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CICLO XXV

**Dissecting TAZ biology:
mechanisms and regulations**

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(Platone, Apologia di Socrate)

INDEX

ABSTRACT (english)	1
ABSTRACT (italiano)	3
PUBLICATIONS	5

Part I

Mechanisms of TAZ regulation and activity in breast cancer stem cells	7
INTRODUCTION	9
1. TAZ AND YAP	9
1.1. <i>TAZ and YAP in mammalian stem cells</i>	9
1.2. <i>TAZ and YAP in cancer</i>	10
1.3. <i>Upstream regulators of TAZ and YAP</i>	11
1.4. <i>TAZ and YAP as transcriptional co-activators</i>	13
2. CANCER STEM CELLS	14
2.1. <i>The cancer stem cell concept</i>	14
2.2. <i>Cancer stem cells in breast cancer</i>	15
2.3. <i>Cancer stem cells and the epithelial-to-mesenchymal transition</i>	16
SUMMARY OF PREVIOUS DATA AND AIM OF THE PROJECT	17
RESULTS	18
1. UPSTREAM REGULATORS OF TAZ IN BREAST CANCER STEM CELLS	18
1.1. <i>EMT promotes self-renewal through TAZ in breast cancer cells and in non-transformed mammary epithelial cells.</i>	18
1.2. <i>TAZ is activated by Scribble deregulation.</i>	20
1.3. <i>Scribble regulates TAZ through the Hippo pathway</i>	21
2. MOLECULAR EVENTS DOWNSTREAM OF TAZ IN BREAST CANCER CELLS	23
2.1. <i>TAZ downregulates the expression mir-200 family members</i>	23
2.2. <i>Overexpression of mir-200c opposes TAZ biological activity in MII cells</i>	24
2.3. <i>An experimental plan to investigate TAZ transcriptional program</i>	26
DISCUSSION	30
1. TAZ activity is promoted by EMT	30
2. Scribble is a novel upstream regulator of Hippo signaling in mammals	31
3. A cell-specific requirement of mir-200 downregulation for TAZ activity	32
4. Investigating the mechanism of TAZ activity	33

<u>Part II</u>	
Role of TAZ in Wnt biological responses	35
INTRODUCTION	37
1. The canonical Wnt signaling pathway	37
2. Wnt signaling in stem cells	38
3. Wnt/ β -catenin signaling cascade and TAZ/YAP: a comparison	39
SUMMARY OF PREVIOUS DATA AND AIM OF THE PROJECT	41
RESULTS	42
1. ROLE OF TAZ IN WNT-DEPENDENT DIFFERENTIATION OF MESENCHYMAL STEM CELLS	42
1.1. <i>TAZ stabilization is part of the “osteogenic program” promoted by Wnt</i>	42
1.2. <i>Fat differentiation requires TAZ degradation by the destruction complex in a “Wnt OFF” state</i>	43
2. ROLE OF TAZ DOWNSTREAM OF ONCOGENIC WNT SIGNALING IN APC-MUTANT COLORECTAL CANCER CELLS	44
2.1. <i>Oncogenic Wnt signaling regulates TAZ protein levels</i>	44
2.2. <i>TAZ contributes to the biological properties of APC-mutant colorectal cancer cells</i>	45
2.3. <i>Two transcription factors mediate Wnt gene responses in colorectal cancer cells</i>	46
3. β -CATENIN AS TAZ INHIBITOR IN BREAST CANCER CELLS	47
DISCUSSION	49
1. TAZ is a mediator of Wnt signaling	49
2. β -catenin as an active repressor of TAZ activity	50
CONCLUSION	51
MATERIALS AND METHODS	53
REFERENCES	59
FIGURES	67
Table 1 - siRNA SEQUENCES	99
Table 2 - ANTIBODIES FOR WESTERN BLOT	100
Table 3 - PCR PRIMER SEQUENCES	101

ABSTRACT

(English)

The central theme of this thesis is TAZ, a transcriptional co-activator that works - together with its homologue YAP - as transducer of the mammalian Hippo pathway.

Since the beginning of my PhD training, I have been involved in the investigation of TAZ function and regulation in breast cancer stem cells. Our findings established TAZ as a molecular determinant of self-renewal and tumorigenic potential in breast cancer cells. Indeed, the vast majority of high grade breast tumors (known to be enriched with cancer stem cells) display a strong positive staining for TAZ, compared with normal mammary tissue and low grade tumors. Experiments described in the first part of this thesis show that TAZ protein is stabilized in cells that have undergone an epithelial-to-mesenchymal transition, or simply lost the apico-basal polarization proper to epithelial cells. Indeed, in polarized epithelial cells the basolateral determinant Scribble activates the Hippo kinases to inhibit TAZ; in depolarized cells (including cells in malignant carcinomas) this inhibitory mechanism is disabled and TAZ can accumulate. How TAZ overexpression modifies the cell to turn it into a cancer stem cell is the object of ongoing work. Part of these results was published in (Cordenonsi et al., 2011).

During the last year, I have collaborated on a second project, dealing with TAZ regulation by Wnt signaling (Azzolin et al., 2012). We have found that Wnt signaling activates TAZ along with β -catenin. This happens because, in the absence of Wnt signals, phosphorylated β -catenin drives TAZ degradation; Wnt stimulation thus leads to the parallel stabilization of β -catenin and TAZ. Experiments presented here show that β -catenin effectively inhibits TAZ activity in the absence of Wnt signaling, and that TAZ stabilization is instrumental in both physiological and pathological Wnt biological responses.

To facilitate reading, these two stories are presented separately, as Part I and Part II of this thesis.

ABSTRACT

(Italiano)

Il tema centrale di questa tesi è TAZ, un co-attivatore trascrizionale che – insieme al suo omologo YAP – funge da trasduttore della Hippo pathway nei mammiferi.

Dal primo anno di dottorato, ho partecipato allo studio della funzione e la regolazione di TAZ nelle cellule staminali del tumore al seno. I risultati ottenuti dimostrano che TAZ è un determinante molecolare della capacità di auto-rinnovamento (*self-renewal*) e delle proprietà tumorigeniche di cellule di tumore mammario. La maggioranza dei tumori di alto grado istologico (che contengono un numero elevato di cellule staminali tumorali) esprimono livelli di TAZ più alti rispetto al tessuto sano della ghiandola mammaria e ai tumori di basso grado istologico. Gli esperimenti descritti nella prima parte di questa tesi dimostrano che TAZ è stabilizzato – a livello di proteina – in cellule epiteliali che hanno subito una trasformazione mesenchimale, o hanno perso la polarità apico-basale tipica delle cellule epiteliali. Infatti, in cellule epiteliali polarizzate Scribble, un determinante basolaterale, attiva le chinasi della Hippo pathway ed inibisce TAZ; in cellule depolarizzate (come molte cellule nei carcinomi maligni) questo meccanismo è inattivo e TAZ si accumula. Come la stabilizzazione di TAZ modifichi le cellule per trasformarle in cellule staminali tumorali è oggetto di studi attualmente in corso. Parte di questi risultati è stata pubblicata in (Cordenonsi et al., 2011).

Durante l'ultimo anno, ho collaborato ad un secondo progetto, dedicato alla regolazione di TAZ da parte di Wnt/ β -catenina (Azzolin et al., 2012). I segnali Wnt attivano TAZ insieme a β -catenina. Ciò è dovuto al fatto che, in assenza di Wnt, β -catenina fosforilata porta TAZ al proteasoma; la stimolazione con Wnt determina quindi la stabilizzazione parallela di β -catenina e TAZ. Gli esperimenti qui presentati dimostrano che β -catenina inibisce l'attività biologica di TAZ in assenza di Wnt, e che la stabilizzazione di TAZ ha un ruolo funzionale nelle risposte biologiche (sia fisiologiche che patologiche) ai segnali Wnt.

Per facilitare la lettura, i due progetti sono presentati separatamente, come Parte I e Parte II di questa tesi.

PUBLICATIONS

Azzolin L, Zanconato F, Cordenonsi M, Bicciato S, Forcato M, Bresolin S, Basso G, Piccolo S (2012) – **Role of TAZ as mediator of Wnt Signaling.** *Cell*, 2012 Dec 21;151(7):1443-56

Inui M, Montagner M, Ben-Zvi D, Martello G, Soligo S, Manfrin A, Aragona M, Enzo E, Zacchigna L, Zanconato F, Azzolin L, Dupont S, Cordenonsi M, Piccolo S (2012) - Self-regulation of the head-inducing properties of the Spemann organizer. *PNAS*, 109(38):15354-9

Montagner M, Enzo E, Forcato M, Zanconato F, Parenti A, Rampazzo E, Basso G, Leo G, Rosato A, Bicciato S, Cordenonsi M, Piccolo S (2012) - SHARP1 suppresses breast cancer metastasis by promoting degradation of hypoxia-inducible factors. *Nature*, 487(7407):380-4

Cordenonsi M, Zanconato F, Azzolin L, Forcato M, Rosato A, Frasson C, Inui M, Montagner M, Parenti AR, Poletti A, Daidone MG, Dupont S, Basso G, Bicciato S, Piccolo S (2011) - **The Hippo transducer TAZ confers cancer stem cell-related traits on breast cancer cells.** *Cell*, 147(4):759-72

Dupont S, Morsut L, Aragona M, Enzo E, Giulitti S, Cordenonsi M, Zanconato F, Le Digabel J, Forcato M, Bicciato S, Elvassore N, Piccolo S (2011) – Role of YAP/TAZ in mechanotransduction. *Nature*, 474(7350):179-83

The work presented in this thesis was ideated and coordinated by Prof. Michelangelo Cordenonsi and Prof. Stefano Piccolo. I performed experiments under their supervision, together with Dr. Luca Azzolin. Dr. Azzolin is the first author of the work on TAZ regulation by Wnt.

Important contributions to our work were given by our collaborators: Prof. Silvio Bicciato and Dr. Mattia Forcato for bioinformatics, Dr. Antonio Rosato for experiments with mice, Prof. Anna Parenti and Dr. Vincenza Guzzardo for histological analysis, Prof. Giuseppe Basso, Dr. Chiara Frasson and Dr. Silvia Bresolin for cytofluorimetry and microarray experiments.

PART I

**MECHANISMS OF TAZ REGULATION AND
ACTIVITY IN BREAST CANCER STEM CELLS**

INTRODUCTION

1. TAZ and YAP

TAZ (transcriptional co-activator with PDZ-binding motif) and YAP (Yes-associated protein) are two homologous transcriptional co-activators. TAZ and YAP control important biological processes, such as stem cell self-renewal and fate decision – both in physiological and pathological contexts – with partially overlapping and partially separable functions (Pan, 2010).

1.1. TAZ and YAP in mammalian stem cells

TAZ and YAP are crucial regulators of embryonic development. This is underscored by the severe phenotypes associated with ablation of TAZ and/or YAP genes. Only a fraction of TAZ knockout (KO) mice is viable, and they progressively develop pathological changes in the kidney and lung that resemble the common human diseases polycystic kidney disease and pulmonary emphysema; part of the fetuses die *in utero* with an uncharacterized phenotype (Makita et al., 2008). Deletion of YAP results in embryonic lethality at approximately embryonic day 8.5 with multiple defects in extra embryonic structures, accompanied by a lack of embryonic axis elongation (Morin-Kensicki et al., 2006). The double knockout of YAP and TAZ has an even more dramatic phenotype: embryos die before the morula stage at embryonic day 2 due to a pre-implantation defect (Nishioka et al., 2009).

TAZ and YAP have key roles in the maintenance of stem cell populations. They are required for human and mouse embryonic stem cells (ESCs), respectively, to maintain their pluripotency *in vitro* (Varelas et al., 2008) (Lian et al., 2010). Mouse ESC differentiation is accompanied by YAP inactivation, and is inhibited by YAP ectopic overexpression. Moreover YAP is activated in induced pluripotent stem cells (iPSCs) and increases reprogramming efficiency (Lian et al., 2010).

The importance of YAP in organ development and tissue homeostasis is well exemplified by the skin. Skin-specific deletion of YAP impairs epidermis formation: YAP conditional KO mouse embryos had a thinner and fragile skin, with no epidermal tissue covering the distal limbs. This phenotype was attributed to the

gradual loss of epidermal stem/progenitor cells and their limited capacity to self-renew (Schlegelmilch et al., 2011). Conversely, activation of YAP in the skin resulted in severe thickening of the epidermal layer, due to amplification of undifferentiated interfollicular stem and progenitor cells (Schlegelmilch et al., 2011) (Zhang et al., 2011). Similarly, deletion of YAP in the embryonic mouse heart caused lethal myocardial hypoplasia due to impaired cardiomyocyte proliferation, whereas cardiac-specific activation of YAP lead to a dramatic cardiomegaly due to an increase in cardiomyocyte number and proliferation (Xin et al., 2011) (Heallen et al., 2011). In the adult intestine, endogenous YAP expression is typically restricted to the crypt compartment (where stem cells reside); overexpression of YAP induced an expansion of the undifferentiated progenitor compartment, without an obvious enlargement of organ size (Camargo et al., 2007). Studies in intestine-specific YAP KO models revealed that YAP is dispensable for normal intestinal homeostasis, but it is required for the regeneration of the intestinal epithelium after injury (Cai et al., 2010).

TAZ and YAP are also involved in the regulation of cell fate decisions. The clearest example may be in the pre-implantation mouse embryo: in morula-stage embryos, TAZ and YAP are activated in the outer cells, where they promote the expression of *Cdx2*, thus specifying these cells to a trophectoderm lineage; the inner cells, where TAZ and YAP inactive, will form the inner cell mass (Nishioka et al., 2009). TAZ also has a crucial role in regulating mesenchymal stem cell differentiation, through interactions with lineage-specific transcription factors. TAZ binds and activates the transcription factors *Runx2* and *MyoD*, thus promoting the terminal differentiation of osteoblast and myoblast precursors, respectively; TAZ binding to the transcription factor *PPAR γ* , instead, results in its inhibition and blocks adipocyte differentiation (Hong et al., 2005) (Jeong et al., 2010).

1.2. TAZ and YAP in cancer

The capacity of TAZ and YAP to direct cell proliferation and stem cell fate decisions provides these proteins with oncogenic potential (Pan, 2010). Evidence from transgenic mice supports this prediction: prolonged YAP overexpression in the liver of adult mouse leads not only to hepatomegaly, but also to the development of hepatocellular carcinomas (HCC) (Camargo et al., 2007) (Dong et al., 2007). In line, YAP was found to be overexpressed in about 50% of human HCC samples (Zhao et

al., 2007) and it was determined to be an independent prognostic marker for overall and disease-free survival (Xu et al., 2009).

Amplification of the YAP gene locus has been reported at varying frequencies in a wide spectrum of human tumors, such as medulloblastomas, oral squamous-cell carcinomas, and carcinomas of the lung, pancreas, esophagus, and liver. Consistently, comprehensive survey of the most common solid cancer types revealed widespread and frequent YAP overexpression in lung, ovarian, pancreatic, colorectal, hepatocellular, and prostate carcinomas (Pan, 2010). However, YAP is also reported to have proapoptotic activity by co-activation of p73 in some colorectal cancer cell lines (Strano et al., 2005).

A comprehensive survey of TAZ protein expression across multiple tumor types is unavailable at present. Enhanced expression of TAZ has been found in both breast cancer cell lines and tissues (Chan et al., 2008), and in non-small cell lung cancer (NSCLC) cell lines (Zhou et al., 2011). More recently, TAZ was found to contribute to glioblastoma through regulating differentiation into the malignant mesenchymal phenotype (Bhat et al., 2011).

Of note, both TAZ and YAP overexpression in immortalized mammary epithelial cells (MCF10A cells) were reported to induce epithelial-to-mesenchymal transition and to promote proliferation, anchorage-independent growth and cell migration *in vitro* (Chan et al., 2008) (Overholtzer et al., 2006). This kind of evidence strongly suggests that TAZ and YAP confer cellular behaviors typical of tumor cells; hence, they are considered oncogenes in the mammary gland. However, whereas TAZ depletion in some breast cancer cell lines impairs their tumorigenicity (Chan et al., 2008), YAP depletion was reported to have the opposite effect in other cellular contexts; this, and the fact that YAP is deleted in a subset of luminal breast cancers, rather suggests that YAP might be a tumor suppressor, instead (Yuan et al., 2008).

1.3. Upstream regulators of TAZ and YAP

The Hippo pathway. TAZ and YAP are negatively regulated by a conserved kinase cascade, the Hippo pathway, that was first delineated in *Drosophila melanogaster* as a master regulator of organ growth. In mammals the core components of the Hippo cascade are the kinases MST1/2 and LATS1/2, and their co-activators WW45 (also named Sav) and Mob1/2. MST1/2 and LATS1/2 are sequentially activated by phosphorylation, and eventually phosphorylate TAZ and

YAP. When the Hippo kinases are inactive, unphosphorylated TAZ and YAP accumulate in the nucleus; when the Hippo kinases are active, phosphorylation of TAZ and YAP by LATS leads to their sequestration in the cytoplasm and ubiquitination-dependent proteasomal degradation (fig. 1A). Therefore, the Hippo pathway acts to restrict the availability/functionality of TAZ and YAP in the nucleus (Pan, 2010) (Hong and Guan, 2012).

The initial steps of Hippo signal transduction are poorly understood. No specific extracellular ligands or membrane receptors are known upstream of the Hippo kinases. The Neurofibromatosis2 gene product Nf2 (also known as Merlin) is considered an upstream component of the Hippo pathway both in *Drosophila* and in mammals (Pan 2010). Genetic evidence supports this view: indeed, liver-specific Nf2 KO leads to liver enlargement and YAP hyperactivation (Zhang et al., 2010), phenocopying the conditional deletion of MST1/2 and WW45, and the overexpression of YAP (fig. 1B) (Lee et al., 2010) (Song et al., 2010) (Dong et al., 2007). However, how the membrane-associated Nf2 protein signals to MST or other downstream components remains undefined.

Recently, the activity of LATS1/2 and TAZ/YAP was reported to be regulated by G-protein coupled receptors (GPCRs) and their extracellular ligands, with different classes of G-proteins mediating activating or inhibitory effects (Yu et al., 2012).

Cell-cell contact. Increasing evidence indicates that cell-cell contact (and, thus, junctional proteins) regulate TAZ and YAP. Indeed, the Hippo pathway patterns the subcellular localization of TAZ and YAP in response to cell density both in tissue culture and in the early mouse embryo. In cultured cells, high cell density induces YAP/TAZ phosphorylation and cytoplasmic translocation (Zhao et al., 2007); and in mouse morula, the less compacted outer cells display prominent nuclear YAP/TAZ localization, whereas the more compacted inner cells display cytoplasmic YAP/TAZ (Nishioka et al., 2009). In both cases, LATS activity was suggested to translate the “density information” into YAP/TAZ localization, but the mechanism of LATS modulation is unknown.

Recent studies have proposed that junctional proteins may directly inhibit YAP and TAZ. For example, α -catenin and angiomin (two components of adherent and tight junctions, respectively) have been shown to interact with YAP/TAZ and to recruit them to the cell cortex, thus preventing YAP/TAZ nuclear activity with a

mechanism that does not require canonical Hippo signaling (Boggiano and Fehon, 2012) (Schlegelmilch et al., 2011) (Wang et al., 2011).

Apico-basal cell polarity. Polarized epithelial cells consist of distinct apical and basolateral membrane domains, that are separated by cell-cell junctions (Macara, 2004). Polarity proteins have been linked to the Hippo pathway in *Drosophila*. Mutation of the apical protein Crumbs or the basolateral protein Lgl (Lethal giant larvae) lead to extra cell proliferation, relocalization of Yorkie (YAP/TAZ homologue) from the cytoplasm to the nucleus and up-regulation of Hippo target genes (Halder and Johnson, 2011). The role of Crumbs as upstream regulator of TAZ and YAP appears to be conserved in mammals; Crumbs and other components of the complex were reported to bind to TAZ and YAP, and regulate their phosphorylation and cytoplasmic retention (Varelas et al., 2010b).

Mechanical cues. YAP/TAZ/ localization is also linked to cell morphology and to mechanical cues that impact upon the cytoskeleton. Increased cell tension promotes the localization of TAZ and YAP to the nucleus, whereas loss of cellular tension increases their cytoplasmic localization. This regulation does not involve the Hippo kinases, but it rather depends on the activity of Rho-GTPase proteins and the actin cytoskeleton (Dupont et al., 2011).

1.4. TAZ and YAP as transcriptional co-activators

Both TAZ and YAP possess a strong transcriptional activation domain, but no DNA binding domain. Thus, they rely on the interaction with DNA binding-platforms to form functional transcription factor complexes and drive the expression of their target genes (Mauviel et al., 2012). TAZ and YAP primarily interact with the TEAD/TEF family of DNA-binding proteins. The TEAD transcription factors are widely expressed in most tissues and organs, with at least one TEAD isoform expressed in every adult tissue in mammals (Pan, 2010). The TEAD proteins are required for many activities of TAZ and YAP, including contact inhibition, epithelial-mesenchymal transition and trophectoderm development (Ota and Sasaki, 2008) (Nishioka et al., 2009) (Zhao et al., 2008) (Zhang et al., 2009).

TAZ and YAP regulate the transcriptional activity of numerous other transcription factors, including, Runx2, SMADs, Pax3, Tbx5, and PPAR γ for TAZ, and Runx2 and p73 for YAP. This greatly increases the number of genes whose expression can be specifically regulated by TAZ and YAP and strongly supports the notion that

TAZ and YAP have both redundant and non-overlapping functions (Hong and Guan, 2012).

