansion. Indeed, the Hippo pathway is largely deregulated and YAP is often over-expressed and it has aberrant activity in breast cancer as well as in several other human carcinomas.

The aim of this thesis was to identify new pathways able to influence and regulate YAP functions in breast cancer and to study mechanisms through which this regulation occurs. To this aim we tested a library of FDA-approved drugs for their ability to regulate YAP nuclear localization in a model of metastatic breast cancer. Since the identified compounds are well-characterized in their targets and intracellular signaling, it was then possible to elucidate the molecular mechanisms through which they regulate YAP activity.

# RESULTS

## Identification of drugs regulating YAP protein levels in cancer cells

Aiming at identifying novel small-molecules able to regulate YAP functions in cancer cells we tested, by a high-content screening, the effect of a collection of 640 clinically-used compounds on YAP protein levels and in the breast cancer cell line MDA-MB-231. All the drugs used in this screening have already received the FDA-approval and therefore have been characterized for their pharmacokinetic and pharmacodynamic properties as well as for their molecular mechanisms of action and molecular targets. Cells were seeded in 384-well plates and cultured at low density. After 24 hours each compound of the library was transferred robotically from library stock plates to the plates containing the cells. Drugs were added to the culture medium at two different concentrations (1 and 10 µM). 24 hours after treatment, cells were fixed and processed immediately for YAP-specific immunofluorescence analysis (Figure 1a). The effect of each compound in modulating the total YAP protein levels was assessed by detecting the YAP-specific staining and by quantifying the YAP-relative fluorescence intensity at single-cell level. The image acquisition was performed using an automated high-content screening fluorescence microscope (Molecular Devices) at a 10x magnification. The screening was performed in duplicate and 4,500 cells were analyzed per experimental condition and replicate.

Several compounds were able to significantly modify, although at different degrees, YAP protein levels. Among them Statins that based on our recent studies emerged as a class of compounds with strong YAP inhibitory effect (Sorrentino et al., 2014). These drugs belong to the mevalonate pathway inhibitors class and are used to reduce the cholesterol levels in patients with cardiovascular diseases (Opie et al., 2015). Interestingly as shown in Figure 1b, statins emerged as the most effective drugs in reducing the YAP protein levels thus confirming the reliability of our screening. However, analysing the results of the screening we become interested also on a group of molecules that strongly increased YAP fluorescence intensity (Figure 1b).

Of note, most of the identified hits, Betamethasone, Prednisolone, Dexamethasone, Fluocinolone, belong to the same pharmacological class of Glucocorticoids (Figure 1b, c and d and Table 1). Therefore among the 640 FDA-approved drugs tested in our assay, we identified Glucocorticoids as the main class of compounds that significantly increased the YAP protein levels thus suggesting that the Glucocorticoid Receptor signalling pathway could regulate the biological activity of YAP.

## Breast Cancer cells are fully responsive to glucocorticoids stimulation

The expression of GR has been reported in various breast cancer cell lines (Skor et al., 2013). Therefore we first tested whether the MDA-MB-231 cells were responsive to pharmacological activation of GR. The activation of endogenous GR was assessed monitoring the transcriptional activation of GR in cells using the pGL4.36 luc2P/MMTV/Hygro luciferase reporter assay. The vector contains MMTV LTR (Murine Mammary Tumor Virus Long Terminal Repeat) that drives the transcription of the luciferase reporter gene luc2P in response to transcriptional activation of the Glucocorticoid Receptor. As shown in Figure 2a, after administration of Betamethasone (BM, a synthetic glucocorticoid agonist) for 24 hours, MDA-MB-231 cells showed a strong enhancement of the GR-induced luminescent signal. Moreover, when cells were co-treated with Mifepristone (RU486, a GR antagonist) the signal decreased to basal levels, meaning that the increase in luminescence signal was specifically due to GR activation and that MDA-MB-231 cells show an intact GR signalling pathways. This finding indicates that the MDA-MB-231 cell line expresses endogenous GR, which is fully responsive to glucocorticoid stimulation.

Inactive GR proteins are localized in the cytoplasm bound to chaperone proteins (e.g. HSP90) and following agonist stimulation they translocate to the nucleus where exert their transcriptional activity (Nicolaides et al., 2010). Thus, we examined the nuclear translocation of Glucocorticoid Receptor in MDA-MB-231 cells after treatment with Betamethasone. For this purpose we overexpressed a construct composed by the cDNA of the GR fused to that of the green fluorescent protein (GFP-GR) and analysed its subcellular localization by fluorescence microscopy. As expected, in absence of stimulation, the GR localized in the cytoplasm, while

addition of Betamethasone clearly promoted its accumulation into the nucleus (Figure 2b). Similar results were obtained by monitoring the translocation of endogenous GR by immunofluorescence (data not shown).

These data demonstrate that the triple negative breast cancer MDA-MB-231 cell line is fully responsive to endocrine stimulation by glucocorticoid and therefore can be used as a cellular model to test the role of Glucocorticoid Receptor signalling in regulating YAP protein levels and activity.

## Glucocorticoids receptor directly increase YAP levels and activity

According to the results of the screening previously described, Glucocorticoids represent the main class of drugs able to significantly increase YAP fluorescence intensity. In order to validate the screening and to assess if the increase in fluorescence intensity corresponded to an increase in total YAP protein levels, MDA-MB-231 cells were treated with Betamethasone 1 µM for 24 hours and then analysed for YAP protein amount by western blot. As shown in Figure 2c, treatment with Betamethasone led to a significant increase in YAP protein levels both in MDA-MB-231 and in another breast tissue derived cell line, MCF10A-M2 (Cordenonsi et al., 2011). Importantly, blocking the GR activation by co-administrating RU486, almost totally abolished the increase of YAP levels in the cells, meaning that YAP protein levels are specifically influenced by GR activation. Interestingly, the levels of TAZ were unchanged upon Betamethasone administration, indicating that glucocorticoids regulate specifically the intracellular levels of YAP protein leaving unaltered its homolog protein TAZ (Figure 2c).

Being YAP a transcriptional co-activator, we sought to test whether the increase in its protein levels corresponded to an increase in its biological functions by monitoring the mRNA levels of a canonical YAP target gene. One of the most-well-characterized YAP target genes that mediate several YAP biological functions is the ankyrin repeat domain 1 (*Ankrd1*) gene (Dupont et al., 2011; Sorrentino et al., 2014; Yu et al., 2012). This gene is mainly known to encode for the cardiac ankyrin repeat protein (CARP) and to be involved in pathogenesis of hypertrophic cardiomyopathy (Arimura et al., 2009). However, it has also been characterized as a YAP target gene (Dupont et al., 2011; Yu et al., 2012) in breast cancer cells and in ovarian cancer,

where its expression correlates with chemotherapy sensitivity and with clinical outcome (Scurr et al., 2008). Interestingly, treatment with Betamethasone 1 µM for 24 hours led to a dramatic up-regulation of ANKRD1 protein (Figure 2d). As co-treatment with RU486 blunted this effect, we concluded that up-regulation of ANKRD1 was specifically triggered by the GR activation. Moreover, to formally prove that GC induced ANKRD1 expression through YAP transcriptional activation, we knocked down YAP by siRNA transfection for 48 hours before Betamethasone treatment. Strikingly, knocking-down YAP completely prevented the ANKRD1 increase prompted by Betamethasone, strongly suggesting that glucocorticoid signalling enhances YAP transcriptional activity (Figure 2d). Altogether, these findings suggest that Glucocorticoid Receptor activate a signalling able to increase YAP nuclear activity in breast cancer cells.

## Glucocorticoid receptor directly regulates YAP mRNA transcription

Glucocorticoid receptor belongs to the nuclear receptor superfamily of transcription factor proteins and functions as a ligand-dependent transcription factor that is able to bind the promoter of glucocorticoid-responsive genes regulating positively or negatively their expression (Frankfurt and Rosen, 2004; Smith and Cidlowski, 2010).

To find out whether the increase in YAP protein levels was an effect of direct glucocorticoid receptor activity on YAP mRNA expression, MDA-MB-231 cells were treated with betamethasone for 24 hours and then analysed for the amount of YAP mRNA transcript. As reported in Figure 2e YAP mRNA expression was increased upon treatment with betamethasone and the concomitant administration of RU486 prevented this transcriptional induction suggesting that glucocorticoids are able to increase YAP expression at the transcriptional level.

We then hypothesized that GR could directly recognize and bind the YAP promoter thus triggering the transcription of its mRNA. To identify predicted GR binding site we inspected the promoter sequence of YAP gene using the LASAGNA algorithm (Lee and Huang, 2013). This analysis revealed a DNA region located at -1150 to -900 bp upstream of the first YAP exon with statistically significant prediction to be bound by the GR (Figure 2f). Chromatin immunoprecipitation (ChIP) assay was then performed in MDA-MB-231 cells treated with vehicle or betamethasone 100 nM for 6 hours

(Polman et al., 2012) to confirm the recruitment of GR to the YAP promoter. As shown in Figure 2g GR was recruited on YAP promoter after Betamethasone treatment while the negative control (heterochromatin region) doesn't show any recruitment, thus confirming that YAP is a *bona fide* glucocorticoid-responsive gene in breast cancer cells.

To further demonstrate the effect of GR activation on YAP mRNA levels and biological activity in vivo, we performed experiment in vivo using the Drosophila model and analysing the biological activity of the YAP Drosophila ortholog Yorkie (YKI). As shows in figure 2h, treatment of the developing flies with Betamethasone led to a significant increase of YKI mRNA levels in the ovary tissue (Figure 2h). In line, the mRNA levels of the YKI target genes, Drosophila inhibitor of apopotosis protein-1 (DIAP1), and Expanded (Ex), were concomitantly increased in the same tissue (Figure 2h).

Our findings suggest that glucocorticoid receptor directly promotes YAP mRNA transcription *in vitro* and *in vivo* in response to glucocorticoid stimulation.

## Glucocorticoids regulate YAP nuclear localization by promoting its de-phosphorylation and nuclear accumulation

Being a transcriptional co-activator, YAP is strongly influenced by its sub-cellular localization, which correlates with its biological activity (Zhao et al., 2007). Indeed, when YAP is localized in the cytoplasm it is phosphorylated and bound to 14-3-3 in an inactive state and is actively degraded in a proteasome-dependent manner. In the nucleus YAP is instead protected from phosphorylation and degradation and can interact with several transcription factors modulating their activity (Piccolo et al., 2014). The evidence that GCs not only increased YAP protein levels but also its transcriptional activity suggests that GCs might not only increase the amount of YAP mRNA and protein, but could also trigger the YAP nuclear translocation essential for its transcriptional activity. Thus, we assessed the YAP subcellular localization in cells treated with GCs. At first, we re-analysed the data obtained by the high content screening described above extracting information about the effect of the drugs on YAP nuclear localization. Interestingly, Glucocorticoids clustered together as drugs

able to significantly increase the YAP nuclear localization (Figure 2a and b), meaning that GC are able not only to increase the YAP protein levels, but also to promote its nuclear accumulation.

To demonstrate that the increased YAP nuclear localization observed in the screening was not an unspecific consequence of the GC-induced increase of YAP protein levels, we first transfected YAP in MDA-MB-231 cells. As shown in Supplementary Figure 1a YAP overexpression led to a strong increase of YAP mRNA levels. Interestingly enough, the increase of YAP protein levels did not correlate with any increase of YAP nuclear localization (Supplementary figure 1b). Indeed, the majority of transfected cells showed cytoplasmic-localized YAP meaning that other signals, in addition to the general increase of YAP protein levels, are required to foster YAP nuclear localization.

Among post-translational signals controlling the cytoplasmic retention and degradation of YAP, phosphorylation (in particular on Ser127), is a critical one. In fact dephosphorylated YAP entirely accumulates in the nucleus. Of note, conversely to the overexpressed WT form of YAP, which showed mainly cytoplasmic localization, the overexpressed unphosphorylable YAP mutant (YAP-5SA)(Pan, 2010) was mainly nuclear (Supplementary figure 1b).

We thus tested whether Glucocorticoids could promote YAP de-phosphorylation in addition to increasing its protein levels. Western blot analysis of protein lysates from MDA-MB-231 and MCF10A-M2 cells treated with betamethasone showed a dramatic reduction of the levels of Serine 127 phosphorylated YAP and this effect was totally prevented by RU-486 (Figure 3c). As expected, Betamethasone was able to promote YAP nuclear localization in three different breast cancer cell lines and RU-486 efficiently prevented this effect (Figure 3d and e).

Of note, GR-induced YAP nuclear accumulation was seen also in HeLa cells meaning that the hormonal control of YAP nuclear localization might not be restricted to the breast tissue (Figure 3d).

Altogether these results demonstrate that GCs trigger YAP transcriptional activation through the combined effect of increasing its mRNA levels and of inducing its nuclear accumulation by specifically reducing its inhibitory phosphorylation.

## Glucocorticoids promote YAP nuclear accumulation by controlling actin cytoskeleton dynamics

One of the most established inputs controlling YAP nuclear localization is the activation of the Hippo pathway. In particular LATS1 kinase activation is responsible for YAP phosphorylation and cytoplasmic retention downstream of the Hippo pathway (Johnson and Halder, 2014). To test if LATS1 was involved in the GC-induced YAP de-phosphorylation we checked the levels of LAST1 and its active phosphorylated form (pLATS1) after BM and RU486 treatment. As shown in supplementary figure 1c we could not observe any change in LATS1 and pLATS1 levels after treatments suggesting that this kinase is not involved in the regulation of YAP phosphorylation by GC treatment.

Recently, several studies pointed out that mechanical forces and physical cues, such as perturbation in extracellular matrix (ECM) stiffness and in cell geometry contribute to development, differentiation, stemness and tumour progression (Jaalouk and Lammerding, 2009; McBeath et al., 2004). In the last years, an important upstream mechanism controlling YAP activity has emerged: YAP is under the control of mechanical signals exerted by ECM rigidity and cell shape (Dupont et al., 2011; Yu et al., 2012). In this contest, the activity of Rho GTPases, a family of proteins that control actin cytoskeleton dynamics and cell geometry has emerged as a fundamental input in controlling YAP phosphorylation and nuclear localization (Dupont et al., 2011; Sansores-Garcia et al., 2011; Wada et al., 2011). Similarly, metabolic cues have been linked to YAP activation through Rho GTPases activation via geranylgeranylation (Sorrentino et al., 2014). Importantly, the regulation of YAP phosphorylation and activity by cytoskeleton rearrangements does not involve the activation of LATS1/2 kinases (Dupont et al., 2011).

By microscopy analysis we noticed that cells receiving Betamethasone underwent marked morphological changes with a clear increase in cell-size and spreading, suggesting that GCs could promote a strong rearrangement of the actin cytoskeleton leading to cell shape alteration (Figure 4a).

Thus, we tested if Glucocorticoids could affect YAP nuclear localization acting directly through mechanical cues. To this aim, cells were treated with Betamethasone 1 µM for 24 hours. To verify whether the treatment caused an

alteration of actin cytoskeleton we analysed the actin cytoskeleton through Phalloidin staining (which is commonly used to highlight the actin microfilaments) by fluorescent microscopy. The immunofluorescence images show that Betamethasone treatment dramatically increased the formation of F-actin stress fibers in MDA-MB-231 cells (Figure 4b).

To formally link the effect of GCs to YAP nuclear localization and activity through cytoskeletal cues, we decided to pharmacologically prevent the actin polymerization by treating cells with Latrunculin-A, an F-actin destabilizing toxin that binds actin monomers and prevents them from undergoing polymerization (Dupont et al., 2011). As shown in Figure 4c, with this experimental set-up, Betamethasone totally failed to increase the amount of F-actin stress fibers. More importantly, Latrunculin-A treatment prevented the induction of YAP nuclear localization and transcriptional activity (as assessed by monitoring the levels of its target gene ANKRD1) triggered by Betamethasone (Figure 4f and g), suggesting that F-actin polymerization is required for YAP activation by GCs. Of note, similar results were also obtained by using statins, which inhibit several Rho-GTPases (Sorrentino et al., 2014), essential component of the actin polymerization machinery (Figure 4g).

Hence, these data indicate that the ability of GCs to induce actin polymerization plays a major role in promoting YAP nuclear accumulation and activity. Conversely, in conditions in which actin cannot be polymerized (e.g. after Latrunculin-A treatment), Glucocorticoids fail to activate YAP.

Interestingly, the control of YAP exerted by glucocorticoid at transcriptional level (Figure 4e) is clearly uncoupled from the cytoskeleton-mediated one. Indeed cells treated with betamethasone in presence of latrunculin-A still show increased YAP mRNA and protein amount (Figure 4e) but this is not sufficient to sustain its nuclear function due to the lack of YAP de-phosphorylation (Figure 4e and f).

Therefore these data indicate the existence of two independent mechanisms of YAP regulation by glucocorticoids: on one hand glucocorticoid receptor increases YAP mRNA expression leading to YAP protein accumulation within the cell, on the other glucocorticoids promote actin polymerization leading to YAP de-phosphorylation, nuclear accumulation and activity.

## Glucocorticoids are serum factors that counteract the GPCRs-mediated negative regulation of YAP

When our senses perceive an environmental stress such as danger or a threat, cells in the nervous and endocrine systems work closely together to prepare the body for action. Often referred to as the “fight or flight” or “stress response”, this remarkable example of cell communication elicits instantaneous and simultaneous responses throughout the body. In this context, Epinephrine and Cortisol are secreted by the same glands during stress; Epinephrine is involved in the “fight-or-flight” response and temporary increase in energy production while Cortisol is an important mediator in long term stress response.

Recently it has been demonstrated that YAP is regulated by a large number of G-protein-coupled receptor (GPCR) that can either activate or inhibit the Hippo-YAP pathway depending on the coupled G protein (Yu et al., 2012). As consequence, the YAP nuclear activity can be regulated by several serum components, such as lysophosphatidic acid (LPA), sphingosine 1-phosphophate (S1P), glucagon or epinephrine acting through GPCRs signalling.

Rho-GTPases are known downstream mediators of GPCRs and orchestrate actin cytoskeleton rearrangement upon their activation (Yu et al., 2012). Interestingly, the control of YAP nuclear activity by GPCRs agonists/antagonists has been found to be mediated by Rho-GTPases and requires actin cytoskeleton remodelling (Fa-Xing Yu et al., 2012). GPCRs coupled to alphas G-proteins, are among the most effective YAP regulators. In particular, Isoprenaline and its analogous Salmeterol (Yu et al., 2012) show potent YAP inhibitory effect acting through the beta-adrenergic receptors. Thus we asked whether GC could counteract the inhibitory effect of beta-adrenergic receptor agonists, which are present in the serum by controlling the actin cytoskeleton. To this aim, we decided to treat MDA-MB-231 cells with the known b2-adrenergic agonist Salmeterol. As expected, Salmeterol inhibited YAP nuclear localization by increasing its phosphorylation (Figure 5a and b). Interestingly, treatment with betamethasone efficiently rescued the nuclear YAP localization by preventing its phosphorylation (Figure 5a, b and c). Moreover, RU486 blunted this Betamethasone-induced effect, meaning that inhibition of YAP phosphorylation was specifically dependent on GR activation (Figure 5a, b and c). Of note, betamethasone

could not only rescue the YAP nuclear localization in presence of Salmeterol, but also restore YAP transcriptional activity (Figure 5d).

Thus, we can conclude that adrenergic and glucocorticoid stress signalling can exert opposite effect in regulating YAP biological activity in cancer cells.

## Glucocorticoids sustain breast cancer stem cells by activating YAP

Cancer stem cells (CSCs) are a sub-population of cancer cells known to play a major role in breast cancer development, metastasis, drug resistance and tumour recurrence (Dittmer and Rody, 2013). The most widely used assay to monitor the number of breast cancer stem cells in mixed populations of stem and differentiated cells is an in vitro cultivation system known as mammosphere assay (Dontu et al., 2003). This in vitro assay is suitable for testing peculiar characteristics of stem cells, such as self- renewal and ability to generate differentiated progeny. In order to perform this assay, cells are removed from culture plates and then cultured in a specific stem-cell growth medium in "ultra- low attachment" plates that prevent cell adherence. These experimental conditions allow for growth only of mammary stem cells (and progenitor cells in an undifferentiated state), based on their ability to proliferate in suspension and to develop non-adherent spheres. Once formed, these spheres are disrupted and plated for a second time in the same conditions (secondary generation mammospheres). Importantly, transient amplifying or differentiated cells have a limited ability to form mammospheres, whereas stem cells are able to generate spheres that can be passaged beyond multiple generations (from primary to secondary generation) (Figure 6a). Thus, considering the number of secondary and following mammospheres, it is possible to evaluate the cancer stem cells self-renewal capability and, therefore, the presence and the amount of cancer stem cells.