2. CANCER STEM CELLS

2.1. The cancer stem cell concept

Central to the cancer stem cell (CSC) concept is the observation that not all cells in tumors are equal. The CSC hypothesis postulates that, similar to regenerative tissues such as bone marrow, skin or intestinal epithelium, tumors follow a hierarchical organization; at the top of the hierarchy, a small subset of CSCs fuel tumor growth and generate the heterogeneous population of rapidly proliferating as well as postmitotic, differentiated cells that constitute the bulk of the tumor. CSCs are believed to be responsible for sustaining tumorigenesis, therapy resistance and tumor relapse, and metastatic dissemination (Clevers, 2011) (Visvader and Lindeman, 2012).

CSCs are defined by a series of functional traits. They can undergo unlimited self-renewing divisions and, at the same time, they can spawn differentiated progeny, in the same way that normal adult stem cells have the life-long capacity to self-renew and generate diverse differentiated cell types (Bacelli and Trumpp, 2012). Indeed, CSCs are tacitly believed to have acquired the molecular armaments of normal stem cells and to exploit the same molecular signals that orchestrate self-renewal and proliferation in healthy stem cell compartments (Clevers, 2011) (Visvader and Lindeman, 2008). That said, CSCs do not necessarily originate from the transformation of normal stem cells; CSCs may originate from restricted progenitors or more differentiated cells that have acquired self-renewing capacity. Furthermore, during disease progression, new CSC clones can arise either from existing CSCs or from the differentiated progeny via mutation-mediated partial reprogramming (Bacelli and Trumpp, 2012).

Typically, the presence of human CSCs within a cell population is experimentally addressed by transplantation of isolated tumor cells into immunocompromised mice; the CSC-containing population should re-establish the phenotypic heterogeneity evident in the primary tumor and exhibit self-renewing capability on serial passaging (Visvader and Lindeman, 2008). At limiting dilutions, the number of cells required

to seed new tumors provides an estimate of the fraction of CSCs within a population of cancer cells (Gupta et al., 2009). Clearly, these assays have some limitations: cells are transplanted in a milieu that does not recapitulate the original tumor microenvironment, and several experimental parameters (such as the method of cell dissociation or mouse strain) affect the result. However, transplantation assays using isolated tumor cells provided evidence for the existence of CSCs in a variety of solid tumors, including breast, brain, colon, pancreas, prostate, lung, and head and neck tumors (Clevers, 2011) (Visvader and Lindeman, 2008). Only recently the existence of CSCs has been proven *in vivo* through lineage-tracing experiments in mouse models of skin papilloma, colon adenoma and glioblastoma (Schepers et al., 2012) (Driessens et al., 2012) (Chen et al., 2012).

2.2. Cancer stem cells in breast cancer

The presence of CSCs in breast tumors was first reported by Clarke and colleagues in 2003 (Al-Hajj et al., 2003). They showed that breast tumors comprise heterogeneous populations of cancer cells, and that tumorigenic cells could be prospectively identified and isolated by flow cytometry based on cell surface marker expression. The population of cells with a $CD44^+CD24^{-/LOW}$ immunophenotype displayed the functional properties of CSCs: it was highly tumorigenic (as few as 100 cells with this phenotype were able to form tumors in mice, whereas tens of thousands of cells with alternate phenotypes failed to form tumors), and the tumors it generated could be serially passaged (Al-Hajj et al., 2003).

An easier way to assess the presence of CSCs within a population of breast cancer cells is the mammosphere-forming assay. When normal mammary epithelial cells are cultured in a serum-free medium on a surface to which they do not adhere, stem/progenitor cells form a three-dimensional cell cluster which is called mammosphere; cells within mammospheres can self-renew (as spheres can be serially passaged) and they are capable of differentiation along multiple lineages (Dontu et al., 2003). Similarly, within a population of breast cancer cells, a fraction of cells can seed mammospheres; these spheres contain CSCs that display a $CD44^+CD24^{-/LOW}$ marker profile and can seed tumors with high efficiency (Ponti et al., 2005).

In breast cancer, CSCs are believed to generate not only intra-tumor heterogeneity, but also inter-tumor phenotypic differences. Indeed, Pece et al.

reported that human well differentiated (histological grade 1) breast tumors contain fewer CSCs than poorly differentiated cancers (histological grade 3). This finding implies that the number of CSCs in human breast cancers has a discernible impact on clinical and pathological features (Pece et al., 2010).

2.3. Cancer stem cells and the epithelial-to-mesenchymal transition

Epithelial-to-mesenchymal transition (EMT) is a cellular program during which epithelial cells lose their polarized organization and cell–cell junctions, undergo changes in cell shape and in cytoskeletal organization and acquire mesenchymal characteristics, such as fibroblast-like cell morphology and increased cell migration and invasion. The EMT program is activated at multiple steps of embryonic development and, in adult organs, as a physiological response to injury, during wound-healing and tissue regeneration; on the other hand, EMT is also an important element in some pathological conditions, such as organ fibrosis and cancer progression (Thiery et al., 2009).

EMT has a key role in the acquisition of invasive and migratory traits by many types of carcinoma cells. Cells at the invasive edge of tumors have been observed to undergo EMT (Thiery et al., 2009). This is thought to enable tumor metastasis, as EMT provides tumor cells with the capacity to break away from neighboring cells, invade adjacent tissues and disseminate to distant sites (Yang and Weinberg, 2008). Disseminated cancer cells would have some common traits with CSCs: in fact, the initiation of a macroscopic metastasis in a new organ can be assimilated to *de novo* tumor growth (Brabletz et al., 2005). A few years ago, Mani and colleagues reported that the induction of EMT in transformed mammary epithelial cells generates populations of cells that are highly enriched for CD44⁺CD24^{-LOW} CSCs. Cells that have undergone an EMT are able to grow as mammospheres, and they are highly tumorigenic when xenografted at limited dilution in immunocompromised mice. A similar correlation exists between EMT induction and the acquisition of stem-like traits in immortalized non-tumorigenic mammary epithelial cells (Mani et al., 2008). This opens the possibility that CSCs may arise from fully differentiated cells that have been reprogrammed to an aberrant stem state via a process closely allied to an EMT (Gupta et al., 2009).

SUMMARY OF PREVIOUS DATA AND AIM OF THE PROJECT

Recent research in our laboratory has revealed that TAZ is a molecular determinant of biological properties associated with CSCs in breast tumors.

Through bioinformatic analysis of breast cancer datasets (which provide gene expression data from tumor samples, together with clinical information about the patients) we have found that TAZ/YAP target genes are frequently overexpressed in high grade breast tumors compared with more benign lesions. Furthermore, we could detect high levels of TAZ protein in ~80% of high grade tumor samples (fig. 1C). Thus, high TAZ expression and activity correlates with an elevated number of CSCs in breast tumors. The functional relevance of this association was validated in breast cancer cell lines. We have shown that TAZ controls self-renewal and tumorigenic properties of breast cancer cells. TAZ overexpression in MII cells (a model of low-grade breast tumors, expressing low levels of TAZ) enhanced CSC properties: TAZ overexpression expanded the CSC-enriched subpopulation (characterized by the CD44^{HIGH}/CD24^{LOW} surface phenotype) and boosted the capacity to seed mammospheres *in vitro* (a readout of self-renewal) and to seed “high-grade” tumors *in vivo*. Conversely, in cells expressing high levels of TAZ and displaying an aggressive behavior (MIV cells), TAZ depletion impaired both mammosphere formation and tumor initiation.

Experiments presented here investigate two complementary aspects of TAZ function in breast CSCs. The first is the mechanism of TAZ activation in breast CSCs. Indeed, whereas TAZ protein levels are elevated in a large fraction of high grade human breast tumors, the amplification of TAZ locus or overexpression of TAZ mRNA were not as frequent. We thus reasoned that a post-translational mechanism must explain TAZ protein stabilization in most malignant breast tumors. Then, we addressed the molecular mechanisms activated by TAZ in breast cancer cells to foster CSC properties.

RESULTS

1. UPSTREAM REGULATORS OF TAZ IN BREAST CANCER STEM CELLS

1.1. EMT Promotes Self-Renewal through TAZ in breast cancer cells and in non-transformed mammary epithelial cells.

We noticed that the biological activity of TAZ in breast cancer cells presents some intriguing analogies with the cellular traits acquired after an EMT (Thiery *et al.*, 2009). Both TAZ and EMT promote the acquisition of CSC-related traits, as suggested by the following evidence:

- both overexpression of TAZ (S89A) and induction of EMT determine an expansion of the subpopulation of cells with the immunophenotype of breast CSCs (CD44^{HIGH}/CD24^{LOW});
- both TAZ and EMT enhance the capacity of cells to form mammospheres when cultured in suspension, a property tightly linked to the presence of self-renewing stem or progenitor cells;
- finally, both TAZ overexpression and induction of EMT increase the frequency of tumor-initiating cells (Mani *et al.*, 2008).

Moreover, we observed that cells expressing high TAZ levels display key mesenchymal features. MCF10A-T1k (MII) cells (a MCF10A derivative that is tumorigenic but not metastatic) can be sorted into two subpopulations according to the different expression of the cell-surface markers CD44 and CD24. As shown in figures 2A and 2B, the CD44^{HIGH}/CD24^{LOW} compartment (the only one endowed with self-renewal ability) displays high TAZ levels, it expresses the mesenchymal marker Vimentin but no junctional E-cadherin; on the other hand, cells belonging to the CD44^{LOW}/CD24^{LOW} subpopulation (with low TAZ levels and poor self-renewal potential) are clearly epithelial. In MIV cells, a malignant derivative of MII cells (Santner *et al.*, 2001) with enhanced stem properties, TAZ protein levels are higher and, in spite of an apparent epithelial morphology, E-cadherin fails to localize in cell-cell contact areas (fig. 2C-D). Finally, MDA-MB-231 cells, highly metastatic

breast cancer cells characterized by high TAZ levels, display an overt mesenchymal morphology, with no E-cadherin expression at all (fig. 2C-D).

To test if a causal link exists between EMT induction and TAZ upregulation, we transduced epithelial cells with lentiviral vectors encoding EMT-inducing transcription factors. The same experiment was performed in normal (immortalized, but not transformed) mammary epithelial cells (HMLE cells – fig. 3A) and in breast cancer cells (MII cells – fig. 3C). Both Twist overexpression in HMLE cells and Snail overexpression in MII cells induced EMT (as shown by E-cadherin downregulation); furthermore, EMT was accompanied by a strong increase in TAZ protein levels, but not in *TAZ* mRNA (fig. 3B). TAZ target gene CTGF was upregulated, too, indicating a functional activation of TAZ. It is interesting to note that, in spite of the ability of TAZ itself to trigger an EMT in some cell types, TAZ upregulation is not relevant for the acquisition of the mesenchymal morphology after Twist or Snail overexpression: indeed, TAZ depletion by siRNA did not rescue E-cadherin expression in HMLE-Twist cells nor in MII-Snail cells (fig. 3A and 3C).

We then asked whether TAZ upregulation downstream of EMT mediates its pro-stemness effects. As expected, HMLE-Twist cells displayed a > 5-fold increase in mammosphere-forming capacity compared with empty vector control cells (Figure 2C) (Mani et al., 2008). Crucially, TAZ knockdown significantly reduced Twist-induced mammosphere-forming ability (fig. 3D).

Similarly, in MDA-MB-231 cells loss of TAZ dramatically affected the expression of TAZ target genes CTGF and Survivin (fig. 3E) as well as primary and secondary mammosphere formation (indicating a depletion of the pool of cells endowed with self-renewal capacity – fig. 3G). However, loss of TAZ did not change the mesenchymal morphology of MDA-MB-231 cells nor induced E-cadherin expression (fig. 3E-F). Thus, TAZ is required for self-renewal, but not for the morphological properties of mesenchymal breast cancer cells.

We conclude from these experiments that the levels of endogenous TAZ protein increase during EMT and that TAZ is a relevant mediator of the mammosphere-forming capacity promoted by EMT both in non tumorigenic mammary epithelial cells and in breast cancer cells.

1.2. TAZ is activated by Scribble deregulation.

Although full EMT is rare in human tumors, most carcinomas show evidence of a partial EMT (Savagner, 2010). One hypothesis is that cancer cells do not need to reach a completely mesenchymal state, but they rather exploit only the onset of the EMT program, entailing loss of apico-basal polarity and destabilization of junctional complexes (Thiery et al., 2009). Thus, we set out to verify if disturbing cell polarity is sufficient to achieve TAZ stabilization.

Among the proteins involved in the establishment of epithelial polarity, we focused our attention on the basolateral determinant Scribble (Macara, 2004), because:

- Scribble is frequently disabled during mammary tumorigenesis and also serves as a tumor suppressor in *Drosophila* (Zhan et al., 2008);
- in fly tissues, the overgrowth of *scribble* mutant clones is dependent on *yorkie* (Grzeschik et al., 2010); yet, the molecular mechanisms of this genetic interaction remain unknown.

We thus interfered with the function of the basolateral polarity module by depleting Scribble. As shown in figure 4A and 4B, Scribble knockdown in MII cells resulted in a robust upregulation of TAZ protein (but not mRNA) levels and it also enhanced the expression of TAZ target CTGF; these effects were observed in the absence of evident changes in E-cadherin localization (fig. 4C). Notably, Scribble inhibits TAZ only in epithelial cells; after Snail-induced EMT, Scribble knockdown didn't produce any further increase in the levels of TAZ or its target genes (fig. 4D). This suggests that Scribble is somehow inactivated during the EMT program, thus allowing TAZ upregulation.

In human breast cancer Scribble is deregulated by both downregulation and mislocalization (i.e. in 50% of tumor samples Scribble is localized to the cytoplasm, and not restricted to cell-cell junctions) (Zhan et al., 2008). In our cellular models, EMT only had modest effects on Scribble protein levels (fig. 4D, compare lanes 1 and 3) but dramatically changed its subcellular localization: in MII and HMLE epithelial cells, Scribble is localized in the basolateral membrane and it occupies cell-cell contact areas, but after expression of Snail or Twist, it becomes predominantly cytoplasmic (fig. 4E). Scribble displacement from the plasma membrane is a common trait of cells characterized by high TAZ levels and the

expression of stem properties, such as the CD44^{HIGH}/CD24^{LOW} subpopulation of MII cells (fig. 4F), and MIV and MDA-MB-231 cell lines (fig. 4G).

We thus asked if Scribble localization is relevant for TAZ regulation in breast cancer cells. To test this, we introduced a point mutation in the LRR domain of Scribble, that anchors it to the plasma membrane: this mutation results in a protein (Scrib P305L) that fails to localize to the plasma membrane and even disrupts membrane recruitment of endogenous Scribble (Audebert et al., 2004) (fig. 5A). In MII cells transduced with Scrib P305L TAZ is stabilized and activated, as judged by target gene expression (fig. 5B). We also fused Scribble coding sequence with the C-terminal CAAX motif of Ras (Lowy and Willumsen, 1993): this short sequence is palmitoylated to anchor proteins to the plasma membrane. We overexpressed Scrib-CAAX in MII-Snail mesenchymal cells (where endogenous Scribble is delocalized to the cytoplasm): TAZ expression and activity were strongly inhibited in the presence of membrane-tethered Scribble (fig. 5C-D). Thus, membrane-bound Scribble inhibits TAZ, and the mere displacement of Scribble to the cytoplasm impairs its inhibitory control on TAZ, fully phenocopying Scribble depletion.

At the functional level, expression of ScribP305L increased the self-renewal potency of MII cells, as assayed by mammosphere formation; this effect was dependent on TAZ upregulation, as it was abolished by TAZ depletion (fig. 5E). Thus, an early event in the EMT program, loss of apico-basal polarity (here mimicked by Scribble delocalization) can well recapitulate the effects of EMT-inducing transcription factors on TAZ levels and biological activity.

1.3. Scribble regulates TAZ through the Hippo pathway

Data presented so far indicate that, in mammary epithelial cells, Scribble exerts a constitutive inhibition on TAZ; such regulation is subverted by the induction of EMT, Scribble depletion or Scribble mislocalization. What is the mechanism of TAZ regulation by Scribble?

TAZ is regulated by the kinases of the Hippo pathway. Four LATS phosphorylation sites are present in TAZ aminoacidic sequence. Particularly, phospho-Ser89 is a docking site for 14-3-3 proteins and mediates TAZ cytoplasmic sequestration (Kanai et al., 2000). Phosphorylation of Ser311, instead, triggers TAZ proteasomal degradation. After phosphorylation on Ser311 by LATS, TAZ is phosphorylated by CK1 ϵ on Ser314. These two consecutive phosphorylation events

generate a functional phosphodegron that is recognized by the SCF β -TrCP E3 ubiquitin ligase complex (Liu et al., 2010).

To assess whether disturbing cell polarity through Scribble deregulation affects the activity of the Hippo pathway on TAZ, we monitored TAZ phosphorylation on one of LATS target sites, Ser89. TAZ was immunoprecipitated from lysates of control MII cells, Scribble-depleted MII cells and post-EMT cells (MII Snail). TAZ phosphorylation levels dropped after Scribble depletion or EMT induction, in spite of higher total protein levels (fig 6A). The same results were obtained in HMLE cells transfected with Scribble siRNA or transduced with the EMT-inducing transcription factor Twist (fig. 6B). Thus, the absence of Scribble or its displacement from the plasma membrane (in MII Snail and HMLE Twist cells) reduces TAZ phosphorylation by LATS.

To confirm this hypothesis, we generated some mutant murine TAZ cDNAs (mTAZ), encoding proteins with Serine to Alanine substitutions in the two key LATS target sites described above, Ser89 and Ser306 (corresponding to Ser311 in the human protein – fig. 6C). Wild-type (WT) and mutant cDNAs were subcloned into retroviral vectors and stably transduced in MII cells. As shown in figure 6D, the expression level of the S89A mutant was comparable to WT protein; however, it was more active (as indicated by the upregulation of TAZ target gene CTGF). TAZ S306A (which escapes proteasomal degradation) was much more stable than WT TAZ, but it wasn't more active (probably indicating that it is excluded from the nucleus due to phosphorylation on Ser89). The combined mutation of the two residues results in a protein that cannot be recognized by 14-3-3 proteins, or by β -TrCP and is very stable and active (TAZ S89/306A). We then compared the effect of Scribble depletion on WT TAZ and "LATS-insensitive" TAZ S89/306A. Exogenous wild-type mTAZ appeared to be controlled by the same mechanisms that regulate the endogenous human protein, as Scribble depletion led to its stabilization and activation (see CTGF expression) (fig. 6E, lanes 1-2). mTAZ S89/306A, instead, wasn't affected by Scribble depletion (fig. 6E, lanes 3-4). Overall, data indicate that in epithelial cells there exists an active mechanism that keeps TAZ levels repressed; this mechanism depends on TAZ phosphorylation by LATS and on the correct maintenance of cell polarity.

Further support to this interpretation was provided by co-immunoprecipitation experiments: we found that endogenous TAZ co-immunoprecipitates with Scribble,

MST, LATS; the complex also includes β -TrCP (fig. 6F, lane 1 and fig. 6G, lane 1). In other words, in epithelial cells we could detect a biochemical complex containing TAZ, Scribble and TAZ “degradation machinery” (comprising the Hippo kinases and β -TrCP). After Scribble depletion, the interaction between TAZ and LATS was weakened, and the association between TAZ and MST or β -TrCP was barely detectable (fig. 6F, lane 2 and fig. 6G, lane 2). Moreover, in control cells LATS was phosphorylated by MST on Ser909 inside the protein complex, whereas in Scribble-depleted cells LATS was mainly unphosphorylated, and thus inactive (fig. 6F-G, lanes 1-2). This observation is in line with the diminished levels of phospho-TAZ in cells transfected with Scribble siRNA (see figures 6A and 6B). Thus, Scribble is required for the assembly of a complex between TAZ, LATS and MST and for MST-dependent activation of LATS, resulting in TAZ phosphorylation and recognition by β -TrCP.

The induction of EMT blocked TAZ interaction with Scribble and phenocopied the effect of Scribble depletion on the interaction between TAZ, the Hippo kinases and β -TrCP (fig. 6F, lane 3 and fig. 6G, lane 3).

As we have previously demonstrated that in MII-Snail and HMLE-cells Scribble is delocalized from the plasma membrane (fig. 4E), we speculate that membrane-bound Scribble promotes the activity of the Hippo kinases in epithelial cells, leading to TAZ degradation; Scribble displacement to the cytoplasm in mesenchymal cells prevents the functional association between TAZ and its “degradation machinery” and leads to TAZ stabilization, phenocopying Scribble knockdown.

Taken together, data indicate that Scribble regulates the Hippo pathway to modulate TAZ expression and activity in normal mammary epithelial cells and breast cancer cells.

2. MOLECULAR EVENTS DOWNSTREAM OF TAZ IN BREAST CANCER CELLS

2.1. TAZ downregulates the expression miR-200 family members

We have found that TAZ upregulation in breast cancer cells profoundly changes their nature. For example, differentiated cells isolated from MII cell line are

“reprogrammed” to a CSC-like state following TAZ overexpression; they acquire a new phenotype and new properties (such as self-renewal capacity) that do not usually belong to differentiated cells.

Throughout development similar state transitions are often orchestrated by microRNAs (miRNAs), short non-coding RNAs (21-23 nucleotides) involved in post-transcriptional silencing of gene expression (Bartel, 2004).

We thus decided to perform a large scale analysis of microRNA expression to search for microRNAs regulated by TAZ overexpression in MII cells. For this purpose, we used the TaqMan MicroRNA Human Array (Applied Biosystems), a high-throughput platform for the detection of mature microRNAs by real time RT-PCR with TaqMan probes. The array we used (panel A) allows the amplification of 377 among the most expressed and well-characterized human microRNAs.