In this context, TAZ activity has been shown increased in basal breast cancers that show a more stem-cell-like phenotype (Cordenonsi et al., 2011). Indeed, TAZ hyperactivity promotes tumorigenic potential by the acquisition of additional cancer cell phenotypes and, among these, enhancing stem-cell-like properties plays a central role. More recently, also YAP has been found to be determinant in maintaining stem cell phenotypes and in conferring CSC properties to several

different cell types (Cao et al., 2008; Lian et al., 2010; Song et al., 2014; Yimlamai et al., 2014; Zhao et al., 2014). This evidence suggests that stemness could be pivotal in YAP oncogenic behaviour.

Considering the results previously obtained, we aimed to determine whether YAP induction, promoted by glucocorticoid treatment, might play a causal role in defining CSC traits. To this aim, we used derivatives of the non-tumorigenic human mammary MCF10A cell line: RAS-transformed MCF10A-T1k cells (M2 cells, Figure 6b and c) (Cordenonsi et al., 2011). Cells were grown in a specific stem-cell growth medium and cultured for two mammosphere generations with or without Hydrocortisone 0.5 µg/ml. About two weeks later, only mammospheres that resulted to be over 200 µm were considered for analysis. As shown in Figure 6b glucocorticoid treatment led to dramatic induction of secondary mammospheres, meaning that GR-signalling is essential for cancer stem cell self-renewal. To formally demonstrate that GCs led to CSCs expansion by inducing YAP biological activity, endogenous YAP was knocked down through siRNA transfection for 48 hours before performing the assay. Strikingly, knocking down YAP in M2 cells almost totally prevented the ability of Hydrocortisone to induce CSCs self-renewal, proving that YAP is a key executor of the GCs-induced stem cell traits in cancer cells (Figure 6b and c).

We thus reasoned that if YAP is a downstream nuclear effector responsible for the ability of GCs to sustain CSCs self-renewal, we can expect that cells expressing a constitutively nuclear-localized form of YAP (YAP-5SA), should be totally capable to form mammospheres even in absence of GR-signalling. Strikingly, expressing this nuclear form of YAP, CSCs completely bypassed the requirement of GR-signalling (Figure 6b and c). We could thus prove that YAP plays a central role in sustaining mammary cancer stem cell expansion and that the positive role of GCs on YAP accounts for the ability of these hormones to control CSCs traits.

As previously said, cancer stem cells (CSCs), are defined as the fraction of tumour cells specifically endowed with self-renewal and tumour-seeding potential and ability to spawn non-CSC progeny (Visvader and Lindeman, 2008); CSCs are responsible of metastasis, drug resistance and tumour recurrence (Dittmer and Rody, 2013). Due to the results obtained before we asked whether glucocorticoid receptor inhibitor RU486 could be able to blunt the expansion of cancer stem cells.

To this aim, M2 cells were treated for 24 hours, then the cells were grown in a specific stem-cell growth medium (see experimental procedures section) and cultured for two mammosphere generations with or without RU486. As shown in Figure 6e, RU486 treatment dramatically reduced the number of secondary mammospheres generated by MCF10A-M2 cells. In line, RU486 treatment led to strong reduction of the population of cells expressing specific breast cancer stem cells marker CD44+/CD24– (Fillmore, 2007) (Figure 6d and supplementary figure 1d). To test whether this effect was due to a general effect of RU486 in reducing cell viability and proliferation we performed viability assay after treatment of MCF10A-M2 cells (pleated in petri dishes) with two RU486 doses. Strikingly, after 5 day of treatment RU486 treatment didn't show any effect on cell viability (Figure 6f) thus suggesting that its inhibitory effect was specific for cancer stem cells. These results suggest that RU486 is a promising FDA-approved drug that can be used for targeting selectively the cancer stem cells pool in breast cancer.

## DISCUSSION

In the last decades, research on the complex biology of cancer has increased substantially and has led to a deeper knowledge about the molecular mechanisms underlying cancer formation and expansion. The Hippo signaling cascade has emerged as an evolutionally conserved tumour suppressor pathway involved both in organ size control and cancer development. It plays a crucial role in regulating the transcription of several genes, involved in fundamental cellular processes, such as proliferation and apoptosis, organ growth, embryonic development, differentiation and stem cell functions. Consistently, dysregulation of the Hippo signalling and hyper-activation of its major downstream effector Yes-associated protein (YAP) have been associated with cancer onset in a broad range of different human tumours (Johnson and Halder, 2014) (Harvey et al., 2013). Increasing data, in fact, have pinpointed YAP as an established human oncogene and, in particular, recent studies have yielded evidence that YAP is able to confer cancer stem cell features, including epithelial to mesenchymal transition (EMT), chemoresistance and tumour relapse (Kapoor et al., 2014) (Song et al., 2014).

In the present work, we reveal for the first time that YAP is finely regulated by hormonal signals and, in particular, by the Glucocorticoid Receptor (GR) pathway.

We were able to identify a class of well-known compounds, namely Glucocorticoids (GCs), which significantly increased YAP protein levels and activity. After demonstrating that MDA-MB-231 cells were fully responsive to GR-signalling, we could analyze the mechanisms through which Glucocorticoids regulate YAP, at a molecular level. We demonstrate that GCs are able to boost YAP signaling by i) increasing YAP mRNA expression leading to YAP protein accumulation within the cell and ii) by promoting actin polymerization with the consequent YAP de-phosphorylation, nuclear accumulation and activity. Interestingly, we showed that this regulation is specific for YAP and not for TAZ protein.

Importantly, cytoskeleton acts through a complex network of distinct mechanisms to relay mechanical signals that regulate YAP activity (Dupont et al., 2011; Halder et al., 2012). Even if our results have not revealed how Glucocorticoids induce actin-polymerization yet, we have pioneered for further investigation.

In summary, our findings indicate GCs, via the GR-signaling, as a new hormonal-dependent pathway that significantly impacts in YAP regulation and activity. Moreover, our results implicate an important role of YAP as a key executor of the biological program exerted by Glucocorticoids in breast cancer and likely in other tissues controlled by GR action. We might also speculate that YAP behaves as a "stress-responsive" protein that is recruited by Glucocorticoids to execute their physiological processes.

From a clinical point of view, these results may have important implications. Glucocorticoids are a widely prescribed class of drugs. The anti-inflammatory and immune-modulatory effects of Glucocorticoids and the GC-induced apoptosis have led to their use in many clinical settings. In particular, GCs have largely been exploited for their pro-apoptotic activity in the treatment of haematological malignancies (Frankfurt and Rosen, 2004; Vilasco et al., 2011). Indeed, Glucocorticoids are extremely important and a first line of defense in the therapy of hematopoietic cancers. Although the apoptotic effects of Glucocorticoids in the immune system cells have been well studied over the years, molecular mechanisms of action of GC-induced apoptosis have constantly been updated (Gruver-Yates and Cidlowski, 2013). A recent study has reported YAP over-expression as an activator of ABL1/p73-mediated apoptosis in the context of haematological malignancies (Cottini et al., 2014). Previous work has also demonstrated that, in response to DNA damage, YAP interacts with p73 and promotes a p73-dependent apoptotic program (Lapi et al., 2008). Considering the major role played by GC-induced apoptosis in treating haematological cancers, it is conceivable that GCs might mediate part of their pro-apoptotic effect by up-regulating YAP and thus activating its p73 dependent pro-apoptotic function. Experiments to prove this hypothesis are however required.

GCs are given at varying doses to alleviate acute toxicity and to protect healthy tissue (e.g., bone marrow) against the long-term effects caused by chemotherapeutic drugs. As a result, the use of GCs has widely been endorsed for a supportive-care role in cancer patients with solid tumours, including breast cancer (Frankfurt and Rosen, 2004; Vilasco et al., 2011).

In contrast to the effects seen in hematological malignancy mounting clinical evidence has suggested that GR activation by GCs could induce therapy-resistance in

several solid tumours (Zhang et al., 2006), however the underlying molecular mechanisms of this cell/tissue type-specific GC signaling is still not completely understood.

The data presented in this thesis suggest a new mechanism to explain the effects of Glucocorticoids on cancer growth and how they can lead to chemoresistance. These results in fact demonstrate that GC-signaling directly controls the level and the biological activity of YAP, with consequent increase cell proliferation and survival of cancer cells. YAP has been shown to contribute to cancer stem cell growth and expansion (Cao et al., 2008; Lian et al., 2010; Song et al., 2014; Yimlamai et al., 2014; Zhao et al., 2014). Here, we have clearly shown that GCs have a direct role in triggering breast CSCs expansion, by promoting YAP nuclear localization and activity. Since CSCs are directly linked to chemoresistance and tumour regeneration (Dean et al., 2005; Visvader et al., 2009) it is conceivable that GCs could decrease chemotherapeutical response of breast cancers (and possibly in other tumors) through YAP-dependent CSCs expansion.

Importantly Glucocorticoid doses used during our experimental *in vitro* procedures were comparable to the GC plasma concentrations detectable *in vivo* in cancer patients receiving GCs as supportive therapy (Brady et al., 1987; Nakade et al., 2008). Consistently, we can speculate that Glucocorticoids might preserve the ability to stimulate YAP activity also in murine models and in clinical setting. Thus, harmful effects induced by Glucocorticoids on patients' response to anti-cancer treatment could be justified by this new hormonal regulation of YAP.

Further *in vivo* experiments and clinical experimentations are however necessary to support this hypothesis.

In conclusion, our results suggest that Glucocorticoids co-administration as a supportive care during chemotherapy or radiation treatment in breast cancer patients might, in part, have a detrimental role for the therapeutic success. Moreover, since Glucocorticoids are extensively used in cancer clinical settings, our findings could have clinical relevance: we suggest the Hippo/YAP pathway could be a potential target to be taken into account to prevent, or at least limit, negative effects shown by Glucocorticoids on chemo-sensitivity in breast cancer patients.

## EXPERIMENTAL PROCEDURES

### CELL LINES AND CULTURE CONDITIONS

Cell lines used in this thesis were:

- MDA-MB-231 cell line: human epithelial cells derived from triple negative breast cancer subtype; MDA-MB-231 cells were cultured in DMEM (LONZA) supplemented with 10% FBS (Fetal Bovine Serum) and with 1% antibiotics (penicillin 100U/mL and streptomycin 10µg/mL).
- SUM-149 cell line: human epithelial cells derived from triple negative breast cancer subtype; SUM-149 cells were cultured in DMEM/F12 (LONZA) (1:1) supplemented with 5% HS (Horse Serum) and with 1% antibiotics.
- HeLa cell line: human epithelial cells derived from cervical carcinoma; HeLa cells were cultured in DMEM supplemented with 10% FBS and with 1% antibiotics.
- MII cell line (RAS-transformed MCF10A-T1k cell line): non-tumorigenic human mammary MCF10A cell line, transformed with RAS-transfection and derived from xenograft-passaged MCF10-AneoT cells138; MII cells were cultured in DMEM/F12 (1:1) supplemented with 5% HS and 1% antibiotics.

### HIGH CONTENT SCREENING

For the screening experiments, MDA-MB-231 cells (3.0×103 per well) were seeded on black clear-bottom 384-well plates (PerkinElmer). Twenty-four hours later, the FDA- approved drugs were transferred robotically from library stock plates (0.1mM and 1mM in DMSO) to the plates containing the cells; controls were added to columns 1, 2, 23 and 24 of each plate. Cells were fixed at 48 h after plating, i.e. 24h after addition of drugs, and processed immediately for immunofluorescence. Briefly, cells were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.5% Triton X-100 in phosphate buffered saline (PBS) solution for 10 min, followed by 30 min blocking in 3% FBS. Cells were then incubated with a mouse antibody against YAP/TAZ (Santa Cruz Biotechnology) diluted in blocking solution for 1 h. Cells were further washed with PBS and incubated for 1h with a secondary antibody conjugated

to Alexa Fluor-568 (Life Technologies), and stained with Hoechst 33342 (Life Technologies).

Image acquisition was performed using an ImageXpress Micro automated high-content screening fluorescence microscope (Molecular Devices) at a 10x magnification; a total of 16 images were acquired per wavelength, well and replicate, corresponding to ca. 4,500 cells analyzed per experimental condition and replicate. Image analysis to identify cells presenting predominantly nuclear YAP/TAZ localization was performed using the 'Multi-Wavelength Translocation' application module implemented in MetaXpress software (Molecular Devices).

Screening was performed in duplicate, at two drug concentrations (1µM and 10µM); final concentration of DMSO in the culture medium was 1% (v/v) for all experimental conditions. The screening was performed at the ICGEB High-Throughput Screening Facility (http://www.icgeb.org/high-throughput-screening.html).

**PROTEINS EXTRACTION**

Plated cells were lysed with Lysis Buffer (NP40 1%, Tris-HCL pH=7.5% 50mM, NaCl 300mM, EDTA 1mM) solution, supplemented with protease inhibitors (CLAP 0.1mM and PMSF 1mM) and with phosphatase inhibitors (NaF 5mM and Na3VO4 1mM), and were harvested. Cells were then centrifuged at 10,000 rpm for 10 minutes at 4°C. Concentration of proteins in the lysate was then quantified with the spectroscopic analytical procedure Bradford Protein Assay (Bio- Rad). Samples obtained were denatured in Laemmli Sample Buffer 2X or 6X and boiled for Electrophoresis.

**WESTERN BLOT**

Western blotting allows the antibody detection of specific proteins from extracts made from cells. In order to make the proteins accessible to antibody detection they were moved from within the gel onto a membrane made of nitrocellulose with the blotter Trans-Blot Transfer Cell. The membrane was incubated in Blotto-Tween 20 solution (milk powder 5% w/v in PBS solution, added with Tween20 0.2% w/v) for 30

minutes and then incubated with primary antibody over-night. The next day, membrane was incubated with secondary antibody for at least 30 minutes and finally developed in photographic plates with the solution kits ECL or ECL-Plus (Amersharm).

The antibodies used for western blot were: Anti-YAP/TAZ (1:1000) is sc101199 (Santa Cruz Biothecnology); Anti-pYAP (1:1000) (Ser127) is 4911S (Cell Signaling); anti-ANKRD1 (1:1000) is 11427-1-AP (Proteintech DBA); Anti-actin (1:2000) is C11 (Sigma); Anti-vinculin (1:5000) is V4505 (Sigma); Phalloidin-Rhodamine is R415 (Molecular Probes) (used in immunofluorescence).

**TRANSFECTIONS**

siRNA transfections were performed to knock-down endogenous levels of YAP in MDA-MB- 231 and in MII cells. Transfections were performed with Lipofectamine RNAi-MAX (Life technologies) in antibiotic-free medium according to the manufactured instructions. In detail RNAi-MAX Lipofectamine was diluited in Optimem medium (Invitrogen) and, separately, siRNA-YAP and siRNA-control (siRNA Qiagen 1027281) were also diluited in Optimem medium; after 5 minutes of incubation, RNAi-MAX Lipofectamine solution was added to siRNA solutions and they were incubated for 20 minutes; mixed solutions were finally added to the medium of cells, plated 24 hours before. After 48 hours from transfections, cells were analysed. GFP-GR transfection was performed in MDA-MB-231 cells in order to transiently over-express the construct composed by the GR fused to the green fluorescence protein (GFP-GR). Transfection was performed with Lipofectamine 2000 (Invitrogen) in antibiotic-free medium according to the manufactured instructions. In details, Lipofectamine was diluited in Optimem medium (Invitrogen) and separately another solution with GFP-GR and Optimem was prepared; after 5 minutes of incubation, Lipofectamine solution was added to GFP-GR solution and they were incubated for 20 minutes; mixed solutions were finally added to the medium of cells, plated 24 hours before. After 24 hours from GFP-GR transfection, cells were treated with Betamethasone for 24 hours, and then examined by fluorescence microscopy to detect sub- cellular localization of GR-GFP.

**LUCIFERASE REPORTER ASSAY**

Luciferase assay was used to monitor the transcriptional activation of endogenous Glucocorticoid Receptor (GR) in MDA-MB-231 cells. Luciferase assay was performed in MDA- MB-231 cells with the GR-responsive reporter luc2. Cells were transfected with the MMTV reporter plasmid pGL4.36[luc2P/MMTV/Hygro], (Promega) and with CMV-Renilla to normalize for transfection efficiency. The MMTV reporter plasmid pGL4.36 contains the MMTV LTR which encompasses the natural GRE sequences and drives the transcription of the luciferase Cells were then valued at fluorescence microscopy.

**QUANTITATIVE REAL-TIME PCR**

For total RNA extraction, cells were harvested in Qiazol lysis reagent (a solution of phenol and guanidinium thiocyanate) (Qiagen) and contaminant DNA was removed by specific DNase treatment. Retro-transcription of the extracted RNA was performed using the Quantitec Reverse Transcription kit (Qiagen). The obtained cDNA was properly diluited and used in qRT- PCR reactions. Quantitative Real-Time PCR is a technique used to quantify mRNA of the genes of interest. qRT-PCR reactions were performed with the reagent SsoAdvanced SYBR Green Supermix (Biorad) using CFX96 Touch Real-Time PCR Detection System and analysed with Biorad CFX Manager Software. Expression levels are always given relative to histone H3. Primer sequences used in qRT-PCR are described below:

| GENE | PRIMER NAME | PRIMER SEQUENCE |
|---|---|---|
| H3 | FW | GTGAAGAAACCTCATCGTTACAGGCCTGGT |
| | REV | CTGCAAAGCACCAATAGCTGCACTCTGGAA |
| ANKRD1 | FW | CACTTCTAGCCCACCCTGTGA |
| | REV | CCACAGGTTCCGTAATGATTT |
| YAP1 | FW | GCCGGAGCCCAAATCC |
| | REV | GCAGAGAAGCTGGAGAGGAATG |

**IMMUNOFLUORESCENCE ASSAY**

For the immunofluorescence assay, cells were cultured in 6-multi-well plates with square glass coverslips so that cells could attach to the coverslips. 24h later cells were treated with drugs and control treatments were added. 48h after plating, i.e. 24h after addition of drugs, cells were fixed and processed immediately for immunofluorescence. In detail, cells were fixed to coverslips with 4% paraformaldehyde for 15 min, cell membranes were permeabilized with 0.1% Triton X-100 in Phosphate Buffered Saline (PBS) solution for 10 min, and cells then were incubate in Blocking Buffer (3% Fetal Bovine Serum (FBS) [Gibco] in PBS solution) for 30 minutes. Next, cells were incubated with an antibody against YAP (Santa Cruz Biotechnology) diluted (1:100) and with Rhodamine phalloidin peptide diluted (1:700) in Blocking Buffer solution in a humidified chamber at 37°C for 1 hour. Cells were further washed with PBS and incubated again in the humidified chamber at 37°C for 40 min with a secondary antibody, diluted (1:500) for YAP immunofluorescence in Blocking Buffer solution and conjugated to Alexa Fluor-568 (Life Technologies). Finally cells were stained with Hoechst 33342 (Life Technologies) and coverslips were mounted on glass slides for the analysis with the fluorescence microscopy.

**RNA EXTRACTION AND QRT-PCR FROM DROSOPHILA OVARIES**

Wild type individuals from the Oregon-R strain were maintained for the entire development on the food added with betametasone 1 μM. The ovaries from females treated with betametasone were manually dissected in Ringer's solution (182mM KCl, 46mM NaCl, 3mM $CaCl_2$, 10mM Tris-HCl, pH7.5). Total RNA was extracted from 40 ovaries using the RNAqueos-4 PCR Kit (AMBION). To remove any DNA from the preparation, the samples were incubated with DNase I RNase free (AMBION; 1 $Umg^{-1}$ RNA) at 37 °C for 30 min, in a total volume of 100 μl. After this treatment, the enzyme was inactivated with the DNase inactivation reagent (AMBION). DNase-treated RNA was precipitated at −80 °C overnight and after centrifugation it was dissolved in 50 μl of nuclease-free water. In the first-strand cDNA synthesis, 5 μg of total RNA were used as a template for oligonucleotide dT primed reverse

transcription using SuperScript III RNaseH-reverse transcriptase (Life Technologies). Real-time PCR was performed in the SmartCycler Real-time PCR (Cepheid) using SYBR green (Euroclone) according to manufacturer's protocol. For quantification of the transcripts we used the 2ΔΔCt method.