Among the 377 miRNAs we considered, 233 were detectable by real time PCR and only 34 were differentially expressed in MII and MII-TAZ, with at least a twofold up- or downregulation; in detail, 22 were upregulated and 12 were downregulated by TAZ overexpression (fig. 7A and 7B). The results we obtained clearly state that all the microRNAs of the miR-200 family (miR-200a, miR-200b, miR-200c, miR-141, miR-429 and the closely related miR-205) are strongly downregulated by the overexpression of TAZ (fig. 7B). We confirmed these results with SYBR green-based real time PCR (fig. 7C).

Mature miRNAs are the product of the enzymatic cleavage of longer precursors, called pri-microRNAs. Pri-miRNAs are the primary, unmodified transcripts synthesized by RNA polymerases from miRNA-encoding genes (Bartel, 2004). The five members of the miR-200 family are clustered into two genomic loci, and they are transcribed as two precursors: one contains the mature sequences of miR-200b, miR-200a and miR-429, the other of miR-200c and miR-141 (Korpál et al., 2008). To gain insight into the mechanism of miR-200 downregulation by TAZ, we tested if it occurs at the level of primary transcripts and we found, by RT-PCR, that the levels of the pri-microRNAs drop following TAZ overexpression (fig. 7D). Thus TAZ regulates miR-200 levels mainly by inhibiting the transcription of their precursors.

2.2. Overexpression of miR-200c opposes TAZ biological activity in MII cells

We then asked whether the silencing of miR-200 in MII cells was functional for TAZ-induced enhancement of stem-like properties. To answer this question, we

stably transfected pri-miR-200c in MII-TAZ cells, in order to obtain a continuous overexpression of the mature form of miR-200c (which is the most represented miR-200 family member in MII cells, together with miR-200b). Overexpression of miR-200 didn't impair the levels of TAZ, nor of its target CTGF (fig. 8A). However, exogenous miR-200 reverted the morphology of TAZ-overexpressing cells from mesenchymal to epithelial, rescuing E-cadherin expression and its localization at cell-cell junctions as in parental MII cells (fig. 8B).

We then evaluated the effect of miR-200 rescue on the “stem” properties of MII-TAZ cells, namely the expression of CSC markers, self-renewal and tumorigenic potential. We have previously shown that TAZ overexpression resulted in an enrichment of the CD44^{HIGH}/CD24^{LOW} stem population (with an almost complete depletion of the CD44^{LOW}/CD24^{LOW} fraction usually present in parental MII cells). Overexpression of exogenous miR-200, instead, reverted the CD44/CD24 profile to the one of MII cells, showing two distinct sub-populations characterized by either low or high levels of CD44 (fig. 8C-D); moreover, the contribution of the two groups to the entire population was quite similar in MII and MII-TAZ-miR-200 cells (CD44^{LOW} ~70%; CD44^{HIGH} ~30%) (fig. 8D). In mammosphere assays, TAZ greatly enhanced the number of mammospheres formed by MII cells, but miR-200 overexpression reduced the number of mammospheres generated by MII-TAZ cells to the same levels of parental MII cells (fig. 8E). Finally, we measured tumor initiating capacity by orthotopic transplantation into the fat pad of immunocompromized mice; miR-200 overexpressing MII-TAZ cells not only seeded less tumors (fig. 8F), but the morphology of the lesions resembled benign tumors originating from control MII cells, rather than undifferentiated tumors originating from MII-TAZ cells (fig. 8G).

Overall, these results indicate that, in MII cells, TAZ cannot foster stem properties in the presence of high levels of miR-200. However, this is inconsistent with the behaviours of a different cell line, MIV cells, where the expression of TAZ-dependent CSC properties coexists with high miR-200 levels (data not shown). Thus, we conclude that miR-200 downregulation is not a general, strict requirement for TAZ activity.

2.3. An experimental plan to investigate TAZ transcriptional program

Our findings on TAZ as a molecular determinant of stem-like traits in breast cancer open another issue: how does TAZ work? What are the molecular events activated by TAZ in cancer stem cells?

As TAZ is a transcriptional co-activator, we speculated that it acts through the regulation of one or more genes, whose products are important for the tumorigenic and self-renewal capacity of cells, either as direct effectors or as “intermediary” transcriptional regulators. Thus, we performed gene expression profiling experiments with Affymetrix microarrays to identify TAZ target genes.

As the primary goal of this analysis was to detect genes whose expression depends on TAZ at endogenous levels, it was carried out in MDA-MB-231 cells. These cells express high levels of TAZ and we have previously demonstrated that their ability to self-renew depends on TAZ. We compared the transcriptome of MDA-MB-231 cells stably transduced with control or TAZ shRNAs. Furthermore, we analyzed a second set of samples, including cells transiently transfected with control siRNAs or two different combinations of TAZ and YAP siRNAs. The combined analysis of data obtained from the two sets of experiments helps in excluding “off-targets” due to the targeting strategies and targeting sequences used. We also reasoned that it would be useful to deplete not only TAZ, but also its homolog YAP for the following reasons: (a) TAZ and YAP share the same DNA-binding factors (the TEAD family members) and some well known target genes, thus some of TAZ target genes might be “masked” in the presence of YAP as a consequence of compensatory mechanisms; (b) larger variations in the levels of target genes obtained through the double depletion of TAZ and YAP may be desirable from a technical point of view.

Total RNA was extracted from four replicas for each experimental condition and prepared for hybridization on the microarrays. Hybridization signals were converted to expression values and differentially expressed genes were identified using Significance Analysis of Microarray (SAM) algorithm. We first evaluated the overall effects of TAZ depletion on gene expression. We observed that – as predicted above – double depletion of TAZ and YAP with siRNAs modified the expression of a larger number of probe sets than TAZ shRNA alone, and induced wider fold-changes.

The table below and diagrams in fig. 9A provide an overview of the results we obtained:

Sample set	n of upregulated probe sets* (q-value < 0.05)	n of downregulated probe sets* (q-value < 0.05)
<i>MDA shTAZ vs shGFP</i>		
fold change ≥ 1.5	460	621
fold change ≥ 2	140	93
max fold change	+4,03	-7,59
<i>MDA siTAZ/YAP (mix 1) vs siCONTROL</i>		
fold change ≥ 1.5	4593	3910
fold change ≥ 2	2498	2248
max fold change	+40,75	-43,33
<i>MDA siTAZ/YAP (mix 2) vs siCONTROL</i>		
fold change ≥ 1.5	4952	4842
fold change ≥ 2	2523	2744
max fold change	+33,24	-52,54
<i>Probe sets regulated by both siTAZ/YAP mix 1 and mix2 (vs siCONTROL)</i>		
fold change ≥ 1.5	3080	2750
fold change ≥ 2	1504	1483

* a probe set consists of 11 oligonucleotides designed to hybridize with the same unique mRNA transcript. About half of genes are represented by a single probe set; the other half genes are defined by two or more probe sets.

To understand whether the analysis of gene expression could provide some information about the biological processes affected by TAZ (and YAP) depletion, we went through the list of up- or downregulated probe sets, searching for genes known to be involved in cellular processes that are central to cell life: apoptosis, cell cycle regulation, DNA repair, autophagy and senescence. The most affected process was cell proliferation. We found that probe sets for genes that promote cell cycle progression (examples include CDK2, CDK4, CDK1, Cyclins E, A and B, Cdc20 and 25, E2F), as well as probe sets for genes involved in DNA replication (including several ORC and MCM proteins and DNA polymerase itself) and chromosome segregation were downregulated after TAZ/YAP knockdown. Indeed depletion of TAZ or TAZ/YAP reduced BrdU incorporation (fig. 9B), as well as the fraction of cells in S-phase (fig. 9C), not only in MDA-MB-231 cells, but also in other cell lines we have exploited to characterize TAZ activity. This finding is in line with the observation that TAZ and YAP are known as promoters of proliferation *in vivo*. It

will be interesting to assess whether this holds true when cells are grown in suspension as mammospheres or as xenografts in mice.

The search for relevant TAZ target genes is currently in progress. As first choice, candidate targets have been selected among probe sets downregulated both by TAZ shRNA (fold change < -1.5) and by TAZ/YAP siRNAs (fold change < -2): we have thus obtained a list of 137 probe sets, corresponding to 83 genes. This list encompasses some established TAZ target genes, such as CTGF, Cyr61 and AnkrD1. The downregulation of candidate target genes will be confirmed by qRT-PCR in TAZ depleted MDA-MB-231 cells. Then, we will verify if they are accordingly regulated by TAZ overexpression in MII cells (our established system to study TAZ gain of function). Positive hits represent *bona fide* TAZ transcriptional targets and they will be subjected to a functional validation, to find those genes or groups of genes that can phenocopy TAZ overexpression and are required for TAZ-induced self-renewal. In practical terms, we plan to deplete candidate target genes in MDA-MB-231 cells or in TAZ overexpressing MII cells, and to test them in mammosphere assays. Relevant mediators of TAZ activity are expected to dampen TAZ-dependent mammosphere formation; they may also be sufficient to foster self-renewal capacity of parental MII cells: in this case, their overexpression should phenocopy TAZ upregulation.

The case of CTGF is presented here as an example. CTGF is a well known transcriptional target of TAZ; indeed, the complex TAZ/TEAD can bind three TEAD binding sites in CTGF promoter (Zhang et al., 2009). Furthermore, it has been shown to mediate TAZ-induced resistance to taxol in MCF10A cells (Lai et al., 2011). As shown in the previous paragraphs, CTGF levels drop in TAZ-depleted MDA-MB-231 cells (see fig. 3E); conversely, CTGF is upregulated by TAZ overexpression in MII-TAZ cells (see fig. 8A). However, the regulation of CTGF levels does not seem to play a functional role in TAZ-induced self-renewal: CTGF depletion by siRNAs did not impair the ability of MII-TAZ cells to seed primary and secondary mammospheres, nor could CTGF overexpression phenocopy the upregulation of TAZ (data not shown). This is in line with evidence indicating that CTGF is only partially responsible for YAP-induced anchorage-independent growth of MCF10A cells (the immortalized, not transformed progenitors of MII cells) (Santner et al., 2001), and that expression of CTGF alone could not phenocopy the effects of YAP overexpression in the same cell line (Zhao et al., 2008). Thus, we conclude that

CTGF, in spite of being a very useful and faithful readout of TAZ transcriptional activity, may not be required for TAZ biological activity.

DISCUSSION

1. TAZ activity is promoted by EMT

Low grade and high grade breast tumors differ in CSC content: low grade tumors contain few CSCs, whereas in poorly differentiated, high grade cancers the fraction of CSCs is larger. We have found that this difference is imputable to the upregulation of the Hippo transducer TAZ in malignant tumors. TAZ activity governs the functional properties that define CSCs: self-renewal and tumor seeding capacity; in other words, TAZ is a molecular determinant of CSCs in breast tumors. It is remarkable that TAZ is upregulated in about 80% of high grade breast tumors, with a mechanism that in most cases probably entails TAZ protein stabilization.

The epithelial-mesenchymal transition (EMT) converts both normal and neoplastic epithelial cells into derivatives with a more mesenchymal phenotype. Beyond morphological changes, EMT results in the acquisition of cell-biological traits associated with high-grade malignancy, including motility, invasiveness, and an increased resistance to apoptosis (Thiery et al., 2009). In addition, forced passage of both normal and neoplastic mammary epithelial cells through an EMT confers on the resulting cells many of the properties associated with normal and cancer stem cells (Mani et al., 2008).

We found that TAZ is activated by EMT, and that it is a mediator of the biological effects of EMT. Indeed, TAZ is turned on by overexpression of the EMT-inducing transcription factors Twist or Snail and is required for self-renewal induced by Twist. It is well known that TAZ itself can trigger EMT (Chan et al., 2008); however, the acquisition of mesenchymal traits downstream of Twist in nontransformed mammary cells does not seem to require TAZ, nor does the depletion of TAZ affect the intrinsic mesenchymal phenotypes of malignant cells. Thus, in the context of the EMT program, morphological changes (for which TAZ is dispensable) can be uncoupled from the acquisition of CSC traits (that, conversely, depends on TAZ). As to TAZ-induced EMT, it may act as an embedded self-sustaining mechanism, whereby TAZ assures a continuous stimulus for its own activation.

Our results suggest that neither stabilization of TAZ protein levels nor induction of self-renewal require the entire EMT program. In fact, TAZ activation is achieved

in the absence of overt mesenchymalization when the polarity determinant Scribble is disabled. This finding could solve a critical point about the role of EMT in malignancy, specifically the observation that the transition of epithelial cells towards a full mesenchymal cell fate is rare in carcinomas. However, disruption of cell polarity and tissue organization is recognized to be a fundamental trait present in the majority of human tumors (Chaffer and Weinberg, 2011) (Savagner, 2010) and we have shown that this early section of the EMT program may be sufficient to activate TAZ and promote the acquisition of a malignant phenotype in a large number of breast tumors. Although intriguing, a more definitive demonstration of this possibility still awaits dedicated *in vivo* studies.

2. Scribble is a novel upstream regulator of Hippo signaling in mammals

TAZ and YAP are central mediators of the Hippo pathway, which regulates organ size from *Drosophila* to mammals (Pan, 2010). How this pathway responds to extracellular signals remains unclear. Increasing evidence points to cell adhesion and polarity proteins as likely candidates of Hippo pathway activation, but the underlying biochemical mechanisms are poorly understood (Boggiano and Fehon, 2012). Our results show that in nontransformed and tumorigenic mammary epithelia, the Hippo cascade is activated by Scribble at the cell-cell contact. Scribble serves as an adaptor to assemble a “TAZ degradation complex”, comprising MST, LATS and β -TrCP; in this complex, the sequential phosphorylation of LATS and TAZ triggers TAZ degradation. (Figure 7K).

Scribble (as other polarity proteins) is a tumor suppressor. In *Drosophila* its function as polarity regulator also governs cell proliferation (Zeitler et al., 2004) and this works (at least in part) through the modulation of Yorkie (the homologue of TAZ and YAP). We have found that a similar mechanism is active in mammary epithelial cells, and that it involves the activation of the Hippo kinases. In breast cancer Scribble is frequently inactivated, by either downregulation or delocalization from the cell membranes (Zhan et al., 2008). *In vitro*, both these events lead to LATS inactivation and TAZ accumulation. We thereby hypothesize that in poorly differentiated breast tumors, loss of apico-basal polarity allows TAZ to escape Hippo control and unleashes TAZ pro-CSCs activity (see model in figure 6H).

3. A cell-specific requirement of miR-200 downregulation for TAZ activity

miRNAs are predicted to control the activity of more than 60% of all protein-coding genes. miRNAs silence their cognate target genes by binding specifically to mRNAs and inhibiting their translation or inducing their degradation (Bartel, 2004). MicroRNAs participate in the regulated transitions of progenitor cells from a multipotent, self-renewal status towards differentiation in numerous cell lineages and tissues throughout embryonic development and in organ homeostasis (Ambros, 2011). In fact, miRNAs are particularly suited to the stabilization of newly acquired cell states: regulatory loops exist between some miRNAs and their targets, so that when a cell acquires one of two possible fates the balance between the miRNA and its target make this choice permanent and self-maintained (Inui et al., 2010).

Aberrant miRNA expression is a common trait of human cancers. Not only individual miRNAs (or miRNA families) act as oncogenes or tumor-suppressor; there is a global downregulation of miRNAs in tumors, that is exploited by cancer cells to induce epithelial plasticity and foster invasive and metastatic behaviour (Lu et al., 2005) (Martello et al., 2010).

We found that TAZ overexpression in MII epithelial cells is accompanied by the silencing of miR-200 family members. The miR-200 family plays a role in establishing and maintaining the epithelial phenotype, both during development and in adult tissues. Inhibition of endogenous miR-200 is sufficient to convert epithelial cells into mesenchymal cells, while ectopic expression induces the opposite transformation (a mesenchymal-to-epithelial transition) in normal and cancer cell lines (Gregory et al., 2008). Recently, miR-200 family members have been demonstrated to be downregulated both in normal human and murine mammary stem/progenitor cells, and in human breast CSCs; overexpression of miR-200c suppressed the ability of normal mammary stem cells to form mammary ducts and of breast CSC to drive tumor formation *in vivo* (Shimono et al., 2009). In line with this evidence, in the presence of overexpressed miR-200, TAZ seemed to lose its ability to foster CSC properties: in spite of high levels of an active exogenous TAZ, MII-TAZ-miR-200 cells displayed poor self-renewal capacity and seeded tumors with very low efficiency. Thus, miR-200 family seems to repress stemness, and stemness properties are unleashed only in cells devoid of these microRNAs.

However, we believe that this is not a general rule. Indeed, MIV cells express high levels of miR-200 family members, yet they display marked stem-like

properties that depend on TAZ. Moreover, though we have shown that miR-200 regulation in TAZ overexpressing MII cells occurs at the transcriptional level, it is unlikely that the transcription of miR-200 precursors is under the direct control of TAZ, as TAZ depletion does not lead to miR-200 upregulation (data not shown). Therefore, we consider miR-200 repression an indirect effect of TAZ overexpression in MII cells (maybe secondary to TAZ-induced mesenchymal conversion); silencing of miR-200 creates a permissive milieu for TAZ activity in this cellular context, but it is not a general requirement for TAZ-dependent acquisition of CSC-related traits.

4. Investigating the mechanism of TAZ activity

To understand how TAZ confers CSC properties on breast cancer cells, we are currently investigating TAZ transcriptional program at the transcriptomic level.

A first survey of the gene expression profiles of malignant breast cancer cells MDA-MB-231 suggests that TAZ (together with its homologue YAP) controls the expression of a large number of genes involved in cell cycle progression. This is not unexpected, given the role of the Hippo pathway and its transducers in the regulation of cell proliferation and organ size. However, only a more in-depth analysis of TAZ transcriptional program will reveal how this biological process is regulated by TAZ at the molecular level.

Preliminary results strongly suggest that many cellular genes are probably regulated in concert by TAZ to trigger its biological effects. Indeed, one of the best available readouts of TAZ activity (CTGF) is not required for TAZ-induced self-renewal, nor sufficient to mimic it. Thus, the search for the molecular mediators of TAZ activity will have to be enlarged to the definition of a network of cooperating target genes.

PART II

**ROLE OF TAZ
IN WNT BIOLOGICAL RESPONSES**

INTRODUCTION

1. The canonical Wnt signaling pathway

Long before the delineation of the Hippo pathway, canonical Wnt signaling was recognized as one of the fundamental mechanisms that direct cell proliferation and cell fate determination during embryonic development and adult tissue homeostasis (MacDonald et al., 2009).

The central player of canonical Wnt signaling is a cytoplasmic protein, β -catenin, whose stability is regulated by a dedicated destruction complex. This complex consists of two scaffold proteins, Axin and adenomatous polyposis coli (APC), and the kinases glycogen synthase kinase-3 α/β (GSK3) and casein kinase-1 (CK1). When Wnt receptors are not engaged, newly synthesized β -catenin is captured by the destruction complex and phosphorylated by CK1 at Ser 45, which in turn primes GSK3 phosphorylation of β -catenin on Thr41, Ser37 and Ser33 residues. The resulting phosphorylated footprint recruits a β -TrCP-containing E3 ubiquitin ligase, which targets β -catenin for proteasomal degradation.

Signaling is initiated when Wnt ligands (cysteine-rich secreted glyco-lipoproteins) engage their cognate receptor complex, consisting of a serpentine receptor of the Frizzled family and a member of the LDL receptor family, Lrp5/6. Receptor occupancy disrupts or functionally inactivates the destruction complex by an incompletely understood mechanism that involves Dishevelled. As a consequence, β -catenin accumulates and travels into the nucleus to interact with TCF/LEF transcription factors.

In the nucleus, in the absence of the Wnt signals, TCF/LEF acts as a repressor of Wnt target genes, in a complex with Groucho. β -catenin can convert TCF/LEF into a transcriptional activator of the same genes that are repressed by TCF/LEF alone. In sum, the canonical pathway translates a Wnt signal into the transient transcription of a β -catenin/TCF target gene program (fig. 10A) (MacDonald et al., 2009) (Clevers, 2006).

2. Wnt signaling in stem cells

Wnts are essential for normal embryogenesis, but also actively participate in the regeneration of adult tissues, such as colon, skin, hair follicles and bone by controlling tissue specific adult stem cell function. This aspect of Wnt signaling is intricately connected to the development of disease (Clevers, 2006). Some examples are briefly discussed here.

In the gut, Wnt signaling is required for the establishment of the compartment of precursor cells that reside near the bottom of the crypts; indeed, in TCF4 KO neonatal mice the crypt progenitor compartment is absent (Korinek et al., 1998). In adult life the proliferation of progenitor cells (that continually provides new cells to the regenerating epithelium) is entirely dependent on persistent stimulation of the Wnt pathway, as inhibition of the Wnt receptor complex by transgenic expression of Dickkopf-1 (a soluble Wnt antagonist) induces the complete loss of crypts in adult mice (Pinto et al., 2003). On the other hand, inappropriate activation of the Wnt cascade leads to the expansion of the crypt compartment and the development of adenomas and, eventually, carcinomas. Indeed, colorectal cancer (CRC) almost invariably initiates with an activating mutation in the Wnt cascade. The most common target is the tumor suppressor APC; mutational inactivation of APC is a common trait of most sporadic CRCs, and germ-line mutations cause a hereditary cancer syndrome termed familial adenomatous polyposis (Reya and Clevers, 2005). Loss of APC function leads to the inappropriate stabilization of β -catenin and the formation of constitutive β -catenin-TCF4 complexes, which contribute to the acquisition of the transformed phenotype (van de Wetering et al., 2002).