The primer sequences are:

**diap1 F** 5'GAAAAAGAGAAAAGCCGTCAAGT3'

**diap1 R** 5'TGTTTGCCTGACTCTTAATTTCTTC3'

**yki F** GCGCCTTGCCGCCGGGATG

**yki R** GCTGGCGATATTGGATTCTC

**rp49 F** ATCGGTTACGGATCGAACAA

**rp49 R** GACAATCTCCTTGCGCTTCT

**MAMMOSPHERE ASSAY**

To obtain mammospheres, cells from monolayer cultures were enzymatically disaggregated (0.05% Trypsin-EDTA) to a single cell cultures, passed through a 40 µm single cell strainer, plated a clonogenic density (2500 cells/cm2), grown in "ultra-low attachment" plates that prevent cell adherence, and cultured in a specific stem-cell growth medium that preserves cells in undifferentiated state. In detail, cells were grown for 7-10 days in MEBM (Mammary Epithelial Basal Medium-Serum free) [Lonza] supplemented with B27 (Invitrogen), 20 ng/ml EGF (PROSPEC), 20 ng/ml bFGF (BD Biosciences), 4 µg/ml heparin (StemCell Technologies Inc.), 0.5 µg/ml Hydrocortisone (Sigma) and 5 µg/ml Insulin (Sigma) in low attachment 24 well plates (Coroning) in a humidified incubator at 37°C, 5% CO2. Primary mammospheres (≥200 µm) were obtain, collected, counted and again enzymatically disaggregated as above to re- plate cells at clonogenic densities to obtain secondary mammospheres. Percentages of mammosphere forming efficienties (%MFE) were calculated as number of mammospheres divided by the plated cell number and multiplied by a hundred. Mammospheres were counted with a 20x objective on an Olympus CK30 microscope.

**REAGENTS AND PLASMIDS**

The library of FDA-approved drugs (Screen-Well FDA-Approved Drug Library, 640 chemical compounds dissolved at 10mM in DMSO) was obtained from Enzo Life Sciences (Enzo Life Sciences Inc.,Plymouth Meeting, PA, USA). The following compounds were purchased from Sigma Aldrich:

| | |
|---|---|
| L5163 100UG | Latrunculin A |
| H0888-1G | Hydrocortisone BioReagent, suitable for cell culture |
| F8880-100MG | Fluocinolone acetonide |
| SML0005-5MG | Cerivastatin |
| S5068-10MG | Salmeterol |
| B7005-100MG | Betamethasone |
| M8046-100MG | Mifepristone |

**CHROMATIN IMMUNOPRECIPITATION PROCEDURE**

Proteins were cross-linked to DNA by adding formaldehyde directly to culture medium to a final concentration of 1% and incubate for 10 minutes at 22-25°C. The reaction was blocked by adding glycine (in PBS1X) to a final concentration of 0,125M. Cells are washed using ice cold PBS containing protease inhibitors (1mM phenylmethylsulfonyl fluoride (PMSF), 1microgram/ml aprotinin and 1microgram/ml pepstatin A), scraped, and pellet was obtained by centrifuge for 4 minutes at 2000 rpm at 4°.

Nuclei were resuspended in 5 volumes of lysis buffer [Pipes (piperazine N,N bis zethone sulfonic acid) pH 8 5mM, KCl 85mM, NP40 0,5%] plus protease inhibitors for 20 minute on ice. Then nuclei are Centrifuged for 10min at 2000rpm at +4°C and resuspended in SDS-Lysis Buffer (1%SDS; 10mMEDTA; 50mM Tris-HCl, pH8.1) for 10 minutes on ice.

Then Nuclei are sonicated to shear DNA to lengths between 500 and 800 basepairs Sonicated DNA was seen by gel electrophoresis. DNA was incubated and immunoprecipitated with rabbit anti-GR (Santa Cruz Biotech.) and rabbit anti-IgG using Pierce ChIP-grade Protein A/G magnetic beads (Thermo Fisher Scientific, Rockford, IL, USA).

Primers used for the amplification of the different regulatory regions are:

YAP promoter -1150 to -900 fw: GCTCCTTGTACATCAGGTGCC

YAP promoter -1150 to -900 rev : GGACTCCGTTAATGTGGACTGA

Negative CTR fw: CAACCAAAGCCCATGTCCTC

Negative CTR rev: AGGCACGCTACAGGGCTTC

The promoter occupancy was measured using the CFX96 Touch Real-Time PCR Detection System and analysed with Biorad CFX Manager software. The experiment was repeated two times.

# FIGURES

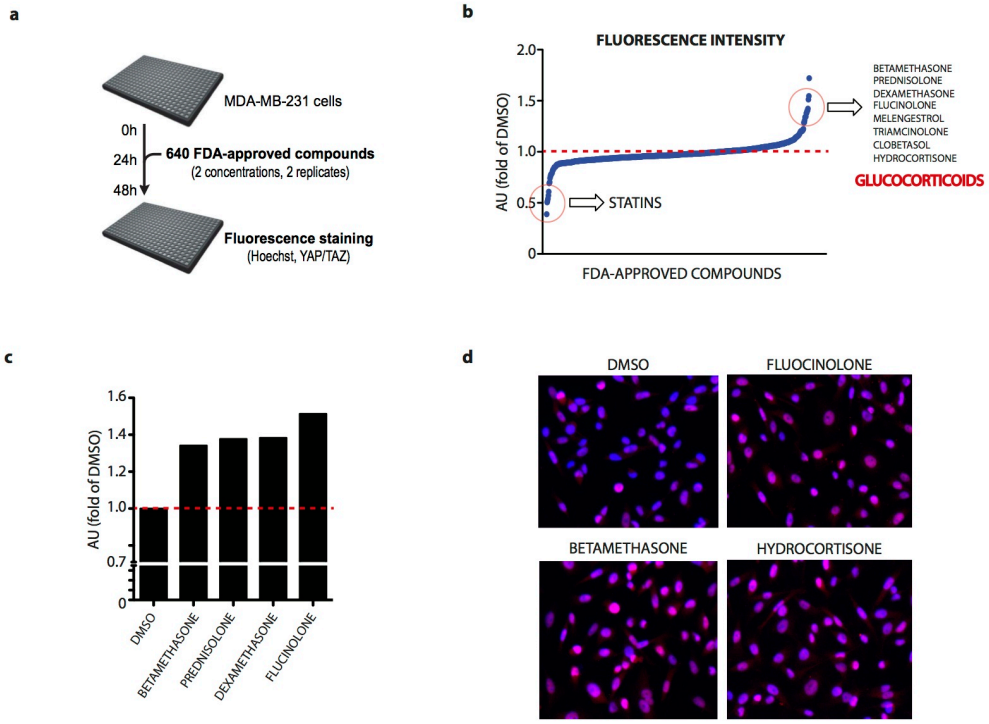



**Figure 1. Identification of drugs regulating YAP protein levels in cancer cells. (a)** Schematic representation of the high-content screening. MDA-MB-231 cells were seeded in 384-well plates and 24h later the FDA-approved compounds were added to cells at 1 or 10µM. 24h after treatment, cells were fixed and processed for immunofluorescence for YAP/TAZ and stained with Hoechst. Automated image acquisition and analysis was then performed to detect the YAP-relative fluorescence intensity at single-cell level and to analyse the subcellular localization of YAP. The screening was performed in duplicate; ca. 4,500 cells were analysed per experimental condition and replicate. **(b)** Results of the screening. Levels of YAP fluorescence intensity are increased by Glucocorticoids and are reduced by Statins. Red dashed line represents the fluorescence levels in cells treated with dimethylsulphoxide (DMSO). AU=arbitrary units. **(c)** Quantification of YAP fluorescence levels from the screening. Representative YAP fluorescence levels in cells treated with DMSO or Glucocorticoids are shown. Values are normalized on fluorescence levels of DMSO (red dashed line; set as 1.0). AU=arbitrary units. **(d)** Representative images of immunofluorescence from the screening. MDA-MB-231 cells stained for Hoechst (blue colour) and YAP (red colour) after treatment with DMSO or with three Glucocorticoids present in the library are shown: YAP-related fluorescence increased with Glucocorticoid treatment.

| *Drug* | *Round 1 intensity* | *Round 2 intensity* | *Average intensity* |
|---|---|---|---|
| Etoposide | 1.74 | 1.70 | 1.72 |
| Cytarabine | 1.76 | 1.49 | 1.63 |
| Topotecan | 1.65 | 1.48 | 1.57 |
| Clobetasol propionate | 1.46 | 1.63 | 1.55 |
| Methylprednisolone | 1.51 | 1.51 | 1.51 |
| Albendazole | 1.71 | 1.23 | 1.47 |
| Triamcinolone | 1.52 | 1.33 | 1.42 |
| Hydrocortisone 21-acetate | 1.34 | 1.48 | 1.41 |
| Mitomycin c | 1.39 | 1.41 | 1.40 |
| Fluocinolone acetonide | 1.50 | 1.26 | 1.38 |
| Dexamethasone | 1.46 | 1.30 | 1.38 |
| Kasugamycin | 1.28 | 1.41 | 1.35 |
| Betamethasone | 1.42 | 1.26 | 1.34 |
| Hydrocortisone | 1.30 | 1.38 | 1.34 |
| Fluoxetine | 1.69 | 0.93 | 1.31 |
| Fenbendazole | 1.47 | 1.10 | 1.29 |
| Melengestrol acetate | 1.20 | 1.37 | 1.29 |
| Prednisolone | 1.31 | 1.14 | 1.22 |
| Vinorelbine | 1.38 | 1.06 | 1.22 |
| Rufloxacin | 1.54 | 0.89 | 1.21 |
| Corticosterone | 1.13 | 1.29 | 1.21 |
| Tolfenamic acid | 1.23 | 1.18 | 1.20 |
| Flubendazole | 1.27 | 1.13 | 1.20 |
| DMSO | 1.00 | 1.00 | 1.00 |

**Table 1. FDA-approved drugs from screening that increase YAP fluorescence intensity.** FDA-approved drugs from the screening that increase YAP fluorescence intensity. List of the main FDA- approved compounds from the library used in the screening that increase YAP fluorescence intensity at high levels. Drugs highlighted in green belong to the Glucocorticoids class. Values indicate relative YAP fluorescence intensity in MDA-MB-231 cells after treatments. Values are normalized on YAP fluorescence levels after treatment with dimethylsulphoxide (DMSO) (highlighted in red), set as 1.0. Round 1 intensity=YAP fluorescence intensity in the first round of the screening; Round 2 intensity= YAP fluorescence intensity in the second round of the screening.