Wnt signaling is essential for specification and morphogenesis of the mammary gland. Later in adult life, Wnt proteins seem to “preserve” the population of stem cells in the mammary epithelium (Zeng and Nusse, 2010) and inappropriate Wnt signaling can result in deregulated self-renewal: mammary tissue from MMTV-Wnt1 transgenic mice harbors a substantially increased number of mammary stem cells and an aberrant population of progenitor cells that express basal markers (such as CK14) and have *in vivo* regenerative activity (two properties of “more” stem, less differentiated cells). Wnt-induced hyperplasia can evolve into mammary carcinoma, albeit after a long latency period, indicating that excessive Wnt signaling initiates a premalignant state at risk of progression to cancer (Visvader, 2009) (Howe and Brown, 2004).

Mesenchymal stem cells (MSCs) are a small population of bone marrow cells capable of extensive self-renewal and multilineage differentiation (Ling et al., 2009). Wnts play an important role in the self renewal of MSCs and, later, in the lineage-specification of mesenchymal precursors into adipocytes, osteoblasts and chondrocytes. Canonical Wnt signaling promotes osteogenesis and blocks adipogenesis (Davis and Zur Nieden, 2008). Significantly, overexpression of Wnt10b from the osteocalcin promoter in transgenic mice increases postnatal bone mass, whereas total bone volume is lower in Wnt10b^{-/-} mice (Bennett et al., 2005); overexpression of Wnt10b in the bone marrow and adipose tissue, instead, determines a 50% reduction of body fat (Longo et al., 2004). Such diverse effects of canonical Wnt signaling are likely determined by the identity of the responding cells, so that cell-type specific intrinsic properties lead to distinct transcriptional output downstream of the same signal.

3. Wnt/ β -catenin signaling cascade and TAZ/YAP: a comparison

Interestingly, there are - at least superficially - clear elements of overlap between β -catenin and TAZ (or YAP). They all are potent transcriptional co-activators that shuttle from the cytoplasm to the nucleus to regulate gene expression in association with specific DNA-binding platforms. Indeed, while none of them can bind DNA, they are built for protein interactions: the armadillo domains in β -catenin, and the WW, coiled-coil and PDZ-binding domains in TAZ and YAP scaffold their association with a wide range of cellular partners (Clevers, 2006) (Hong and Guan, 2012). Both β -catenin and TAZ/YAP are under the negative control of kinases, so that the phosphorylated protein cannot reach the nucleus. Finally they are degraded by the same β -TrCP ubiquitin ligase complex (Aberle et al., 1997) (Liu et al., 2010) (Zhao et al., 2010). Biologically, Wnt/ β -catenin and TAZ and YAP appear to control overlapping processes. The components of the Hippo pathway were first discovered as critical regulators of organ size; later, YAP and TAZ have been implicated in the regulation of self-renewal and expansion of a variety of stem cells, from embryonic stem cells to tissue specific progenitors and cancer stem cells (as briefly explained in the introduction to the first part of this thesis).

Such analogies between Wnt/ β -catenin signaling and TAZ/YAP hint at some form of joined regulation and activity. Indeed, some recent studies suggest that β -catenin

activity is inhibited by the Hippo pathway (Imajo et al., 2012) (Heallen et al., 2011) (Varelas et al., 2010a), whereas Konsavage et al. reported that YAP is under the transcriptional control of β -catenin/TCF complex (Konsavage et al., 2012).

SUMMARY OF PREVIOUS RESULTS AND AIM OF THE PROJECT

The possibility that Wnt signaling regulates TAZ has been the object of recent work in our laboratory. We have found that TAZ protein is stabilized in a variety of cell types by the activation of the Wnt cascade, either by treatment with ligands, receptor overexpression or depletion of the components of the destruction complex. TAZ stabilization downstream of Wnt results in the induction of its transcriptional activity, either in reporter assays or at the level of endogenous target genes. Notably, TAZ regulation by Wnt is independent of Hippo signaling.

TAZ, like β -catenin, is constitutively degraded in the absence of Wnt signaling, and Wnt stimulation stops TAZ degradation. In fact, we have found that β -catenin degradation pathway is exploited by the cell to achieve concomitant TAZ degradation. In the absence of Wnt signaling, cytoplasmic β -catenin (on its own route to proteasomal degradation after phosphorylation in the destruction complex) associates with TAZ and mediates TAZ interaction with β -TrCP, thus working as a “molecular bridge”. Indeed, β -catenin depletion reduces TAZ association with β -TrCP in co-immunoprecipitation assays, and it results in TAZ stabilization. Activation of the Wnt pathway allows β -catenin to escape from the destruction complex and β -TrCP and, consequently, dampens TAZ degradation, leading to concomitant accumulation of β -catenin and TAZ (fig. 10B).

The aim of the experiments presented hereafter is to demonstrate the functional relevance of the new Wnt/ β -catenin - TAZ axis, showing that TAZ is a relevant mediator of Wnt biological responses. We evaluated the role of TAZ in physiological and pathological Wnt responses; moreover, we tested the functional relevance of β -catenin as TAZ inhibitor in the absence of Wnt signaling.

RESULTS

1. ROLE OF TAZ IN WNT-DEPENDENT DIFFERENTIATION OF MESENCHYMAL STEM CELLS

1.1. TAZ stabilization is part of the “osteogenic program” promoted by Wnt

Mouse bone marrow stromal cells (ST-2 cells) are bipotent progenitors that can be driven to differentiate into osteoblasts or adipocytes by adding the appropriate stimuli in the culture medium. Osteoblastogenesis is triggered by β -glycerophosphate and can be stimulated by the activation of Wnt signaling by various means, such as the overexpression of canonical Wnt ligands or GSK3 inhibition (Bennett et al., 2005). We verified that the addition of recombinant Wnt3a to osteogenic differentiation medium (ODM) promotes osteoblast formation. The differentiation of MSC into osteoblasts is evaluated through the activity of Alkaline Phosphatase (ALP), an enzyme that is expressed when committed osteoblast precursors differentiate into pre-osteoblasts and is thus considered a marker of bone differentiation. As shown in figure 11A, Wnt3a strongly induced ALP activity, whereas “background” osteogenesis could only induce very low levels of ALP in untreated cells (compare lanes 1 and 3). Western blots show that Wnt3a-induced osteogenesis was accompanied by a parallel raise of β -catenin and TAZ protein levels (fig. 11B, lane 3). *TAZ* mRNA levels remained unchanged, in agreement with a post-transcriptional mechanism for TAZ regulation by Wnt signaling (fig. 11C). To assess whether TAZ upregulation was instrumental for bone formation, we transfected ST-2 cells with TAZ siRNA before treatment with recombinant Wnt. TAZ depletion was sufficient to impair ALP induction by Wnt3a (fig. 11A, lane 4), in spite of unmodified β -catenin levels (fig. 11B, lane 4), indicating that TAZ is an essential mediator of bone differentiation induced by Wnt in this cellular context.

Like mouse ST-2 cells, cultured human MSCs can be driven towards different fates by the addition of adequate “cocktails” of differentiation factors to the growth medium. When hMSCs are cultured in ODM (that contains ascorbic acid and β -glycerophosphate), they differentiate into osteoblasts, as indicated by the appearance of ALP-positive blue areas in figure 11D. In the ODM, bone differentiation is

supported by autocrine and paracrine Wnt signaling; indeed, addition of recombinant Dkk-1, an extracellular Wnt antagonist, severely impairs ALP activity (Krause et al., 2010) (fig. 11D-E). Interestingly, we found that, in growth medium, TAZ is constitutively repressed by the β -catenin destruction complex, as depletion of GSK3, Axin or APC lead to TAZ stabilization (see fig. 12A). After 10 days of culture in ODM, TAZ protein levels rose, compared with cells maintained in growth medium (fig. 11F, lanes 1-2); moreover, the addition of Dkk-1 to ODM blocked TAZ upregulation (fig. 11F, lane 3). Thus, autocrinous Wnt signaling in ODM results in TAZ stabilization, together with the expected β -catenin stabilization (fig. 11F). Results shown above indicate that TAZ mediates Wnt pro-osteogenic signal in ST-2 cells; if the same holds true in hMSCs, the impairment of bone differentiation in the presence of Dkk-1 should be rescued by TAZ overexpression. To test this hypothesis, we sustained TAZ expression by transducing hMSCs with a TAZ lentiviral vector (fig. 11F, lane 4). Crucially, TAZ reconstitution restored bone differentiation of Dkk-1-treated hMSCs, without affecting β -catenin levels (fig. 11D-F). Interestingly, TAZ targets CTGF and Cyr61 were induced during osteogenic differentiation of MSCs in a manner that was TAZ-dependent, opposed by Dkk-1 and rescued by exogenous TAZ (fig. 11A and 11F). Taken together, the data reveal that Wnt promotes MSC differentiation into osteoblasts through TAZ stabilization.

1.2. Fat differentiation requires TAZ degradation by the destruction complex in a “Wnt OFF” state

hMSCs differentiate into adipocytes following cyclic stimulations with insulin in a complex adipogenic differentiation protocol. Mature adipocytes contain lipid droplets that can be visualized with Oil Red to assess the degree of differentiation.

Whereas Wnt induces osteoblast maturation, the initiation of adipogenesis requires inactivation of canonical Wnt signaling (Ling et al., 2009). Indeed, depletion of Axin, GSK3 or APC (that mimics the inactivation of β -catenin destruction complex downstream of Wnt ligands) severely blunted the formation of lipid droplets in pro-adipogenic conditions (fig. 12B-C). As anticipated above, Axin, GSK3 and APC siRNAs all induced TAZ stabilization (fig. 12A). To verify if this molecular event was relevant to the block of adipogenic differentiation, we knocked down Axin and TAZ at the same time: double depletion of Axin and TAZ restored

adipocyte differentiation (fig. 12D-E). Similar results were obtained by double knockdowns of TAZ and GSK3 or TAZ and APC (data not shown). Thus, the inhibition of adipogenesis in Axin-, GSK3- and APC-depleted cells was mediated by the upregulation of endogenous TAZ, indicating Wnt signaling must be “turned off” to downregulate TAZ and allow the differentiation of hMSCs into adipocytes.

Together these results indicate that the regulation of TAZ by the Wnt pathway is functionally relevant for MSC differentiation.

2. ROLE OF TAZ DOWNSTREAM OF ONCOGENIC WNT SIGNALING IN APC-MUTANT COLORECTAL CANCER CELLS

2.1. Oncogenic Wnt signaling regulates TAZ protein levels

To investigate the regulation of TAZ downstream of aberrant Wnt signaling, we used a colorectal cancer cell line (SW480 cells), that expresses a truncated, inactive form of APC, due to non-sense mutations in both APC alleles (Nishisho et al., 1991). The ensuing stabilization of β -catenin promotes neoplastic growth (Roh et al., 2001). Reconstitution of these cells with wild-type APC dampens β -catenin nuclear activity and the tumorigenic phenotype of SW480 cells (Faux et al., 2004). Indeed, we could verify by western blot that β -catenin protein levels are lower in APC-reconstituted SW480 cells than in APC-mutant SW480 cells (fig. 13A), indicating that the expression of wild-type APC leads to the effective reconstitution of β -catenin destruction complex.

We have shown that the same destruction complex responsible for β -catenin degradation also controls TAZ protein levels. Indeed, western blots in figure 13B show that in APC-mutant SW480 cells TAZ is more stable than in APC-reconstituted cells (compare lanes 1 and 3). The reduction in TAZ protein levels induced by wild-type APC is paralleled by a decrease in its activity as transcriptional co-activator, as demonstrated by CTGF downregulation (fig. 13B, lanes 1 and 3). We conclude that aberrant Wnt signaling in colorectal cancer cells activates TAZ.

According to this view, in APC-mutant cells TAZ levels should already be the highest levels achievable through the activation of the Wnt signaling cascade; thus TAZ should be insensitive to further attempts to activate the pathway downstream of APC. Previous data show that in cellular models where the β -catenin destruction

complex is active, depletion of GSK3 results in TAZ upregulation. But GSK3 depletion by siRNA had no effect on TAZ stability, or on its target CTGF, in APC-mutant SW480 cells (fig. 13B, lanes 1-2). However, the expression of wild-type APC restored TAZ sensitivity to inhibition by GSK3 and an increase in TAZ levels could be observed after the transfection of GSK3 siRNA (fig. 13B, lanes 3-4). These results confirm that TAZ activation in CRC cells depends on the inhibition of β -catenin destruction complex.

2.2. TAZ contributes to the biological properties of APC-mutant colorectal cancer cells

Data presented so far demonstrate that APC mutation leads to the parallel accumulation of β -catenin and TAZ. The first is well known to be a key player downstream of oncogenic Wnt signaling for the growth of CRC cells (van de Wetering et al., 2002). We asked whether TAZ might also play important functions in this context.

We thus performed proliferation assays in SW480 cells to assess the relative contribution of β -catenin and TAZ. Cells were first transfected with control, TAZ or β -catenin siRNAs; one day after transfection, 10^5 cells/well were seeded in multiwell plates. 24 hours after seeding, a similar number of cells ($\sim 10^5$) were present in all the wells (figure 13C, light blue bars), indicating that siRNA treatments did not compromise cell survival. After 4 days (blue bars), knockdown of either TAZ or β -catenin had reduced the proliferation of APC-mutant SW480 cells to a similar extent, and only the dual depletion of β -catenin and TAZ had lead to a complete growth arrest (fig. 13C, lanes 1-4).

APC reconstitution in SW480 cells also led to severe growth suppression, as previously reported (Faux et al., 2004 and fig. 13C, lane 5 vs lane 1). As TAZ depletion affects the growth of SW480 cells, we hypothesized that impaired proliferation of APC-reconstituted SW480 cells might be due (at least in part) to TAZ degradation by the functional destruction complex. If it were the case, TAZ overexpression in APC-reconstituted SW480 cells should be sufficient to enhance cell proliferation. Indeed, forced expression of TAZ rescued the proliferative potential of APC-reconstituted SW480 cells (fig. 13C, lane 6).

Some important controls clarify that the effects of TAZ manipulation on cell proliferation were due to TAZ itself, and not to an indirect modification of β -catenin activity. First, the manipulation of TAZ levels (by gain- or loss-of function) did not influence β -catenin levels (fig. 13D, lanes 2 and 5), suggesting that TAZ does not operate through β -catenin stabilization. Furthermore, loss of TAZ did not modify the expression of *Axin2* and *Cyclin-D1*, two of the most established targets of the TCF/ β -catenin complex (Clevers, 2006), ruling out that TAZ depletion acts through impaired TCF-dependent transcription (fig. 13E).

Taken together, these data suggest that TAZ is essential and instrumental for cell proliferation downstream of aberrant Wnt signaling, cooperating with the well-established function of β -catenin in this process.

Another important trait of transformed cells is the ability to generate clones when seeded at very low cell densities on a variety of substrates (such as cell culture plastic, soft agar or Matrigel, a jelly-like mixture of extracellular matrix proteins).

We first verified whether the status of APC also controls the capacity of SW480 cells to grow as spheroids in 3D conditions. Representative pictures of Matrigel-embedded spheres formed by APC-mutant and APC-reconstituted SW480 cells are shown in figure 13F (left panels). 10 days after seeding, APC-reconstituted SW480 cells had formed a much reduced number of colonies than APC-mutant SW480 cells. These results are in line with the role of Wnt to endow clonogenic potential on colon cancer cells and with previous data obtained in soft agar assays (Niehrs and Acebron, 2012) (Faux et al., 2004).

Given this premise, we tested the relevance of TAZ and β -catenin to the clonogenic properties of CRC cells. Remarkably, TAZ depletion in APC-mutant SW480 cells impaired spheroid formation, whereas knockdown of β -catenin did not (fig. 13F-G). In sum, APC deficiency empowers 2D cell growth in a manner that is dependent both on β -catenin and on TAZ, as well as clonogenic properties, which are prevalently dependent on TAZ stabilization.

2.3. Two transcription factors mediate Wnt gene responses in colorectal cancer cells

Wnt signaling (either physiological or oncogenic) ultimately results in the activation of a transcriptional program. As we placed β -catenin and TAZ side by side

as downstream mediators of Wnt signaling in CRC cells, we wished to assess what is their relative contribution to the global Wnt gene response.

We analyzed the transcriptome of CRC cells to identify genes whose expression is modulated by APC mutation; then we assessed which fractions of these genes are represented by β -catenin or TAZ transcriptional targets. We obtained the Affymetrix gene expression profiles of APC-mutant and APC-reconstituted SW480 cells, and of APC-mutant SW480 cells transfected with control, TAZ or β -catenin siRNAs. The comparison of the gene expression profiles of parental and APC-reconstituted SW480 cells provided a list of about 3000 probe sets whose expression was significantly regulated by APC mutation. By crossing this list with the list of probe sets downregulated by β -catenin depletion, we found that one-third of the probe sets modulated by APC mutation depend β -catenin transcriptional activity (p values $<10^{-32}$). One sixth of APC-regulated probe sets, instead, depend on TAZ for their expression. This indicates that TAZ targets represent a substantial fraction of gene expression driven by oncogenic APC.

3. β -CATENIN AS TAZ INHIBITOR IN BREAST CANCER CELLS

The mechanism we propose for TAZ regulation by Wnt signaling is centred on β -catenin destruction complex and β -catenin itself. In the absence of Wnt signaling, phosphorylated β -catenin associates with TAZ and facilitates its interaction with β -TrCP, thus promoting TAZ degradation (see figure 10B). This mechanism implies that β -catenin plays an active role in keeping TAZ levels and TAZ-dependent biological properties repressed in the absence of Wnt signaling.

To validate this assumption, we performed some experiments in a breast cancer cell line, MII cells. We first verified that in these cells TAZ levels and activity are controlled by β -catenin destruction complex. Indeed, knockdown of GSK3, APC or Axin caused robust TAZ protein stabilization (fig. 14A-F), promoted nuclear accumulation of endogenous TAZ (fig. 14G) and upregulated TAZ target CTGF (fig. 14H).

CTGF is only an example of TAZ targets whose expression is repressed by β -catenin destruction complex. A much wider TAZ-dependent transcriptional program

must be shut down in the “Wnt OFF” state, ready to be activated by Wnt signals. To delineate such Wnt-inducible TAZ-dependent transcriptional program, we compared the Affymetrix gene expression profiles of MII cells transfected with control siRNA, APC siRNA (a tool to inactivate β -catenin destruction complex) or the combination of APC and TAZ siRNAs. We identified a list of 739 probe sets upregulated more than two times after APC depletion, thus entailing Wnt-induced target genes. Strikingly, 54% of these probe sets were upregulated in a TAZ-dependent manner (fig. 14I); if we restricted our analyses to genes upregulated more than 5 times by APC knockdown, out of 132 probe sets, 91 (70%) were TAZ-dependent. These results indicate that β -catenin destruction complex negatively controls a large number of TAZ target genes and that, upon Wnt signaling, a large fraction of Wnt-induced transcriptional responses will be relayed by TAZ in this cellular context.

The role of β -catenin as TAZ inhibitor is clearly demonstrated by TAZ accumulation in β -catenin depleted cells. As shown in figures 15A-C, many independent β -catenin siRNAs lead to TAZ stabilization and nuclear translocation, and to CTGF upregulation. On the transcriptomic scale, β -catenin depletion led to the induction of about 350 probe sets in Affymetrix microarrays. The induction of 74% of these probe sets was dependent on TAZ, as it was reverted by concomitant loss-of-TAZ (fig. 15D). This means that APC-regulated cytoplasmic β -catenin, so far considered transcriptionally irrelevant and only meant to be degraded, is in fact a potent repressor of TAZ transcriptional program.

We finally verified whether the activation of TAZ-dependent gene expression enhanced TAZ activity in β -catenin depleted MII cells. As explained in the first part of this thesis, the MII cell line contains a small fraction of cancer stem cells (CSCs), whose self renewal and tumorigenic potential depends on TAZ; TAZ hyper-activation expands the population of CSCs and enhances the expression of CSC properties. To evaluate TAZ activity, we performed a mammosphere assay, and we found that knockdown of β -catenin in MII cells caused an increase in mammosphere formation that was blunted upon concomitant depletion of TAZ (fig. 15E). Thus, in the absence of Wnt signaling β -catenin-mediated TAZ degradation actively represses TAZ-dependent malignant behavior of cancer cells.

DISCUSSION

1. TAZ is a mediator of Wnt signaling

We have found that TAZ is a new downstream element of the Wnt signaling cascade. By virtue of its interaction with cytoplasmic β -catenin, TAZ is negatively regulated by the Axin-APC-GSK3 destruction complex and it is positively regulated by Wnt ligands (or any other means to activate the Wnt pathway). Results presented here add that TAZ activation plays a role in the transduction of Wnt signals, as it is required for some well known Wnt biological responses.

The differentiation of MSCs is quite an emblematic case. Wnt signaling and TAZ were known - independently - to control the decision between osteogenesis and adipogenesis. Both canonical Wnt signaling and TAZ stimulate bone development while simultaneously blocking the differentiation of mesenchymal stem cells into fat (Hong et al., 2005) (Davis and Zur Nieden, 2008). Such analogies can now be explained as a result of the new functional connection between Wnt signals and TAZ.

TAZ is also activated downstream of oncogenic Wnt signaling in CRC cells. We found that TAZ is as important as β -catenin for the proliferation of APC-mutant SW480 cells, whereas the clonogenic potential of the same cells appears to be dependent exclusively on TAZ, but not on β -catenin.

The above evidence supports a new view of the canonical Wnt pathway. We have demonstrated that β -catenin is not the only effector of Wnt signals, as TAZ protein stabilization and TAZ-mediated transcription also mediate Wnt response in different cellular model systems. This new view widens the spectrum of potential Wnt target genes. In fact, while β -catenin/TCF target genes undoubtedly account for important aspects of Wnt biology (consider, for example, the upregulation of c-myc or Cyclin-D1 in some tumors), their highly temporal- and cell-specific expression can hardly capture the pervasiveness and complexity of Wnt biological effects (Barolo, 2006) (Niehrs and Acebron, 2012). According with our findings, also TAZ target genes can be reckoned Wnt target genes, and might help in resolving at least some aspects of Wnt signaling. Indeed, gene expression profiling revealed that the regulation of a large fraction of Wnt target genes is TAZ-dependent both in mammary epithelial

cells and colorectal cancer cells. Clearly, the specific relevance of β -catenin and TAZ will likely depend on the cellular context. In some cell types, or experimental conditions, the effects of Wnt may primarily depend on β -catenin/TCF- or TAZ-dependent transcription alone. However, in the most likely situation both β -catenin- and TAZ-dependent biological effects will be occurring at the same time in response to Wnt pathway activation (such as in CRC cells).

2. β -catenin as an active repressor of TAZ activity

We have depicted a mechanism for TAZ degradation that entails an active role of β -catenin also in the absence of Wnt ligands. In the Wnt OFF state, phospho- β -catenin is an essential element for continuous TAZ degradation as it serves as critical scaffold for TAZ recognition by the β -TrCP E3 ubiquitin ligase. This configures an unexpected role for cytoplasmic β -catenin: besides being recruited to the destruction complex for its own degradation, β -catenin works as yet another intermediary of the Wnt cascade upstream of TAZ.

Constitutive TAZ degradation mediated by the destruction complex and β -catenin in the absence of Wnt stimulation has important biological consequences: for example, it removes an anti-adipogenic factor from mesenchymal stem cells to allow adipocyte differentiation. Moreover, we provide a proof-of-principle of the functional relevance of β -catenin as endogenous inhibitor of TAZ: in breast cancer cells β -catenin depletion enhances TAZ-dependent mammosphere formation. Furthermore, microarray analysis of the transcriptome uncovered that several TAZ target genes are activated by β -catenin depletion; this means that cytoplasmic β -catenin, per se transcriptionally irrelevant, does nevertheless actively control gene expression, as it restrains the TAZ-dependent gene expression program. Wnt signaling is a stemness factor in the mammary epithelium; however, its activity must be tightly regulated, as excessive Wnt signaling leads to the hyperplasia of the stem cell compartment, a condition that predisposes to malignant progression (Howe and Brown, 2004) (Visvader, 2009). It is easy to speculate that such strict control of Wnt signals is primarily connected with Wnt-mediated control of TAZ activity, as we have obtained evidence that β -catenin nuclear activity might be dispensable for CSC properties of breast cancer cells.

CONCLUSION

In this thesis, two important upstream regulators of TAZ have been discussed: the first is apico-basal polarity in epithelial cells; the second is Wnt/ β -catenin signaling, that can modulate TAZ activity in both epithelial and mesenchymal cells. TAZ inhibition by the basolateral determinant Scribble is mediated by MST and Lats, whereas Wnt/ β -catenin signaling regulates TAZ independently of the Hippo pathway. Both mechanisms control TAZ biological activity: stabilized TAZ confers cancer stem cell-related properties on breast cancer cells that have lost apico-basal polarity; similarly, stabilized TAZ promotes cell proliferation and clonogenicity in colorectal cancer cells downstream of oncogenic Wnt signaling.

The molecular events triggered by TAZ deserve further investigation. Several questions are still open, starting from TAZ targets in cancer stem cells. Evidence suggests that some of them might be cell type-specific (the regulation of miR-200 family is an emblematic case). Yet, some aspects of TAZ biology – such as the regulation of cell growth – seem to be widespread phenomena, and a general mechanism is expected to master them.

Another open issue is the interconnection between the many inputs that control TAZ activity. How are different signals integrated to determine TAZ protein levels? Are they additive or is there a “prevailing” signal depending on the cellular context? A better understanding of how TAZ is regulated would open the possibility of efficiently manipulating its activity.

MATERIALS AND METHODS

Reagents and plasmids.

Recombinant Wnt3A and Dkk-1 were from Peprotech.

pSUPER-RETRO-PURO vectors containing shGFP or TAZ RNAi sequences (listed in Table 1) were used for stable knockdown in MII and MDA-MB-231 cells.

For stable expression of TAZ wild-type and TAZ(S89A), TAZ cDNAs were subcloned from pEF-TAZ-N-Flag and pEF-TAZ-N-Flag (S89A) constructs (Addgene #19025 and #19026) to pBABE-hygro (Addgene #1765). Retroviral constructs coding mTAZ S306A and S89/S306A were generated by mutagenesis from pBABE-mTAZWT and pBABE-mTAZ(S89A), respectively. For expression of TAZ in SW480 cells and hMSC, TAZ cDNAs were subcloned in lentiviral vector pCSII-CMV-MCS-IRES2-Bsd (kind gift of H. Miyoshi). pBABE puro-SNAIL (#23347), pLNCX2-Scrib (#24600) and pLPC-ScribP305L (#24601) were purchased from Addgene. Scribble wild-type cDNA was subcloned from pLNCX2-Scrib to pCS2; HA tag was added at the N-terminus. HA-Scrib retroviral expression vector was then obtained by subcloning HA-Scrib from pCS2-HA-Scrib to pBABE-blasti (derived by substituting the hygromycin resistance of pBABE-hygro with blasticidin resistance). HA-Scrib-CAAX was obtained by adding CAAX Ras membrane-targeting sequence (CAAX aminoacid sequence: KLNPPDESGPGCMSCCKVLS) to its C terminus by PCR.

pri-miR-200c (and shGFP as control) was cloned in the miR-Vec expression vector (Voorhoeve et al., 2006). miRNA expression was confirmed by qPCR.

All constructs were verified by sequencing.

Cell lines

II and MIV cells were a gift from S. Santner (Santner et al., 2001). II cells were cultured in DMEM/F12 (Gibco, Life Technologies) with 5% HS, glutamine and antibiotics, freshly supplemented with insulin, EGF, hydrocortisone, and cholera toxin. MDA-MB-231 and MIV cells were cultured in DMEM/F12 with 10% FBS. HMLE cells were a kind gift from R. Weinberg (Mani et al., 2008) and were cultured in DMEM/F12 supplemented with Insulin, Hydrocortisone, EGF and BPE (Invitrogen).

ST-2 stromal mouse cells were purchased from DSMZ (Braunschweig, Germany) and were cultured in MEM alpha (Gibco, Life Technologies) supplemented 10% FBS, glutamine and antibiotics. Primary human mesenchymal stem cells (hMSC), their growth and differentiation media were from Lonza. HepG2 cells were cultured in MEM (Gibco, Life Technologies) supplemented with 10% FBS, glutamine, antibiotics and non essential aminoacids.

SW480 cells were cultured in RPMI 1640 with 10% FBS, glutamine and antibiotics, freshly supplemented with insulin, hydrocortisone, 1-thioglycerol (traces). SW480 cells stably expressing empty vector or APC were a kind gift of M.C. Faux (Faux et al., 2004) and were maintained with 1.5 mg/mL G418. ST-2 stromal mouse cells were purchased from DSMZ (Braunschweig, Germany) and were cultured in MEM alpha (Gibco, Life Technologies) supplemented 10% FBS, glutamine and antibiotics.

siRNA transfections

siRNA transfections were performed with Lipofectamine RNAi-MAX (Life technologies) in antibiotics-free medium according to manufacturer instructions. Sequences of siRNAs are provided in Table 1.

Production of viral particles and infections

Lentiviral particles were prepared by transiently transfecting HEK293T cells with lentiviral vectors together with packaging vectors (pMD2-VSVG and psPAX2). Retroviral particles were prepared by transiently transfecting HEK293gp cells with retroviral vectors together with packaging vector (pMD2-env). Culture medium containing the viral particles was collected and filtered 48 hours after transfection. Viral infection was performed by incubating target cells with diluted viral supernatant for 24 hours. Infected cells were selected with puromycin, hygromycin or blasticidin.

Mammosphere assay

Confluent monolayers of cells were trypsinized, counted, and plated as single-cell suspensions (1000 cells/cm²) on ultra-low attachment plates (Costar). Mammospheres were counted after 5-8 days; cells were thereafter dissociated and reseeded to generate secondary mammospheres. MII and HMLE cells were grown in serum-free DMEM-F12 supplemented with 5 µg/mL insulin, 10 ng/mL EGF, 0,5 µg/mL hydrocortisone, 52 µg/mL BPE and B27 (Invitrogen). MDA-MB-231 cells were grown in serum-free DMEM-F12 supplemented with 0,4% BSA, 5 µg/mL insulin, 10 ng/mL EGF and 20 ng/mL bFGF, and 4 µg/mL heparin. Statistical analyses were done with Prism soft-ware (GraphPad).

Tumorigenesis assay

For xenograft tumor-seeding studies, the indicated numbers of MII or MIV cells were suspended in 100 µl Matrigel (BD Biosciences) and injected into the fat pads of immunocompromized RAG^{-/-} female mice. Tumor formation was assayed by palpation. After 5 weeks, mice were sacrificed and tumors were explanted for histological analyses.

Fluorescence-activated cell sorting

Cells were detached from plates with TrypLE (Invitrogen), resuspended (5 × 10⁵ cells/ml), incubated in running buffer (PBS 13, BSA 0.5%, and EDTA 5 mM) with anti-human CD44 (clone G44-26, FITC-conjugated, BD Biosciences) and anti-human CD24 (clone ML5, PE-conjugated, BD Biosciences), and finally analyzed on a MoFlo XDP sorter (Beckman Coulter).

Matrigel clonogenic assay

The clonogenic potential of parental or APC-reconstituted SW480 cells was analyzed in 3D non-adherent culture conditions using Growth Factor Reduced Matrigel matrix (BD Biosciences). SW480 cells were counted, resuspended in 50% Matrigel Matrix + 50% growth medium in ice and plated on Matrigel-coated chamber slides (Nunc) (500 cells per well). After solidification, gels were supplemented with an appropriate volume of growth medium, which was changed every 2 days during the experiment. 10 days after seeding, representative fields of the colonies were photographed. The number of spheres within each well was counted after recovery from Matrigel, using the Cell Recovery Solution from BD Biosciences. Statistical analyses were done using the Prism software (Graph Pad).

Growth assay

10⁵ SW480 cells were plated in a 35 mm dish (day 0) in triplicate for each time point. Cells were counted with Scepter 2.0 handheld automated cell counter (Millipore) after detachment with trypsin. Each experiment was repeated at least three times independently.

MSC differentiation assays

For bone differentiation of ST-2 mouse cells, cells were cultured in the presence of 100 mg/ml ascorbic acid (Sigma) and 5 mM β -glycerophosphate (Sigma), alone or in combination with 100 ng/ml Wnt3A (Peprotech) for 72 hours. Cells were then harvested in 0.5% Triton X-100 and alkaline phosphatase (ALP) activity was assessed by measuring spectroscopically at 405 nm the hydrolysis of p-nitrophenyl phosphate (Sigma) to p-nitrophenol, following a 10 minutes incubation at room temperature. Data were normalized to the total protein content, determined by Bradford. For bone differentiation of hMSC, cells were switched from growth medium (GM) to osteogenic differentiation medium (ODM) 24 hours after seeding; medium was then renewed every 2 days for a total of 10 days of differentiation. MSC were then fixed and assayed by ALP staining kit (Sigma 85L2). Adipogenic differentiation of hMSC and quantifications of ALP-positive or Oil Red-positive areas were carried out as in (Dupont et al., 2011).

Western blot and immunoprecipitations

Protein lysates were obtained from cells grown for two days at high density by sonication in lysis buffer (20 mM HEPES (pH 7.8), 150 mM NaCl, 5% Glycerol, 5mM EDTA, 0.5% Np40, protease and phosphatase inhibitors). Equal amounts of protein were separated by SDS-PAGE in commercial 4-12% or 10% Nupage MOPS acrylamide gels (Invitrogen) and transferred onto PVDF membranes (ImmobilonP) by wet electrophoretic transfer. In general, blots were blocked one hour at RT with 0.5% non-fat dry milk (BioRad) in TBSt (0.05% Tween) and incubated o.n. at 4°C with primary antibodies (listed in Table 2). Horseradish peroxidase-conjugated secondary antibodies were incubated 1 hour at RT. Blots were developed with Pico or Dura SuperSignal West chemiluminescent reagents (Pierce).

For immunoprecipitation experiments, cells were lysed by sonication in 20 mM HEPES (pH 7.8), 400 mM KCl, 5% Glycerol, 5 mM EDTA, 0.4% Np40, phosphatase and protease inhibitors, and cleared by centrifugation. Extracts were diluted to 20 mM HEPES (pH 7.8), 100 mM NaCl, 5% Glycerol, 2.5 mM MgCl₂, 1% TritonX100 and 0.5% NP40 and incubated for four hours at 4°C with anti-TAZ/YAP monoclonal antibody (Santa Cruz, sc-101199), bound to Protein-A sepharose beads. Prior to IP, beads were incubated overnight in PBS with 2% BSA and 0.05% CHAPS. After three washes in binding buffer, co-purified proteins were analyzed by immunoblotting.

Immunofluorescence

Cells were plated on Fibronectin (Sigma)-coated glass chamber slides (Nunc) and fixed 10 min at room temperature (RT) with Dent's fixative solution. Slides were permeabilized 10 min at RT with PBS 0.3% Triton X-100, and processed for immunofluorescence (IF) according to the following conditions: blocking in 10% BSA or Goat Serum (GS) in PBST for 1 hr followed by incubation with primary antibody (diluted in 2% BSA or GS in PBST) for 16 hr at 4°C, four washes in PBST and incubation with secondary antibodies (diluted in 2% BSA or GS in PBST) for 1.5 hr at room temperature. Primary antibodies are: anti-Scribble and anti-HA (Santa Cruz; 1:100, blocking with BSA), anti-Vimentin (Santa Cruz, 1:300, blocking with GS), anti-E-Cadherin (BD Biosciences, 1:1000, blocking with GS) and anti-TAZ (BD Biosciences, 1:100, blocking with BSA). Secondary antibodies (Invitrogen) are: chicken anti-goat Alexa488 (1:100, blocking with BSA), goat anti-mouse Alexa488 (1:200, blocking with GS), and goat anti-rabbit Alexa568 (1:200, blocking with GS). After three washes in PBS, cells were stained with propidium iodide (PI), Hoechst (Sigma), or YOYO-1 (Invitrogen) for 30 min in PBS. Confocal images were obtained with a Leica TCS SP5 equipped with a CCD camera.

BrdU Cell proliferation assay

Cells were plated on fibronectin-coated glass slides and transfected with TAZ (or TAZ/YAP) siRNAs. 48 hours after transfection cells were incubated with BrdU for 20 min before fixation. Cell fixation and BrdU detection were performed with 5-Bromo-2'-deoxy-uridine Labeling and Detection Kit I (Roche) according to manufacturer's instructions.

Cell cycle analysis

To determine the fraction of cells in each phase of the cell cycle, subconfluent cells were trypsinized 48 hours after siRNA transfection, washed with PBS and fixed in cold 70% ethanol at -20°C for at least one hour. Fixed cells were washed in PBS and incubated in PBS containing Propidium Iodide and RNase A for 20 min at RT. Stained cells were analyzed on a MoFlo XDP sorter (Beckman Coulter).

RNA extraction and qRT-PCR

Total RNA was extracted with Trizol reagent (Invitrogen) according to manufacturer's instruction. To minimize DNA contamination, total RNA was treated with DNase I (Ambion). 1 ug of DNase-treated total RNA was retrotranscribed with MoMLV Reverse Transcriptase (Invitrogen) and oligo-d (T) primers. Real-time PCR was performed on a RotorGene 3000 (Corbett) using FastStart SYBR Green Master (Roche). Primer sequences are listed in Table 3.

Microarray experiments

For microarray experiments, GeneChips Human Genome U133 Plus 2.0 (Affymetrix, Santa Clara, CA, USA) were used. Total RNA from MDA-MB-231, MII or SW480 cells (four replicas for each experimental condition) was extracted using TRIZOL (Invitrogen) and treated with DNaseI (Ambion). RNA quality and purity were assessed on the Agilent Bioanalyzer 2100 (Agilent Technologies, Waldbronn, Germany); RNA concentration was determined using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc.). In vitro transcription, hybridization and biotin labeling were performed according to Affymetrix One Cycle Target Labelling protocol (Affymetrix). As control of effective gene modulation and of the whole procedure, we monitored the expression levels of *TAZ*, *β-catenin*, *APC* - and of known TAZ or *β-catenin* target genes - by qRT-PCR prior to microarray hybridization and in the final microarray data.

All data analyses were performed in R (version 2.14.2) using Bioconductor libraries (BioC 2.9) and R statistical packages. Probe level signals were converted to expression values using robust multi-array average procedure RMA (Irizarry et al., 2003) of Bioconductor *affy* package. Differentially expressed genes were identified using Significance Analysis of Microarray algorithm coded in the *samr* R package (Tusher et al., 2001). In SAM, we estimated the percentage of false positive predictions (i.e., False Discovery Rate, FDR) with 100 permutations.

To identify genes regulated by TAZ in MDA-MB-231 cells, we considered probe sets with an estimated percentage of false positive predictions lower than 5%. The threshold for fold-change was set at +1.5 and -1.5 for upregulated and downregulated probe sets, respectively, when shGFP and shTAZ were compared. For siRNA-transfected cells, thresholds were set at +2/-2.

To identify genes upregulated by APC or *β-catenin* depletion in MII cells (Figures 1G and 6F, respectively), we set the lower limit for fold change induction to 2. All selected probe sets had an estimated percentage of false positive predictions equal to 0. To identify TAZ-dependent genes, we compared the expression profiles of APC-depleted/TAZ-depleted cells with APC-depleted cells, and of *β-catenin*-depleted/TAZ-depleted with *β-catenin*-depleted cells and selected those probe sets with a negative fold change and an FDR equal to 0.

To identify genes whose expression is modified by APC mutation in SW480 cells, we selected those probe sets whose estimated percentage of false positive predictions was lower than 5%. To identify TAZ- or β -catenin-dependent genes, we refined this selection comparing the expression profiles TAZ- or β -catenin-depleted cells with SW480 controls and selecting those probe sets whose FDR was lower than 5% and expression level consistently regulated by APC mutation.

microRNA expression profiling

We used the stem-loop RT-PCR based TaqMan MicroRNA Human Array (Applied Biosystems). RT-PCR reactions were performed according to the manufacturer's instructions. Briefly, 1 μ g of DNase I-treated total RNA was reverse-transcribed using Megaplex RT Primers and the TaqMan miRNA reverse transcription kit in a total of 7.5 μ l volume. Quantitative real-time PCR was performed using Applied Biosystems 7900HT system and a TaqMan Universal PCR Master Mix with 6 μ l cDNA input per plate. The relative amount of each target was determined with the $2^{(-\Delta Ct)}$ method.

For the detection of mature microRNAs with SYBR-green based real-time PCR, 5 μ g of total RNA was polyadenylated with Escherichia coli PAP polymerase (Ambion). First strand cDNA was generated using a polyT adaptor (Shi and Chiang, 2005). Real time PCR was performed with a universal reverse primer and a forward primer specific for each mature miRNA.

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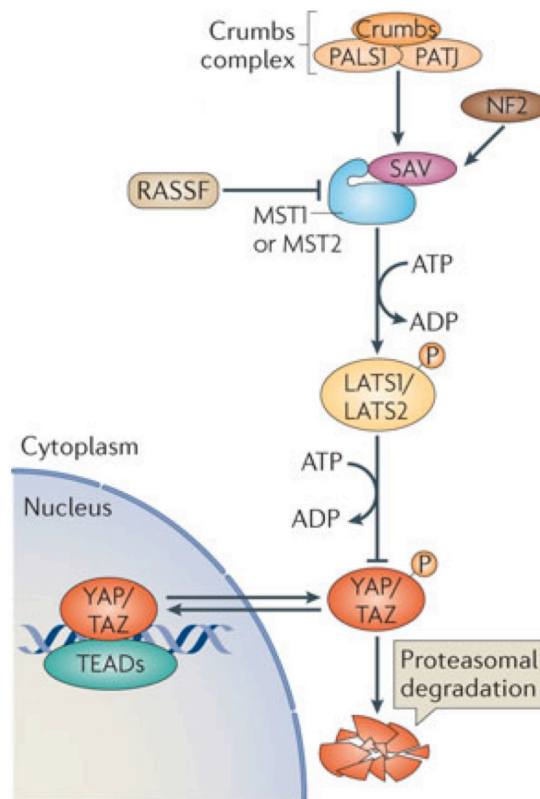
FIGURES

FIGURE 1

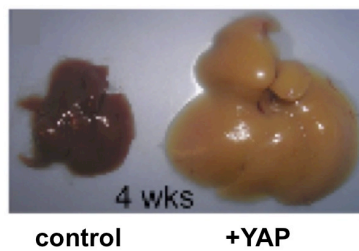
- A.** Schematic representation of the mammalian Hippo pathway. A series of phosphorylation events via MST and LATS ultimately leads to the phosphorylation of TAZ and YAP, that regulates TAZ/YAP localization and degradation.
- B.** A normal (left) and a YAP-overexpressing (right) mouse liver (from Dong et al., 2007).
- C.** Representative immunohistochemistry images for TAZ expression in low grade (G1) or high grade (G3) invasive human breast cancer samples. Nuclei are counterstained with hematoxylin. In the picture on the left, black arrowheads point to TAZ-positive cells in normal mammary ductal tissue (NT) included in the same section with G1 tumor structures; the inset shows very rare TAZ-positive cells in G1 neoplastic tissue (white arrowhead). The histogram on the right depicts the frequency of TAZ-positive G1 or G3 primary human breast cancers: 35/44 G3 tumors and only 1/26 G1 tumor were found positive for TAZ expression by immunohistochemistry (p value < 0.0001).