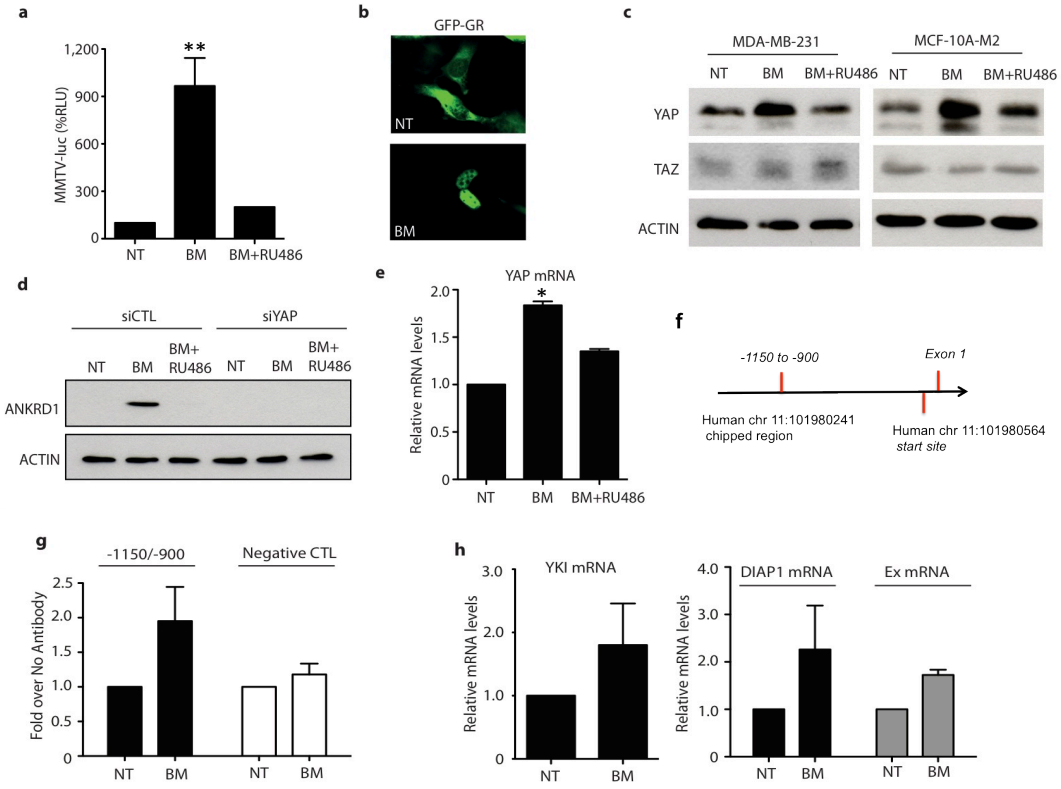



**Figure 2**. **MDA-MB-231 cells are responsive to glucocorticoid treatment. (a)** Effect of Betamethasone on the induction of transcriptional activity of endogenous Glucocorticoid Receptor in MDA-MB-231 cells assayed by luciferase reporter assay. Quantification of GR-induced luminescent signal is shown. Cells were transfected with MMTV-luc and treated with DMSO or 1μM Betamethasone alone or with RU486 (1μM) for 24 hours. NT=not treated (DMSO); BM=Betamethasone; RU486=Mifepristone; RLU=relative light units. Error bars represent mean ± s.d.; experiment repeated three times. Statistical significance is indicated by p-value: **p<0.01 vs DMSO. **(b)** Betamethasone promotes nuclear translocation of the Glucocorticoid Receptor in MDA-MB-231 cells. Cells overexpressing GFP-GR were treated with DMSO or with Betamethasone (1μM) for 24 hours. Representative images of immunofluorescence for GFP-GR subcellular localization are shown: Betamethasone promotes the cytoplasmic-to-nucleus translocation of the fusion protein. NT=not treated (DMSO); BM=Betamethasone; GFP-GR=Green Fluorescence Protein-Glucocorticoid Receptor fusion protein. **(c) Glucocorticoids increase YAP protein levels in MDA-MB-231 and MCF-10A-M2.** Western Blot analysis showing total YAP and TAZ protein levels of MDA-MB-231 and MCF-10A-M2 cells treated with DMSO or 1μM Betamethasone alone or with 1μM RU486 for 24 hours. Values are calibrated on actin levels. NT=not treated (DMSO); BM=Betamethasone; RU486=Mifepristone. **(d) Glucocorticoids increase YAP transcriptional activity and protein level of a well-known target gene ANKRD1.** Western Blot of ANKRD1 protein levels in MDA-MB-231 cells is shown. Cells were transfected with indicated siRNA for 48 hours and then treated with DMSO or 1μM Betamethasone alone or with 1μM RU486 for 24 hours. Values are calibrated on actin levels. siCTL=control siRNA; siYAP=YAP siRNA; NT=not treated (DMSO); BM=Betamethasone; RU486=Mifepristone. **(e) Glucocorticoid receptor induces YAP mRNA transcription (e)** Quantitative PCR analysis of YAP mRNA expression in MDA-MB-231 cells. Values are normalized to YAP mRNA levels in not treated cells (set as 1.0) Cells were treated with DMSO or 1μM Betamethasone alone or with 1μM RU-486. **(f) Glucocorticoid receptor directly promotes YAP mRNA transcription.** Schematic graph illustrating genomic location of YAP gene. The putative promoter region chipped by chromatin immunoprecipitation (ChIP) are also reported. **(g)** ChiP analysis was performed in MDA-MB-231 cells ($3*10^7$ cells) treated for 6 hours and then fixed. Data are normalized to negative CTR, a region of heterochromatin. Error bars represent mean ± s.d. from n = 2 replicates.

**Glucocorticoids promotes YAP mRNA transcription and activity *in vivo*. (h)** Quantitative PCR analysis of YKI DIAP1 and Expanded mRNA expression extracted from 40 female individuals. The ovaries from female were maintained for the entire development on the food added with betamethasone 1 μM. Error bars represent mean ± s.d. from n = 3 biological replicates.

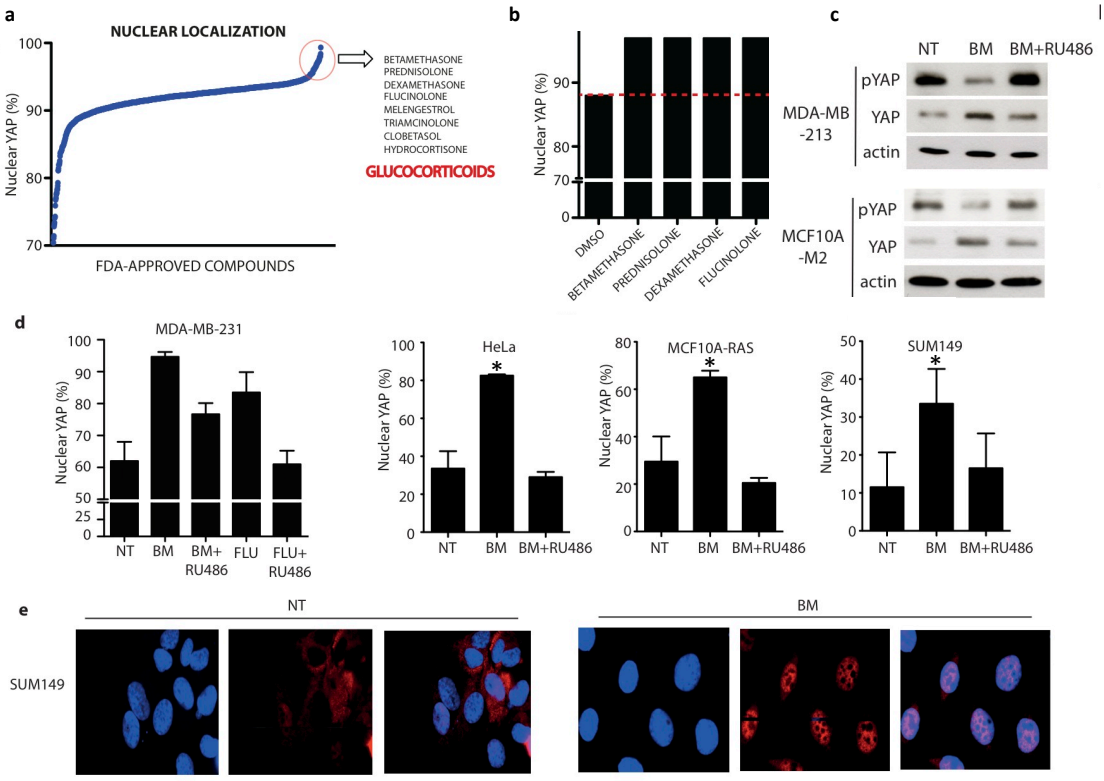



**Figure 3. Glucocorticoids regulate YAP protein localization**. **(a)** Results of the high-content screening. YAP nuclear localization is increased by Glucocorticoids. **(b)** Validation experiments of the screening in MDA-MB-231 cells. Quantification of cells with nuclear YAP. Cells were treated with DMSO or Betamethasone 1µM for 24h; cells were then fixed and processed for immunofluorescence for YAP. Representative blot of immunofluorescence analysis is shown. NT=not treated (DMSO); BM=Betamethasone; RU486=Mifepristone. **Glucocorticoids regulate YAP phosphorylation at S127. (c)** Western Blot of MDA-MB-231 and MCF10A-M2 cells after treatment with DMSO or 1µM Betamethasone alone or with 1µM RU486 for 24 hours. Total YAP protein levels and phospho-YAP protein levels are shown. Values are calibrated on actin levels of cells. NT=not treated (DMSO); BM=Betamethasone; RU486=Mifepristone; pYAP=YAP phosphorylation on residue Ser127. **Glucocorticoids regulate YAP nuclear localization in different cell lines. (d left)** Quantification of MDA-MB-231 cells with nuclear YAP after treatment with DMSO or 1µM Betamethasone or Fluocinolone 1 µM alone or with 1µM RU486 for 24 hours. Data are derived from *n* = 2 independent experiments. Error bars represent mean ± s.d. **(d right)** Quantification of HeLa, MCF10A-RAS and SUM-149 cells with nuclear YAP after treatment with DMSO or 1µM Betamethasone alone or with 1µM RU486 for 24 hours. Data are derived from *n* = 3 independent experiments. Error bars represent mean ± s.d;*p<0.01 vs DMSO. **(e)** Representative images of immunofluorescence in breast cancer cell line SUM-149 are shown. Cells were stained for Hoechst (blue colour) and YAP (red colour): YAP localization is mainly cytoplasmic in not treated (NT) cells, whereas Betamethasone (BM) promotes YAP nuclear accumulation.