FIGURE 1

A The Hippo pathway in mammals



B Mouse liver



C Low grade (G1) breast tumor High grade (G3) breast tumor

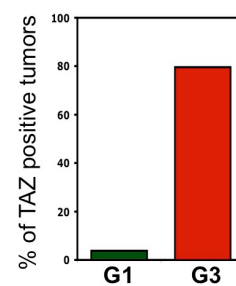
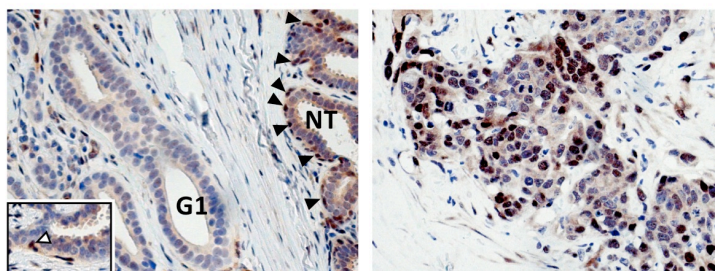
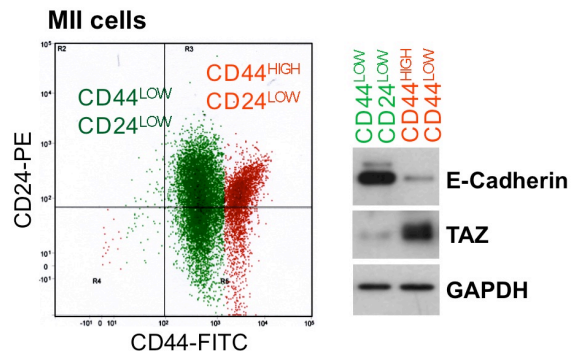


FIGURE 2

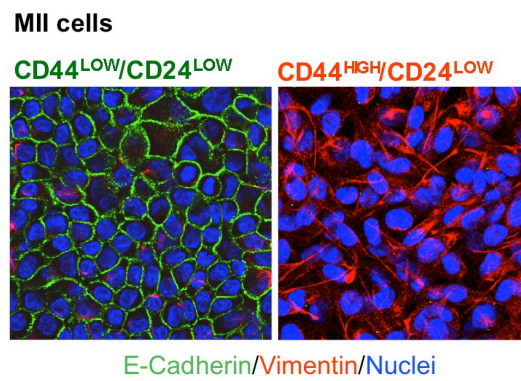
- A.** *Left panel:* representative CD44/CD24 profile of MII cells. Two populations can be distinguished: the CD44^{LOW}/CD24^{LOW} population (green) comprises about 70% of cells; about 30% of cells reside in the CD44^{HIGH}/CD24^{LOW} population (red). *Right panel:* Western blot analysis for E-cadherin and TAZ in the CD44^{LOW}/CD24^{LOW} and CD44^{HIGH}/CD24^{LOW} populations isolated from MII cells by fluorescence-activated cell sorting. GAPDH serves as loading control.
- B.** Immunofluorescence (IF) images for the epithelial marker E-cadherin and the mesenchymal marker Vimentin in the CD44^{LOW}/CD24^{LOW} and CD44^{HIGH}/CD24^{LOW} populations isolated from MII cells by fluorescence-activated cell sorting. Nuclei are stained with Hoechst.
- C.** Comparison of TAZ protein levels in MII, MIV and MDA-MB-231 cell lines. Panels are Western blots; GAPDH serves as loading control.
- D.** Comparison of E-cadherin expression and localization in MII, MIV and MDA-MB-231 cell lines. Panels are representative IF images. Nuclei are stained with Propidium Iodide.

FIGURE 2

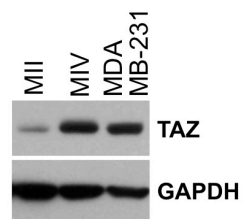
A



B



C



D

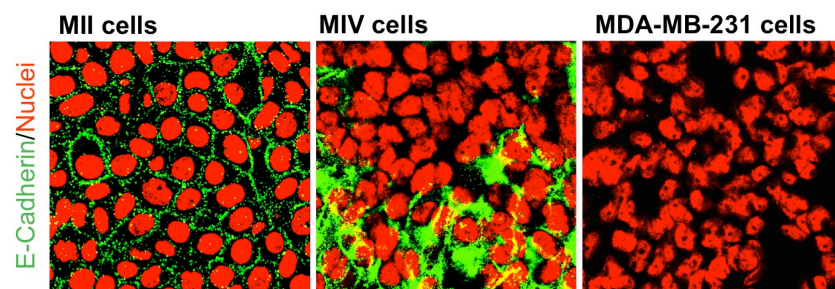


FIGURE 3

- A.** Western blot analysis of lysates from control or Twist-expressing HMLE cells. Where indicated, cells were transfected with a control or three independent anti-TAZ siRNAs. GAPDH serves as loading control. Twist induces E-cadherin downregulation, and upregulation of TAZ and its target genes. TAZ siRNAs in HMLE-Twist cells reduce the expression of CTGF, PAI-1 and Survivin, but does not rescue E-cadherin expression.
- B.** *TAZ* mRNA expression in control and Twist-expressing HMLE cells, as monitored by qRT-PCR. *TAZ* mRNA levels are normalized to GAPDH expression.
- C.** Western blot analysis for the indicated proteins in lysates of MII-empty vector and MII-Snail cells. Where indicated, cells were transfected with a control or two independent anti-TAZ siRNAs. GAPDH serves as loading control. Exogenous Snail causes loss of E-cadherin expression and the upregulation of TAZ and CTGF. Depletion of TAZ in MII-Snail cells abolishes the expression of CTGF but does not rescue E-cadherin expression.
- D.** Quantification of mammospheres formed by control and Twist-overexpressing HMLE cells transiently transfected with control or anti-TAZ siRNAs (mean of six replica experiments + SEM). TAZ is required for Twist-induced mammosphere formation.
- E.** Western blot analysis for TAZ, CTGF, Survivin, and E-cadherin expression in MDA-MB-231 cells stably transduced with retroviral vectors expressing a control (shGFP) or two independent TAZ shRNAs (shTAZ#2 and shTAZ#3). GAPDH serves as loading control. TAZ depletion impairs the expression of TAZ target genes, but does not rescue E-cadherin expression.
- F.** Representative phase-contrast images of control or TAZ-depleted MDA-MB-231 cells. TAZ depletion does not alter cell morphology.
- G.** Quantification of primary and secondary mammospheres formed by the MDA-MB-231 derivatives described in (E). Data are mean+SEM of six replica experiments. TAZ depletion impairs the self-renewal capacity of MDA-MB-231 cells.

FIGURE 3

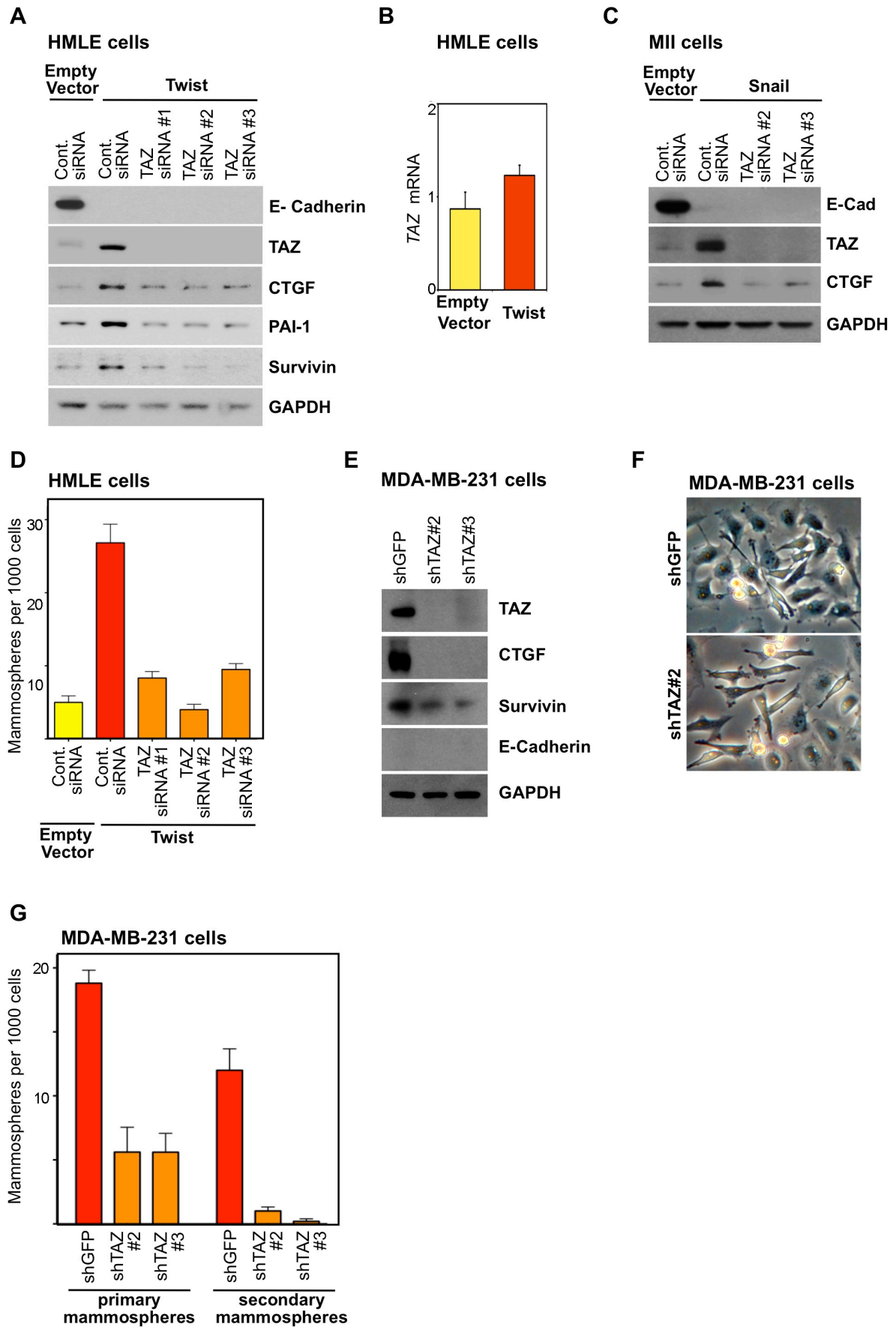


FIGURE 4

- A.** Western blot analysis for Scribble, TAZ, CTGF, and E-cadherin expression in MII cells transfected with control or three independent anti-Scribble siRNAs. GAPDH serves as loading control. Scribble loss increases TAZ protein levels and activity without affecting E-cadherin expression.
- B.** TAZ mRNA expression, monitored by qRT-PCR, in MII cells transfected with control siRNA or SCRIB siRNA#3 (SCRIB siRNA). TAZ mRNA levels are normalized to GAPDH expression.
- C.** IF images showing E-cadherin in MII cells transfected with control siRNA or SCRIB siRNA#3. Nuclei are stained with Hoechst. Scribble downregulation does not change the membrane localization of E-cadherin.
- D.** Western blot for the indicated proteins in lysates of MII-empty vector and MII-Snail cells transfected with control or Scribble siRNA#3 (SCRIB siRNA). GAPDH serves as loading control. Scribble depletion stabilizes and activates TAZ in epithelial cells (MII Empty vector), but not in mesenchymal cells (MII-Snail).
- E.** Confocal images showing the localization of Scribble in control or Snail-expressing MII cells, and control or Twist-overexpressing HMLE cells. For each cell line, panels show XY images (top) and the indicated (red line) Z stacks (bottom). White arrowheads point to lateral membrane domains. Nuclei are stained with Propidium Iodide. Scribble lines the plasma membrane in epithelial cells, whereas it is sparse in the cytoplasm and does not occupy cell-cell contact regions in mesenchymal cells.
- F.** IF images showing the localization of Scribble in FACS-sorted CD44^{LOW}/CD24^{LOW} and CD44^{HIGH}/CD24^{LOW} populations of MII cells. Nuclei are stained with Hoechst. Scribble is localized at the plasma membrane in the CD44^{LOW}/CD24^{LOW} population, whereas it is cytoplasmic in the CD44^{HIGH}/CD24^{LOW} population.
- G.** IF images showing delocalized Scribble in MIV and MDA-MB-231 cells. Nuclei are stained with PI.

FIGURE 4

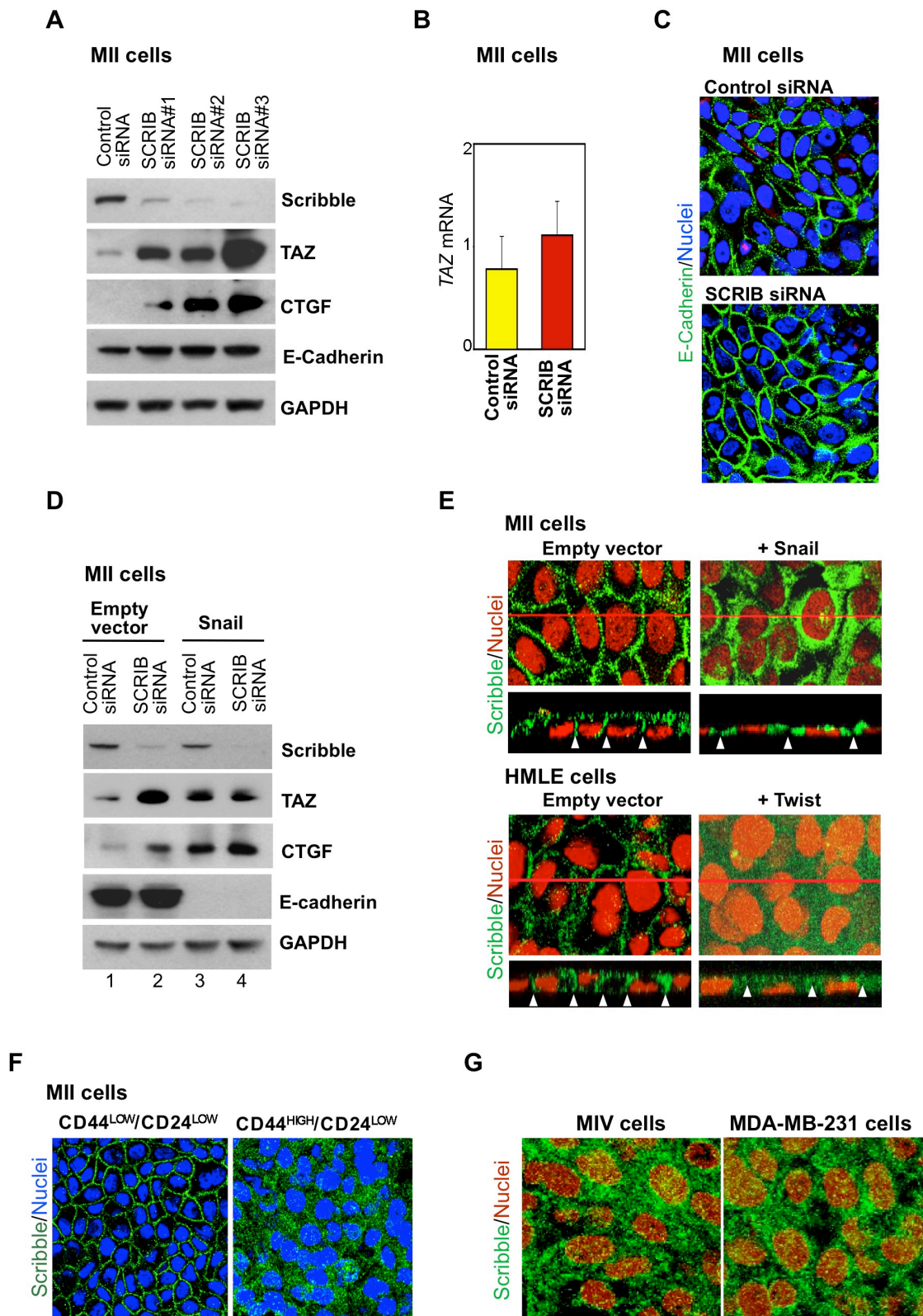


FIGURE 5

- A.** Confocal images showing the localization of Scribble in MII-Empty vector or in MII-ScribP305L. For each cell line, panels show XY images (top) and the indicated (red line) Z stacks (bottom). White arrowheads point to lateral membrane domains. Nuclei are stained with Propidium Iodide. Mutant Scribble cannot localize to the plasma membrane, and also prevents the recruitment of the endogenous protein to the plasma membrane.
- B.** Western blot analysis for Scribble, TAZ, CTGF and Survivin expression in lysates of MII cells expressing either ScribP305L alone or ScribP305L and a TAZ shRNA. GAPDH serves as loading control. Scribble displacement from the plasma membrane stabilizes and activates TAZ.
- C.** Confocal images showing the localization of HA-tagged wild-type Scribble and Scribble-CAAX stably introduced in Snail-expressing MII cells. Nuclei are stained with YOYO-1. Similarly to endogenous Scribble (see fig. 4E), exogenous wild-type Scribble is localized in the cytoplasm in MII-Snail cells, whereas SCRIB-CAAX is mainly anchored to the plasma membrane.
- D.** Western blot analysis for TAZ and its target CTGF in MII-Snail cells stably expressing control (empty vector, lane 1), HA-tagged wild-type Scribble (lane 2), or HA-tagged Scribble-CAAX (lane 3). GAPDH serves as loading control. Overexpression of membrane-tethered Scribble, but not of wild-type Scribble, reduces TAZ expression and activity in MII-Snail cells to levels comparable to parental MII cells (MII-empty vector, lane 4).
- E.** Quantification of primary mammospheres formed by control MII cells and by cells stably expressing ScribP305L or ScribP305L+shTAZ. ScribP305L induces an increase in mammosphere formation that is dependent on TAZ upregulation.

FIGURE 5

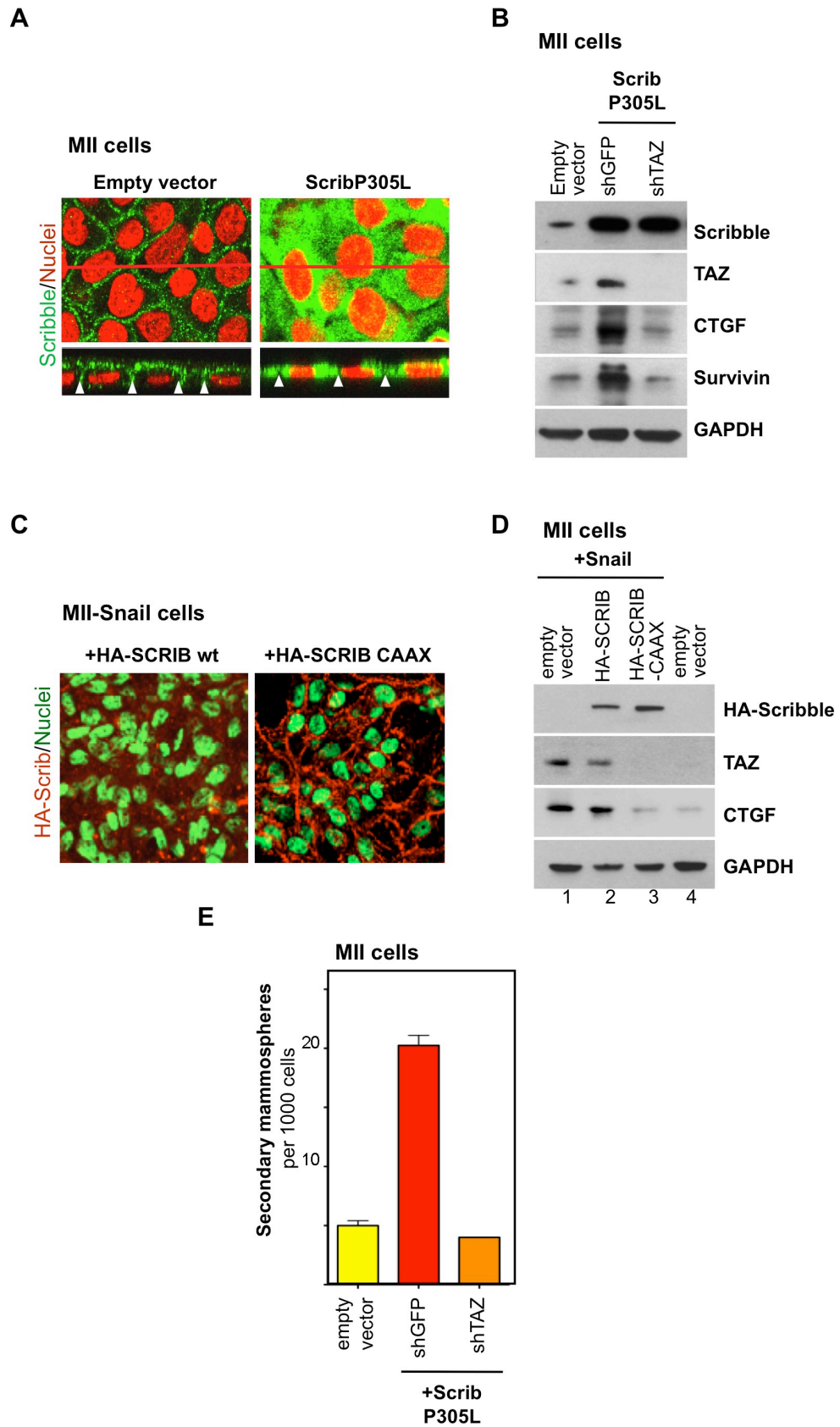


FIGURE 6

- A.** Total TAZ was immunoprecipitated from control MII cells, cells transfected with SCRIB siRNA#3 or MII-Snail cells; phosphorylation on Ser89 was assessed by Western blotting of immunoprecipitated proteins. Both Scribble depletion and EMT induction reduce the levels of phosphorylated TAZ. See fig. 6F for inputs.
- B.** Total TAZ was immunoprecipitated from control HMLE cells, cells transfected with SCRIB siRNA#3 or HMLE-Twist cells; phosphorylation on Ser89 was assessed by Western blotting of immunoprecipitated proteins. As above, TAZ is less phosphorylated in Scribble-depleted and Twist-overexpressing cells. See fig. 6G for inputs.
- C.** Schematic representation of murine TAZ protein, showing Ser89 (that is recognized by 14-3-3 proteins upon phosphorylation by LATS) and Ser306 (whose phosphorylation by LATS primes TAZ for subsequent phosphorylation by CK1 on Ser309; phosphorylated Ser309 is then recognized by β -TrCP). TB: TEAD binding domain; WW: WW domain; TA: Transcriptional Activation domain.
- D.** Western blots for the indicated proteins in lysates of MII cells transduced with empty vector, wild-type mTAZ, or mTAZ variants carrying mutations in some LATS-phosphorylation sites (S89A, S306A, S89/306A). GAPDH serves as loading control. mRNA levels of all the exogenous TAZ isoforms are comparable (data not shown).
- E.** Western blots for the indicated proteins in lysates from MII cells expressing wild-type mTAZ or TAZS89/306A, transfected with control siRNA or Scribble siRNA#3 (SCRIB siRNA). GAPDH serves as loading control. Scribble depletion only stabilizes wild-type, LATS-sensitive TAZ.
- F.** Western blot analyses of whole cell lysates (*left*) and TAZ immunocomplexes (*right*) purified from control MII cells, cells transfected with SCRIB siRNA#3 or MII-Snail cells. Endogenous TAZ co-immunoprecipitates with Scribble, MST, Lats and β -TrCP.
- G.** Western blot analyses of whole cell lysates (*left*) and TAZ immunocomplexes (*right*) purified from control HMLE cells, cells transfected with SCRIB siRNA#3 or HMLE-Twist cells.
- H.** A model depicting the proposed mechanism for TAZ regulation by Scribble and the Hippo pathway.

FIGURE 6

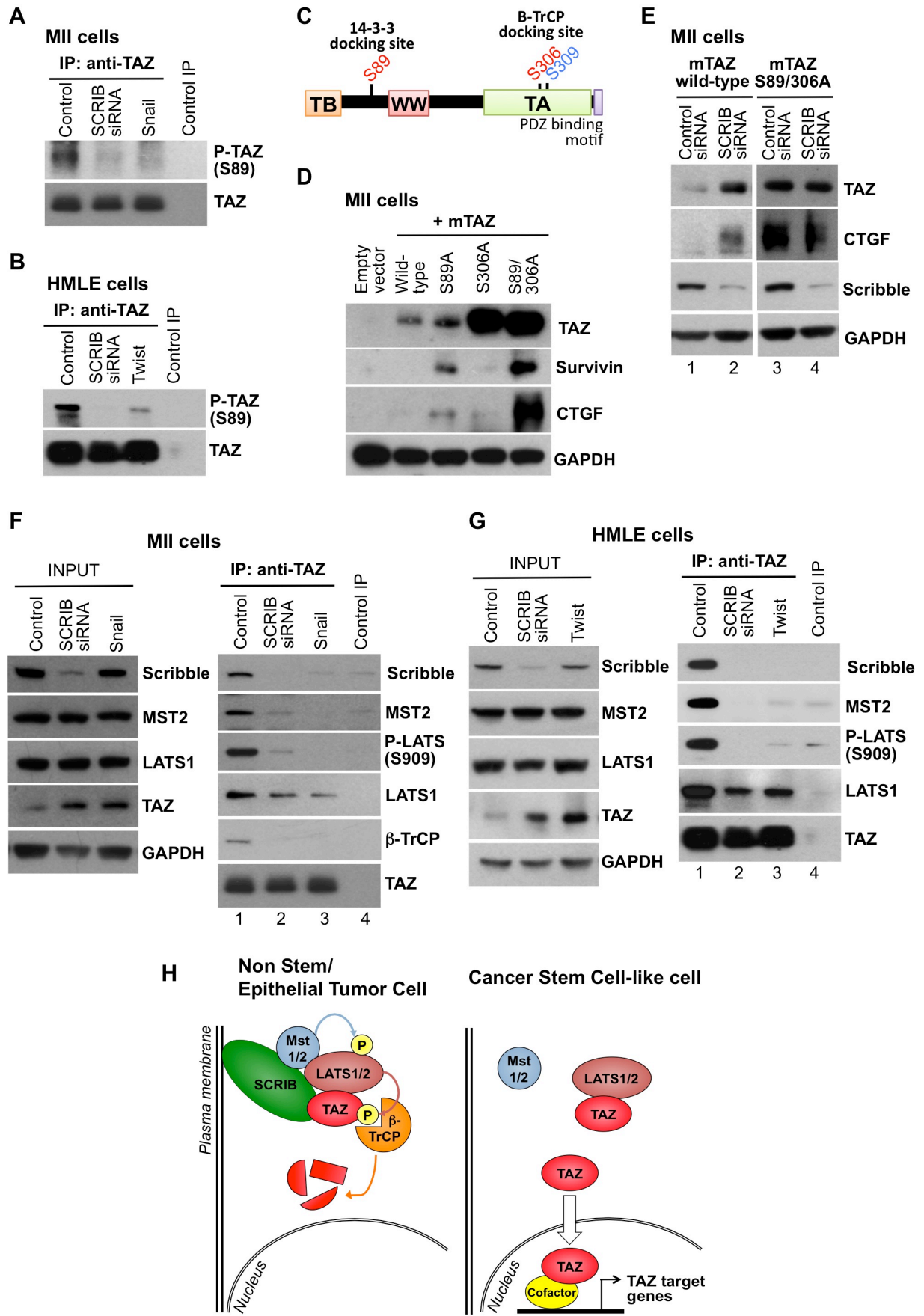


FIGURE 7

- A.** List of microRNAs modulated by TAZ in TAZ(S89A) overexpressing MII cells.
- B.** Graph showing the relative expression levels of TAZ-modulated microRNAs in control and TAZ(S89A) overexpressing MII cells. In the list of regulated microRNAs, miR-200 family members (in particular miR-200b, miR-200c and miR-141) and the closely related miR-205 are among the most expressed microRNAs in MII cells, and the most significantly downregulated microRNAs in MII-TAZ(S89A) cells.
- C.** Analysis of miR-200 family members in MII and MII-TAZ(S89A) cells by SYBR green-based RT-real time PCR. microRNA levels are normalized to snRNAU6.
- D.** Analysis of pri-miR-200b~a~429 and pri-miR-200c~141 by RT-PCR. GAPDH serves as calibrator.

FIGURE 7

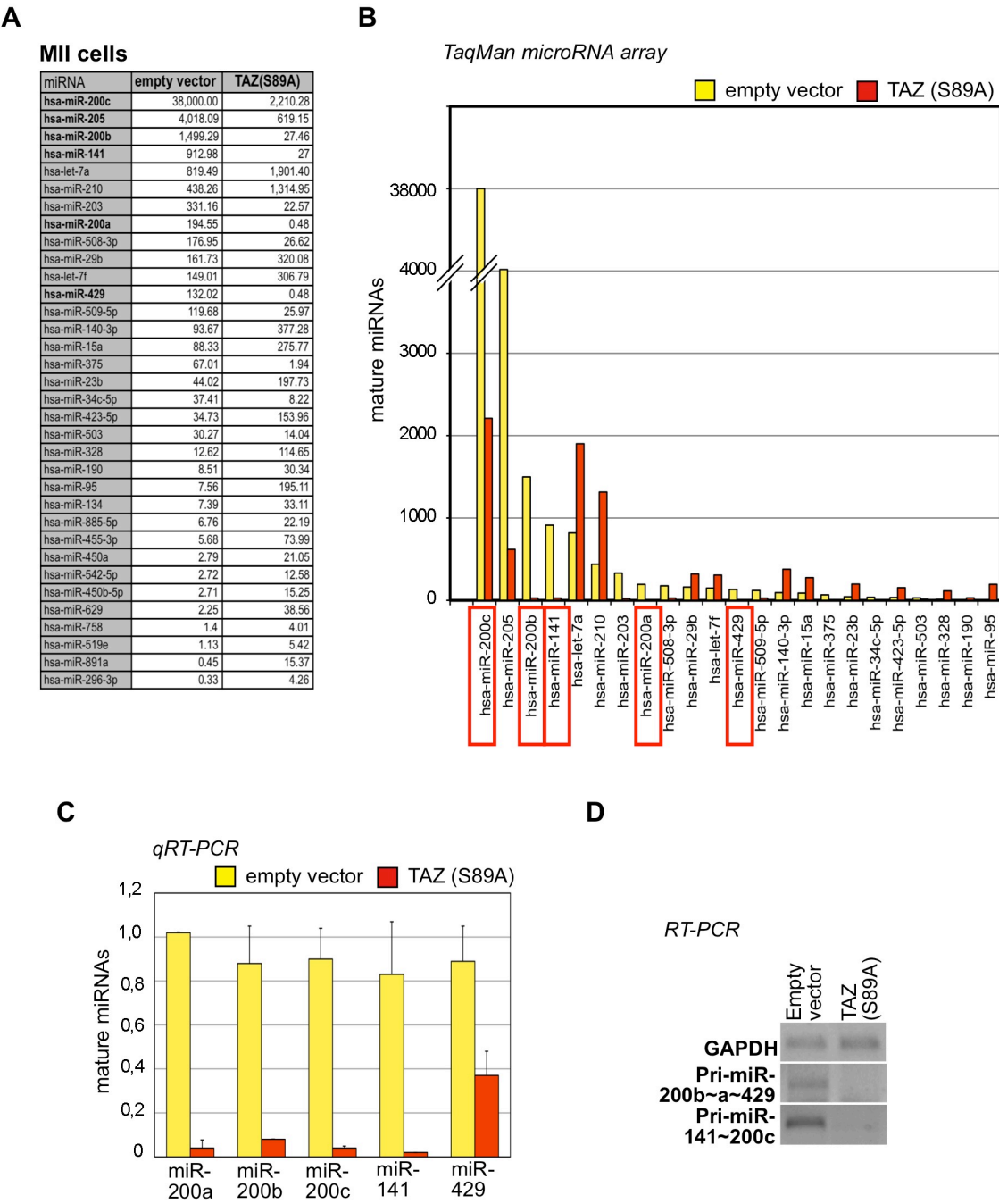


FIGURE 8

- A.** Western blot for the mesenchymal marker Vimentin, the epithelial marker E-cadherin, TAZ and its target in control MII cells and in MII-TAZ(S89A) cells transduced either with a control vector (shGFP) or with miR-200c precursor (miR-200c). GAPDH serves as loading control. Exogenous miR-200c rescues E-cadherin, and abolishes Vimentin expression.
- B.** Representative IP images for E-cadherin and Vimentin in MII, MII-TAZ(S89A)-shGFP and MII-TAZ(S89A)-miR-200 cells. Nuclei are stained with Hoechst.
- C.** Cytofluorimetric analysis of CD24 and CD44 expression in MII, MII-TAZ(S89A)-shGFP and MII-TAZ(S89A)-miR-200 cells. Green rectangles encircle the CD44^{LOW}/CD24^{LOW} sub-populations, red rectangles encircle the CD44^{HIGH}/CD24^{LOW}. miR-200c overexpression reverts the CD44/CD24 profile of MII-TAZ(S89A) cells to the profile of control MII cells.
- D.** Histogram showing the percentage of cells enclosed in green/red areas in the plots in figure C.
- E.** Quantification of secondary and tertiary mammospheres seeded by MII, MII-TAZ(S89A)-shGFP and MII-TAZ(S89A)-miR-200 cells. In the presence of exogenous miR-200c, TAZ activity is inhibited.
- F.** Tumor-seeding ability of MII, MII-TAZ(S89A)-shGFP and MII-TAZ(S89A)-miR-200 cells. The indicated number of cells was injected into the fat pads of RAG^{-/-} mice. The table reports the number of mice developing palpable tumors after 5 weeks.
- G.** Representative hematoxylin and eosin staining of tumors emerging from fat-pad injection of MII, MII-TAZ(S89A)-shGFP and MII-TAZ(S89A)-miR-200 cells. MII and MII-TAZ(S89A)-miR-200 cells form small tubular structures, whereas MII-TAZ(S89A)-shGFP cells form undifferentiated tumor masses.

FIGURE 8

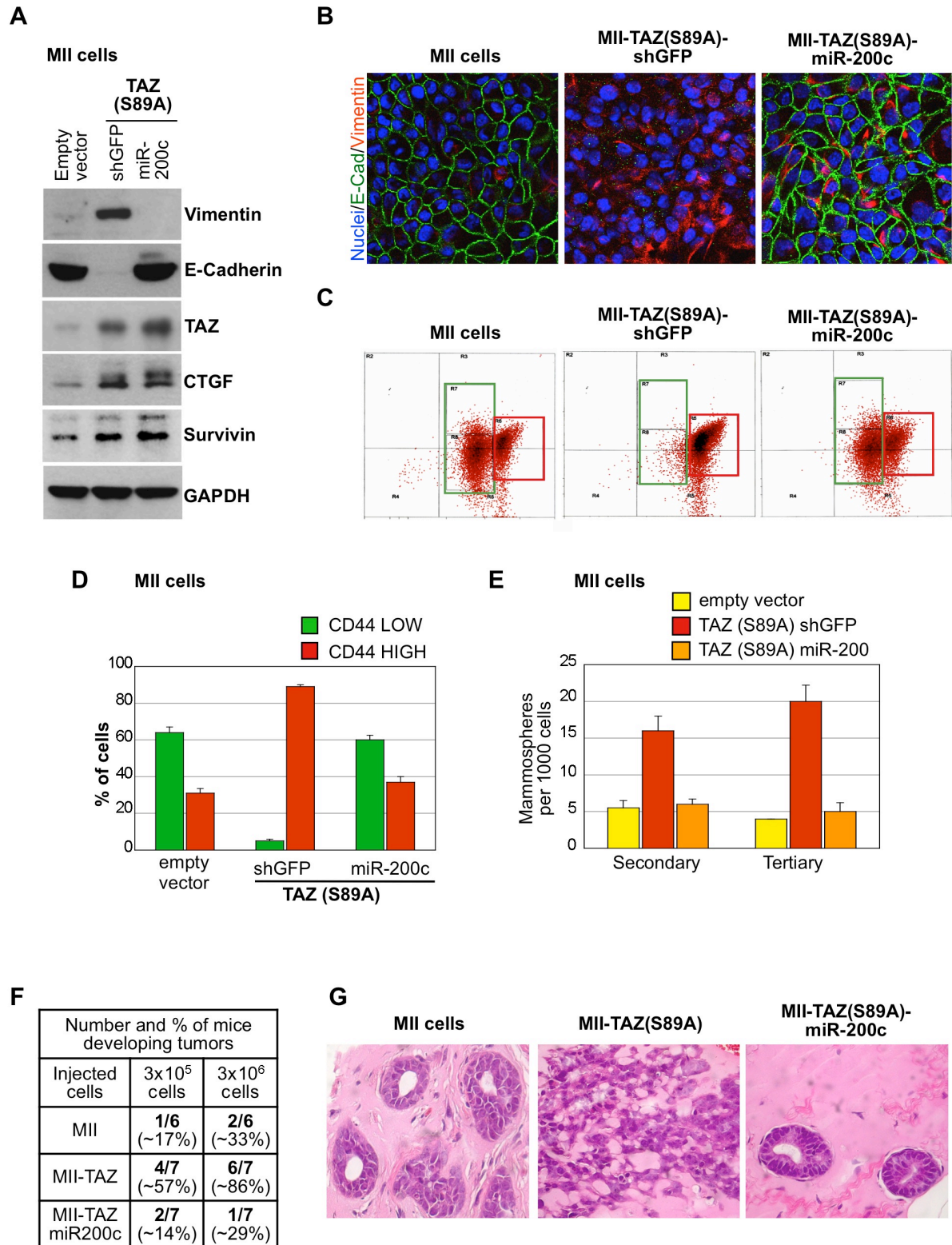


FIGURE 9

- A.** Diagrams report the number of probe sets in Affymetrix microarrays up/downregulated by TAZ depletion with a shRNA or TAZ/YAP depletion with siRNAs in MDA-MB-231 cells. FC: fold change. q-value $\leq 5\%$.
- B.** BrdU incorporation in MDA-MB-231, MIV and MII cells transfected with control, TAZ or TAZ/YAP siRNAs. Cells were labeled with BrdU for 20 min 48h after siRNA transfection. TAZ depletion reduces BrdU incorporation.
- C.** Cell cycle analysis in MDA-MB-231 cells transfected with control, TAZ or TAZ/YAP siRNAs. The fraction of cells in S-phase is lower in TAZ-depleted cells.

FIGURE 9

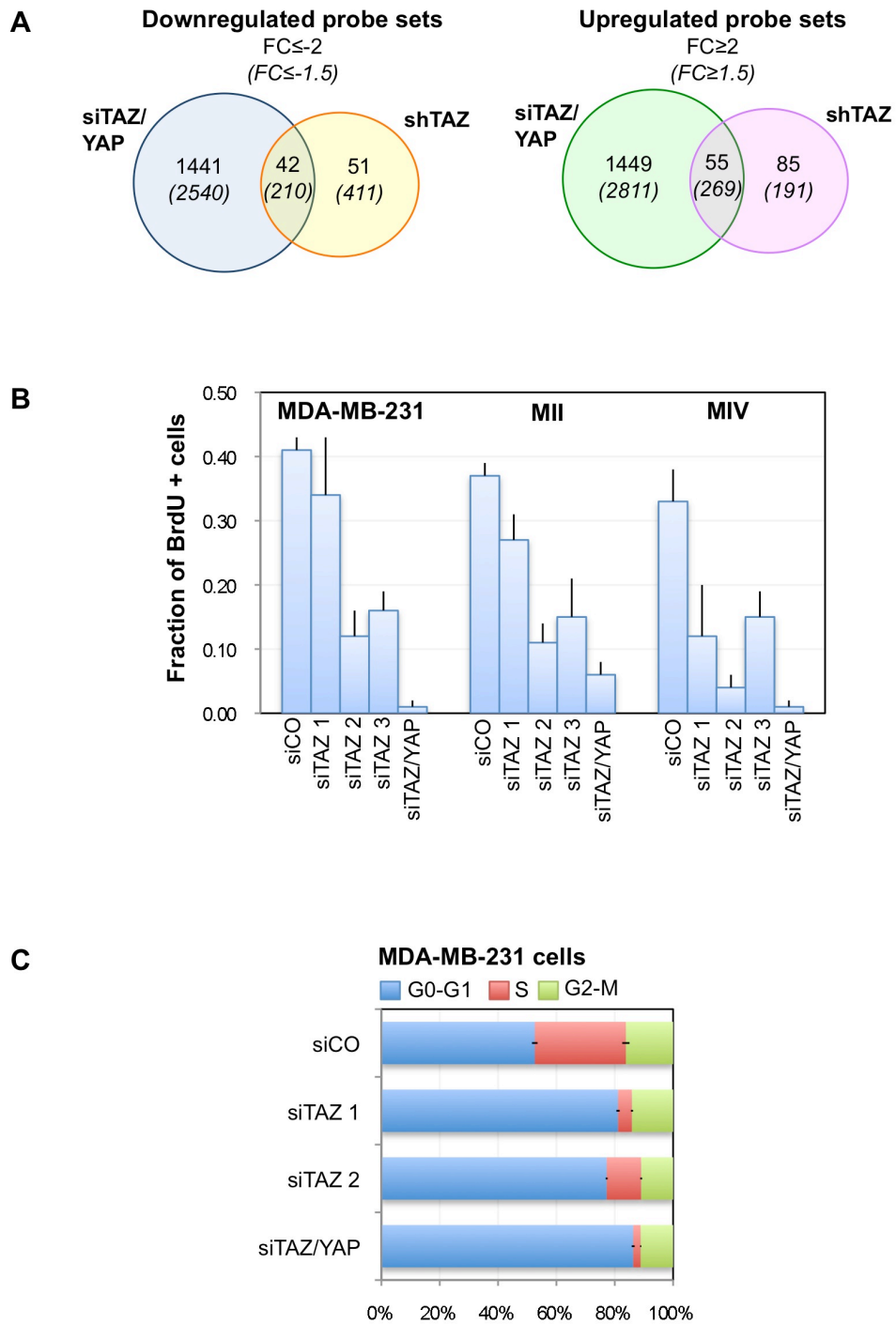


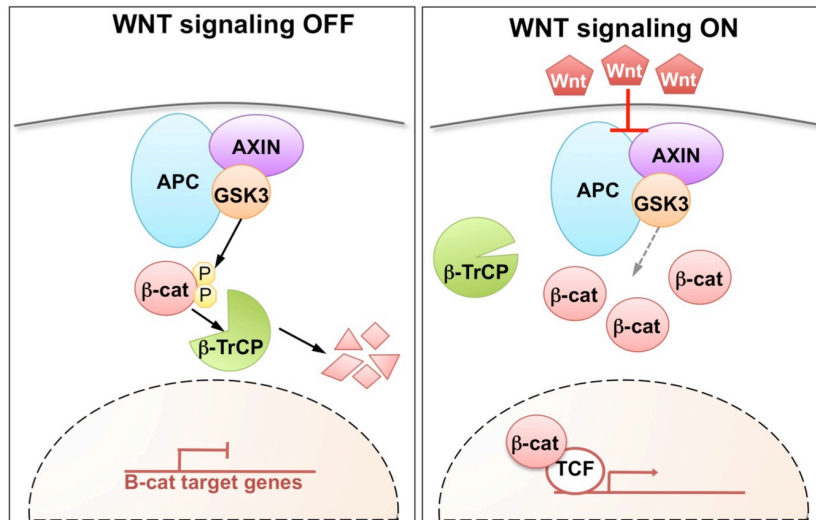
FIGURE 10

- A.** Schematic representation of Wnt signaling pathway. In the absence of Wnt ligands, β -catenin is phosphorylated in the Axin/APC/GSK3 destruction complex and degraded. In the presence of Wnt ligands, the destruction complex is disabled, β -catenin is stabilized and activates the transcription of target genes.
- B.** TAZ is regulated by Wnt signaling. In the absence of Wnt, phosphorylated β -catenin associates with TAZ and escorts it to β -TrCP. Upon Wnt stimulation, TAZ is stabilized along with β -catenin.