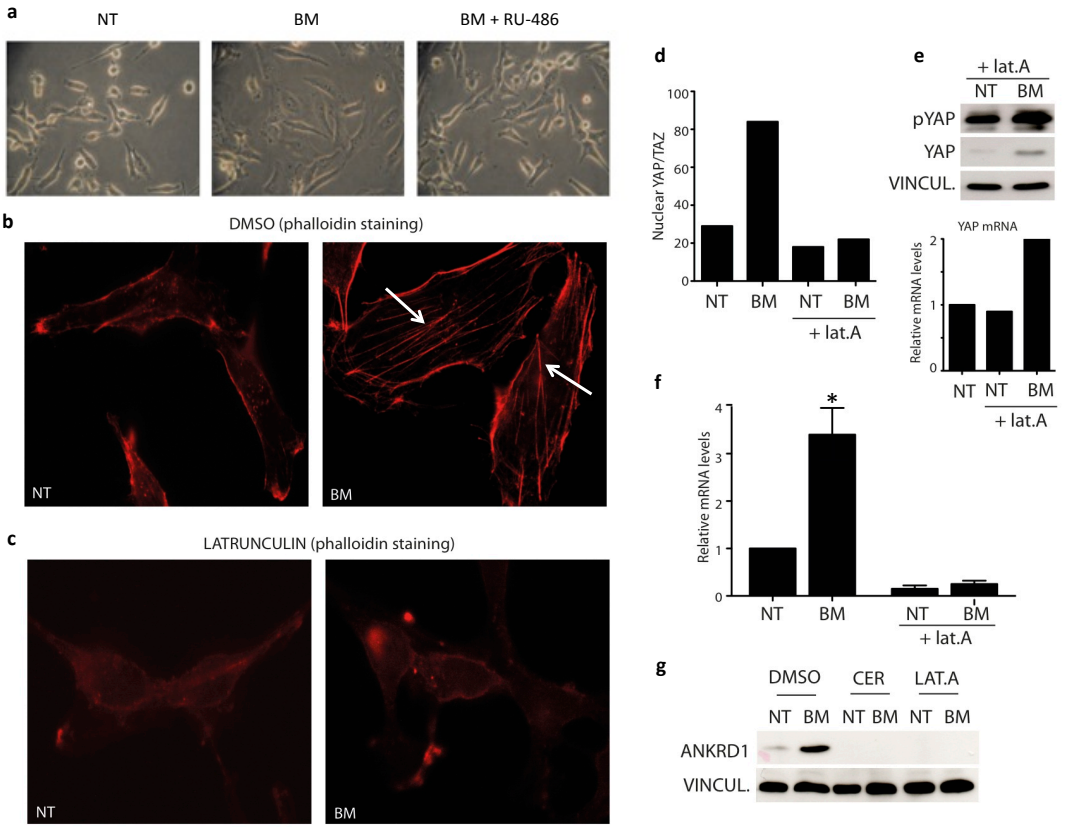



**Figure 4 (a)** Representative image of MDA-MB-231 cell lines treated with DMSO or with Betamethasone (1μM) alone or with RU-486 (1μM) for 24 hours. **Glucocorticoids promote YAP nuclear accumulation by controlling actin cytoskeleton dynamics. (b)** Betamethasone increases the formation of F-actin stress fibers in MDA-MB-231 cells. Cells were treated with DMSO or with Betamethasone (1μM) for 24 hours and then fixed, processed for immunofluorescence and stained for Phalloidin. Representative images of the actin microfilaments (F-actin) within the cells are shown. NT=not treated (DMSO); BM=Betamethasone. **(c)** Latrunculin-A prevents Betamethasone-induced formation of F-actin stress fibers. Cells were treated with 1μM Latrunculin-A alone or with 1μM Betamethasone for 24 hours, and then processed for immunofluorescence and stained for Phalloidin as in a. Representative images of the actin microfilaments within the cells are shown. **(d)** Quantification of nuclear YAP levels in cells treated with DMSO or 1μM Betamethasone alone or with 1μM Latrunculin-A for 24 hours. Representative blots are shown. NT=not treated (DMSO); BM=Betamethasone; latA=Latrunculin-A. **(e)** Western blot of Total YAP protein levels and phospho-YAP protein levels are shown. Values are calibrated on vinculin levels in MDA-MB-231 cells treated with 1μM Latrunculin-A alone or with 1μM Betamethasone for 24 hours. NT=not treated (DMSO); BM=Betamethasone; latA=Latrunculin-A. **(f)** Quantitative PCR analysis of ANKRD1 mRNA expression in MDA-MB-231 cells. Values are normalized to ANKRD1 mRNA levels in not treated cells (set as 1.0) Cells were treated with DMSO or 1μM Betamethasone alone or with 1μM Latrunculin-A for 24 hours. Error bars represent mean ± s.d. from n = 3 biological replicates; *p<0.01 vs DMSO. **(g)** Western Blot of ANKRD1 protein levels in MDA-MB-231 cells is shown. Cells were treated with DMSO or 1μM Betamethasone alone or with 1μM Latrunculin-A or with 1μM Cerivastatin (CER) for 24 hours. Values are calibrated on vinculin levels.

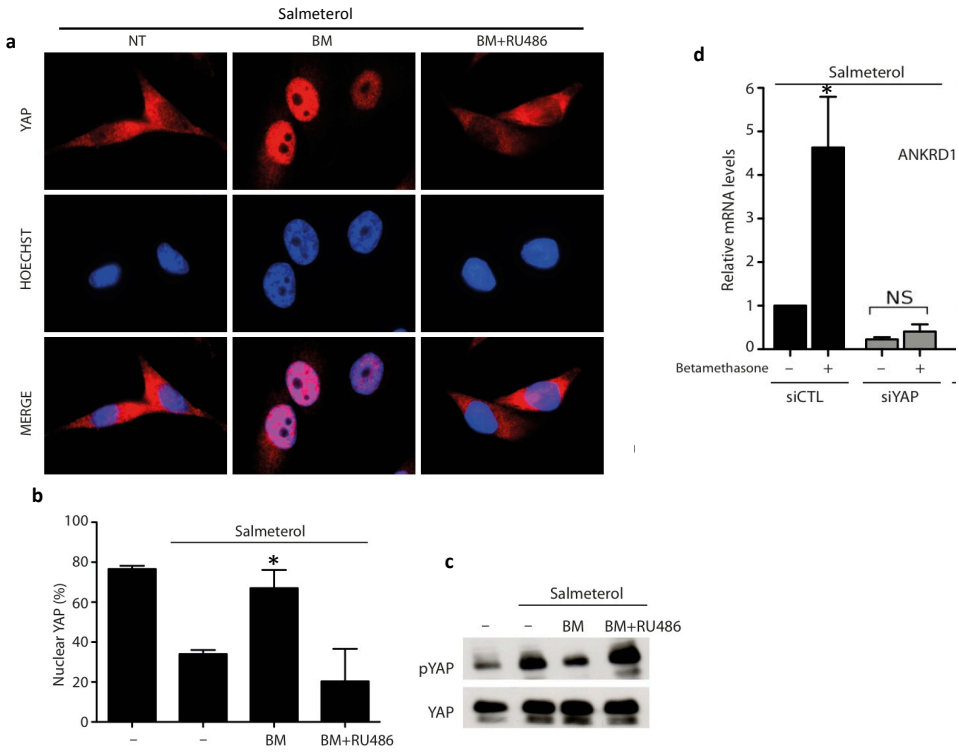



**Figure 5. Glucocorticoids counteract the GPCRs-mediated negative regulation of YAP. (a)** Representative images of immunofluorescence in breast cancer cell line MDA-MB-231 are shown. Cells were stained for Hoechst (blue colour) and YAP (red colour): YAP localization is mainly cytoplasmic after treatment with Salmeterol 10 µM (left) and become nuclear when cells are co-treated with Betamethasone 1µM (middle) alone or with RU486 1µM (right) for 24h. **(b)** Quantification of nuclear YAP by immunofluorescence. Cells treated as in (a). Error bars represent mean ± s.d. from n = 3 biological replicates; *p<0.01 vs DMSO. **(c)** Western Blot of MDA-MB-231 cells treated as in (a). Cel Total YAP protein levels and phospho-YAP protein levels are shown. NT=not treated (DMSO); BM=Betamethasone; RU486=Mifepristone. **(d)** Quantitative PCR analysis of ANKRD1 mRNA expression in MDA-MB-231 cells. Cells are knocked down for YAP through siRNA transfection for 48 hours before Salmeterol 10 uM treatment with or alone betamethasone. Error bars represent mean ± s.d. from n = 3 biological replicates; *p<0.01 vs DMSO.

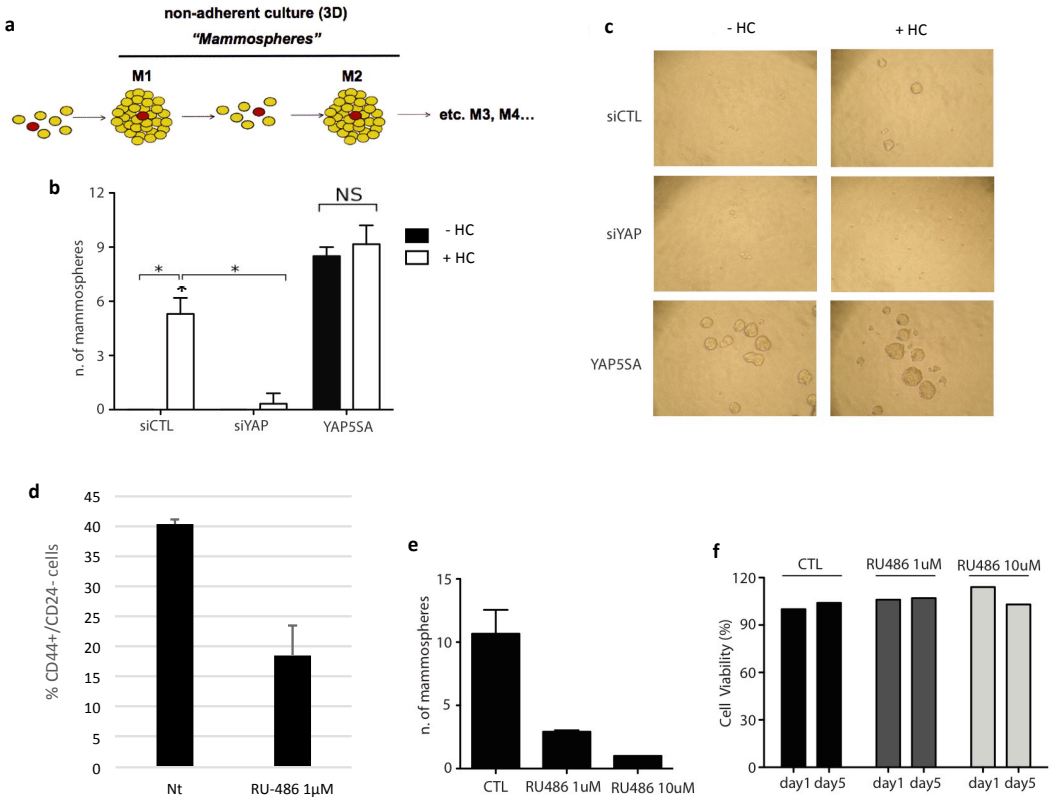
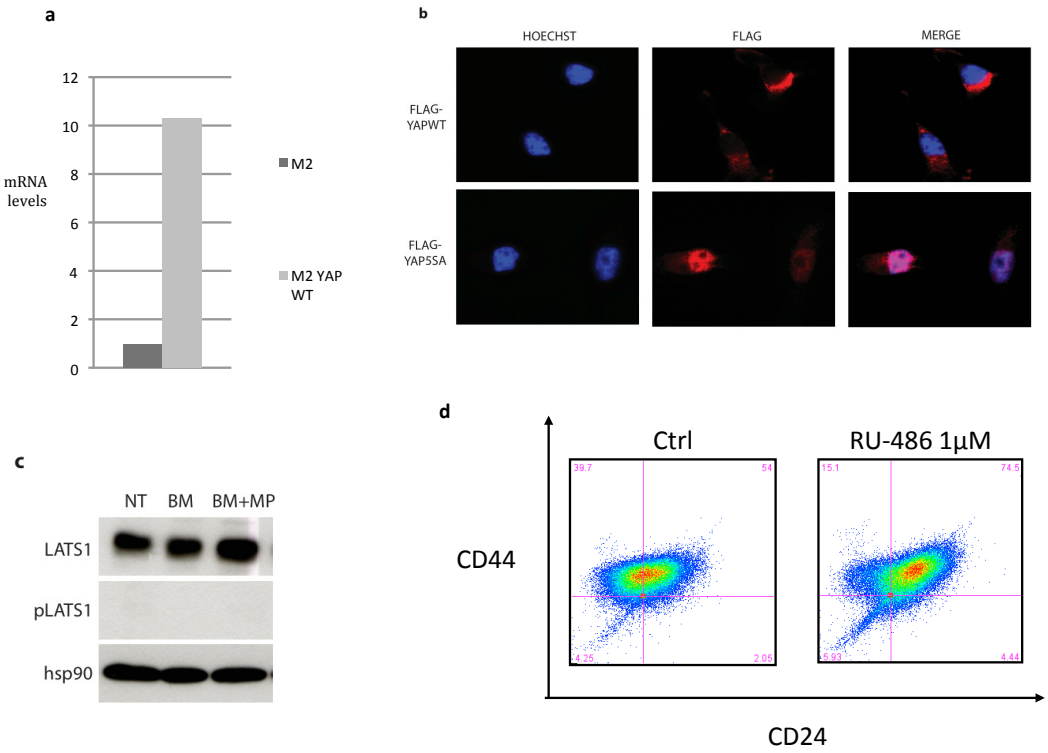



**Figure 6 Mammosphere assay's scheme (a)** Cells are grown in non-adherent conditions in a specific stem-cell growth medium. Primary mammospheres (M1) obtained are enzymatically disaggregated and cells are grown again in the same culture conditions to obtain secondary mammospheres (M2). This process of disaggregation of mammospheres and of specific culture of cells can be repeated several times to obtain tertiary (M3), quaternary mammosphere (M4) etc. **Glucocorticoids induce mammary cancer stem cells self-renewal and CSC features**. **(b)** Hydrocortisone induces formation of mammospheres in M2 cells by activating YAP. The number of secondary mammospheres after treatments and siRNA transfections are indicated. Cells were transfected with the indicated siRNA for 48 hours. Then, M2-transfected cells and 5SA-YAP-overexpressing M2 cells were grown in a specific stem-cell growth medium and cultured in non-adherent conditions with or without Hydrocortisone 0.5 µg/ml. 7 days after, primary mammospheres were quantified, disaggregated and re-plated in the same conditions. After further 7 days, secondary mammospheres obtained were counted. Error bars represent mean ± s.d. from n = 3 biological replicates. * indicates statistical significance: $p<0.01$; NS=not significant. **(c)** Optical microscope photographs of secondary mammospheres obtained after indicated treatments and transfections. HC=Hydrocortisone; siCTL=control siRNA; siYAP=YAP siRNA; YAP5SA=M2 cells that stably express the constitutively nuclear-localized form of YAP. **(d)** CD44/CD24 FACS analysis (fluorescence activated cell sorting) of M2 cell line; histograms representative of $CD44^{+}/CD24^{-}$ M2 cells treated for 1 week with RU-486 1 uM are shown. **(e)** RU-486 reduces the formation of M2 mammospheres formation. Cells were treated with RU-486 1 uM and 10 uM for 48 hours. Then cells were grown in mammospheres as previously described in Figure 6b. Error bars represent mean ± s.d. from n = 3 biological replicates. **(f)** Viability assay of control and MCF-10A-RAS cell lines after treatment with RU-486 1 and 10 µM for 5 days. Data are normalized to untreated. Error bars represent mean ± s.d., from *n* = 3 biological replicates.




**Supplementary Figure 1. (a)** Quantitative PCR of YAP mRNA expression in stable clone of M2 cell line constitutively expressing YAP. **(b)** Representative immunofluorescence of M2 cell line expressing YAP WT and YAP 5SA. **(c)** Western blot of MDA-MB-231 cell line treated with betamethasone alone or with RU-486. **(d)** Facs analysis showing CD44/CD24 markers after treatment with RU-486.

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## APPENDIX

During my PhD I have been involved in the following publications:

Sorrentino, G., Mioni, M., Giorgi, C., Ruggeri, N., Pinton, P., Moll, U., Mantovani, F., and Del Sal, G. (2013). The prolyl--isomerase Pin1 activates the mitochondrial death program of p53. Cell Death Differ *20,* 198---208.

ABSTRACT: In response to intense stress, the p53 tumor suppressor rapidly mounts a direct mitochondrial death program that precedes transcription-mediated apoptosis. By eliminating severely damaged cells, this pathway contributes to tumor suppression as well as to cancer cell killing induced by both genotoxic drugs and non-genotoxic p53-reactivating molecules. Here we have explored the role played in this pathway by the prolyl-isomerase Pin1, a crucial transducer of p53's phosphorylation into conformational changes unleashing its pro-apoptotic activity. We show that Pin1 promotes stress-induced localization of p53 to mitochondria both *in vitro* and *in vivo.* In particular, we demonstrate that upon stress-induced phosphorylation of p53 on Ser46 by HIPK2, Pin1 stimulates its mitochondrial trafficking signal, i.e. monoubiquitination. This pathway is induced also by the p53- activating molecule RITA, and we demonstrate the strong requirement of Pin1 for the induction of mitochondrial apoptosis by this compound. These findings have significant implications for treatment of p53-expressing tumors and for prospective use of p53-activating compounds in clinics.

Sorrentino, G., Ruggeri, N., Specchia, V., Cordenonsi, M., Mano, M., Dupont, S., Manfrin, A., Ingallina, E., Sommeggio, R., Piazza, S., Rosato, A., Piccolo, S., Del Sal, G. (2014). Metabolic control of YAP and TAZ by the mevalonate pathway. Nature Cell Biology, doi:10.1038/ncb2936

ABSTRACT: The YAP and TAZ mediators of the Hippo pathway (hereafter called YAP/TAZ) promote tissue proliferation and organ growth. However, how their biological properties intersect with cellular metabolism remains unexplained. Here, we show that YAP/TAZ activity is controlled by the SREBP/mevalonate pathway. Inhibition of the rate-limiting enzyme of this pathway (HMG-CoA reductase) by statins opposes YAP/TAZ nuclear localization and transcriptional responses. Mechanistically, the geranylgeranyl pyrophosphate produced by the mevalonate cascade is required for activation of Rho GTPases that, in turn, activate YAP/TAZ by inhibiting their phosphorylation and promoting their nuclear accumulation. The mevalonate–YAP/TAZ axis is required for proliferation and self-renewal of breast cancer cells. In *Drosophila melanogaster,* inhibition of mevalonate biosynthesis and geranylgeranylation blunts the eye overgrowth induced by Yorkie, the YAP/TAZ

orthologue. In tumour cells, YAP/TAZ activation is promoted by increased levels of mevalonic acid produced by SREBP transcriptional activity, which is induced by its oncogenic cofactor mutant p53. These findings reveal an additional layer of YAP/TAZ regulation by metabolic cues.