FIGURE 10

A

Canonical Wnt signaling pathway



B

TAZ regulation by Wnt signaling

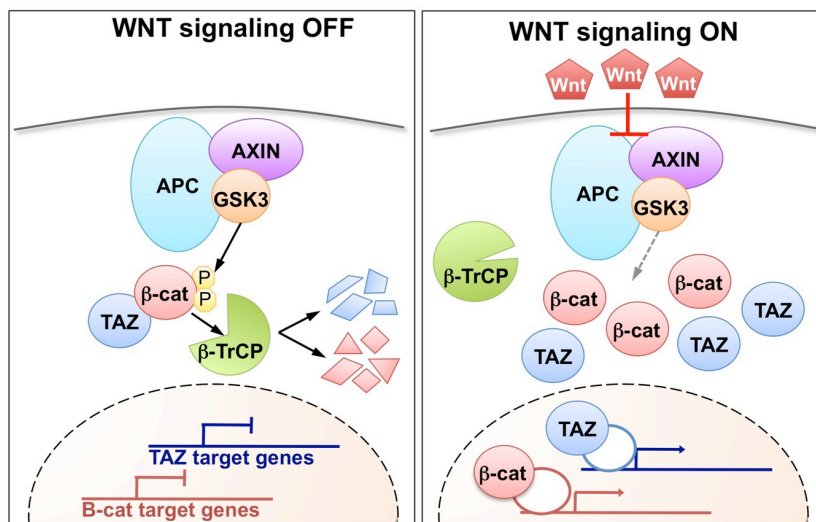


FIGURE 11

- A.** Bone differentiation in mouse ST-2 cells. Sub-confluent ST-2 cells were transfected with control (lanes 1 and 3) or TAZ (lanes 2 and 4) siRNAs; where indicated, cells were exposed for 72 hours to recombinant Wnt3A (lanes 3 and 4, 100 ng/ml). Protein extracts were analyzed for alkaline phosphatase (ALP) activity (expressed as nanomoles of p-nitrophenol/min/ μ g of total protein). Data are presented as mean + SD. Wnt3A promotes bone differentiation of ST-2 cells in a TAZ-dependent manner.
- B.** Western blots for TAZ, its target Cyr61 and β -catenin in ST-2 cells transfected with control or TAZ siRNA, and treated with Wnt3a (as in A). GAPDH serves as loading control. TAZ is stabilized and activated by Wnt3a.
- C.** qRT-PCR for *TAZ* mRNA in ST-2 cells transfected with control or TAZ siRNAs, and treated with Wnt3a (as in A). *TAZ* expression levels are normalized to GAPDH and are presented as mean+SD. *TAZ* mRNA levels do not change upon Wnt stimulation.
- D.** TAZ rescues bone differentiation of Dkk-1-treated human bone marrow mesenchymal stem cells. hMSC, transduced with control or TAZ-encoding lentiviral vectors, were cultured either in growth medium (GM) or osteogenic differentiation medium (ODM); where indicated, Dkk-1 protein (500 ng/ml) was added to the ODM to block autocrine Wnt signaling. After 10 days, cells were fixed for ALP detection (representative fields are shown). Bone differentiation in ODM is inhibited by Dkk-1, but rescued by TAZ overexpression.
- E.** Quantifications of ALP-positive areas in D (mean+SD).
- F.** Western blots for TAZ, Cyr61 and CTGF, and β -catenin in the same samples described in D. GAPDH serves as loading control. TAZ and its targets Cyr61 and CTGF, along with β -catenin, are upregulated when cells are cultured in ODM. Note that gain-of-TAZ does not affect β -catenin levels, that remain inhibited by Dkk-1 in TAZ-expressing MSCs (lanes 3 and 4).

FIGURE 11

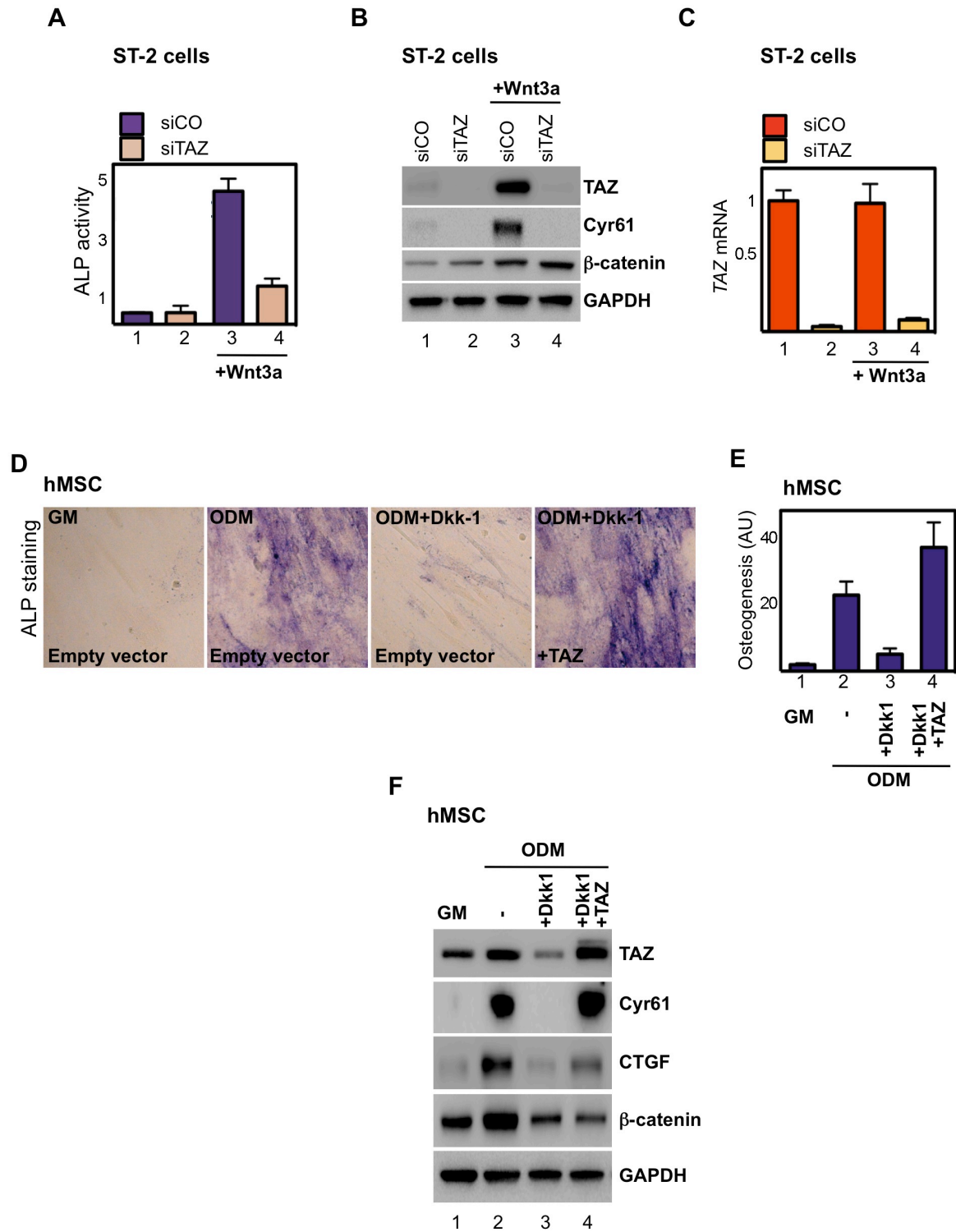


FIGURE 12

- A.** Western blot for TAZ in hMSC transfected with control (siCO), GSK3 (siGSK3), APC (siAPC) or Axin (siAxin) siRNAs. GAPDH serves as loading control.
- B.** Inactivation of the destruction complex inhibits adipogenesis. hMSC were transfected with control, GSK3, APC or Axin siRNAs and induced to differentiate into adipocytes. Pictures represent hMSC stained with Oil Red to visualize lipid droplets. Nuclei are stained with Hoechst.
- C.** Quantification of Oil Red-positive areas (mean + SD) in hMSC described in B.
- D.** Representative pictures of hMSC transfected with control or Axin siRNAs, alone or in combination with TAZ siRNA (siTAZ), and then induced to differentiate in adipocytes. Staining with Oil Red reveals the presence of lipid droplets. Nuclei are stained with Hoechst. TAZ depletion in cells transfected with Axin siRNA rescues adipocyte differentiation.
- E.** Quantification of Oil Red-positive areas (mean + SD) in hMSC described in D.

FIGURE 12

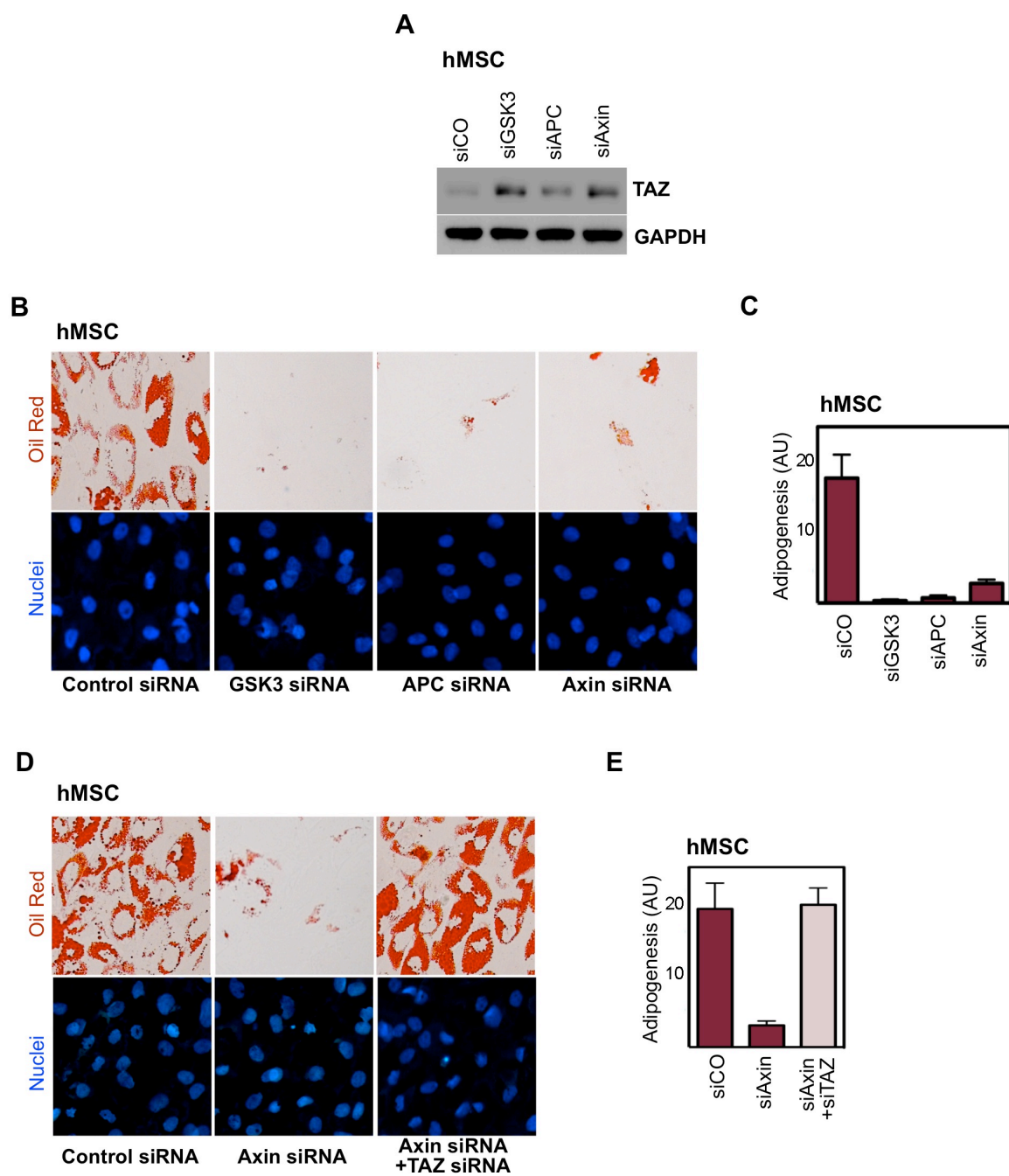


FIGURE 13

- A.** Western blot for β -catenin on protein extracts from APC-mutant (control) or APC-reconstituted (+APC) SW480 cells. GAPDH serves as loading control. APC expression destabilizes β -catenin, indicating the effective reconstitution of the β -catenin destruction complex.
- B.** Western blots for TAZ, CTGF and GSK3 in APC-mutant (lanes 1-2) and APC-reconstituted (lanes 3-4) SW480 cells transfected with control (lanes 1 and 3) or GSK3 siRNAs (lanes 2 and 4). GAPDH serves as loading control. APC-reconstitution downregulates TAZ and restores TAZ sensitivity to inhibition by GSK3.
- C.** Growth assay in SW480 cells transfected with control siRNA (siCo), or siRNAs targeting TAZ (siTAZ), β -catenin (si β cat) or both TAZ and β -catenin (si β cat siTAZ), as indicated. In lane 6, APC-reconstituted SW480 cells were stably transduced with TAZ-expressing lentiviral vector. Bars display the number of SW480 cells counted one day after seeding (light blue bars, showing that a similar number of cells was present in all samples at the beginning of the experiment) or after four days (dark blue bars). Data are shown as mean + SD. Both TAZ and β -catenin depletion impair growth of SW480 cells, with double knockdown leading to complete growth arrest. Cell proliferation is slowed down by APC reconstitution, an effect that is reverted by TAZ overexpression.
- D.** Western blots for TAZ and β -catenin on protein extracts from SW480 cell lines described in C. GAPDH serves as loading control. The manipulation of TAZ levels didn't affect β -catenin expression.
- E.** qRT-PCR for *Axin2* and *Cyclin-D1* mRNAs in APC-mutant or APC-reconstituted SW480 cells transfected with the indicated siRNAs. mRNA expression levels are normalized to GAPDH and are presented as mean+SD. The expression of β -catenin/TCF target genes in SW480 cells is affected by APC reconstitution or by β -catenin depletion but not by TAZ knockdown.
- F.** Representative pictures of Matrigel-embedded spheres formed by the indicated SW480 cell populations. SW480 + APC cells were transfected with control siRNA, whereas SW480 cells were transfected with control, β -catenin or TAZ siRNAs 24-hours before seeding in Matrigel. APC-reconstituted and TAZ-depleted cells display impaired clonogenic capacity, whereas β -catenin depletion has no effect.
- G.** Bars represent the number of spheres formed by the indicated cells. Data are shown as mean + SD.

FIGURE 13

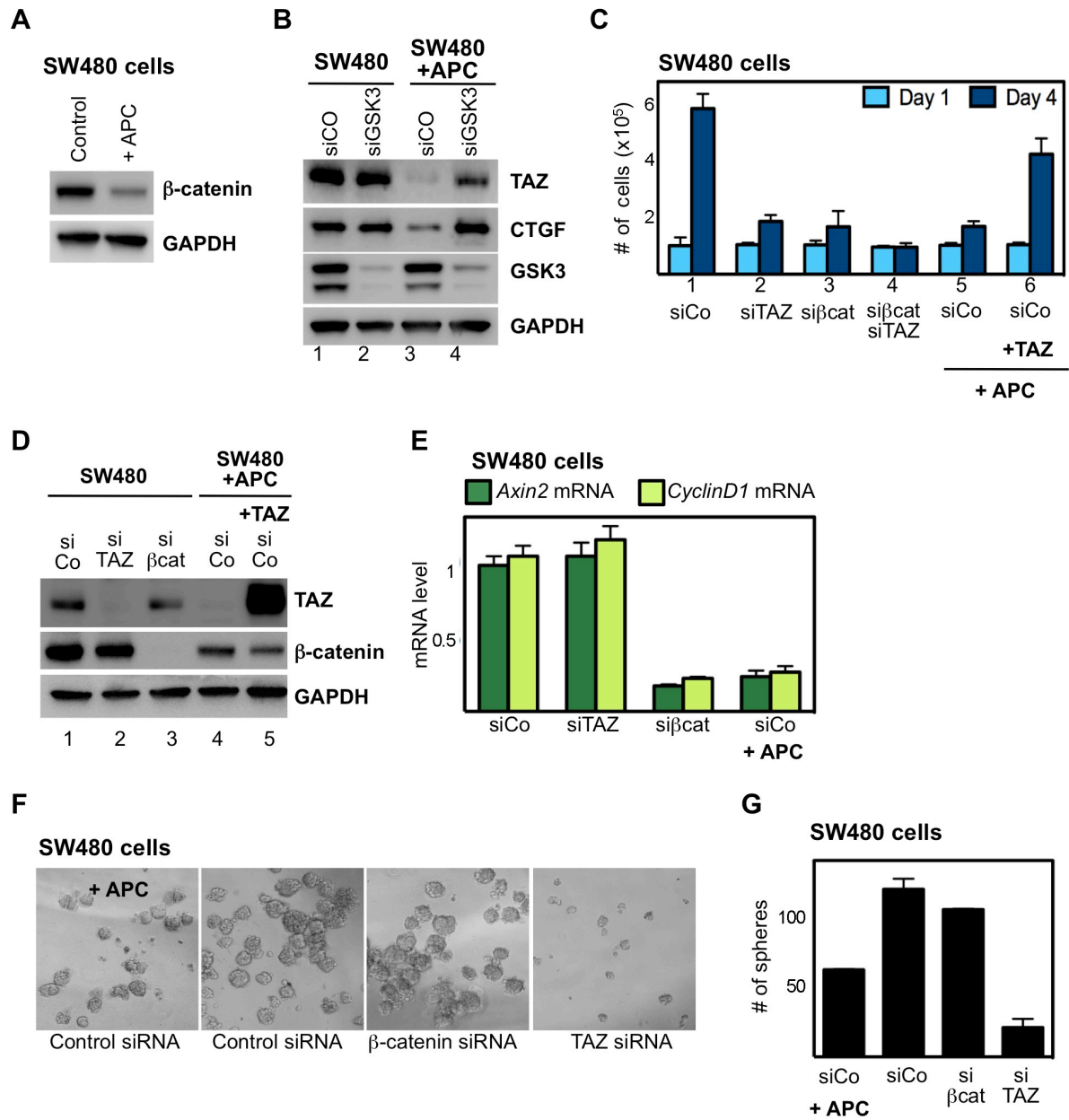


FIGURE 14

- A.** Western blot for TAZ and GSK3 in MII cells transfected with control (siCO) or GSK3 (siGSK3) siRNAs. GAPDH serves as loading control.
- B.** qRT-PCR for *TAZ* mRNA (normalized to GAPDH) in MII cells treated as in A. Bars are mean+SD.
- C.** Western blot for TAZ and β -catenin in MII cells transfected with control or APC (siAPC) siRNAs. GAPDH serves as loading control.
- D.** qRT-PCR for *APC* and *TAZ* mRNA (normalized to GAPDH) in MII cells treated as in C. Bars are mean+SD.
- E.** Western blot for TAZ in MII cells transfected with control or Axin (siAxin) siRNAs. GAPDH serves as loading control.
- F.** qRT-PCR for *Axin1*, *Axin2* and *TAZ* mRNA (normalized to GAPDH) in MII cells treated as in E. Bars are mean+SD.
- G.** Representative IF pictures for TAZ in MII cells, showing TAZ nuclear accumulation upon GSK3, APC or Axin knock-down. Nuclei are stained with Hoechst.
- H.** qRT-PCR analysis of TAZ target gene *CTGF*, normalized to *GAPDH* expression, in MII-shGFP or MII-shTAZ#3 cells transfected with the indicated siRNAs. Bars are mean + SD. Overall, depletion of GSK3, APC or Axin leads to TAZ stabilization and nuclear accumulation, and to the upregulation of TAZ target CTGF.
- I.** Pie chart of U133plus2 Affymetrix probe sets upregulated in MII cells transfected with APC siRNA compared to control siRNA-transfected cells. Dark colored area of the graph represents the fraction of probe sets whose upregulation by APC depletion is reverted by co-transfection of TAZ siRNA.

FIGURE 14

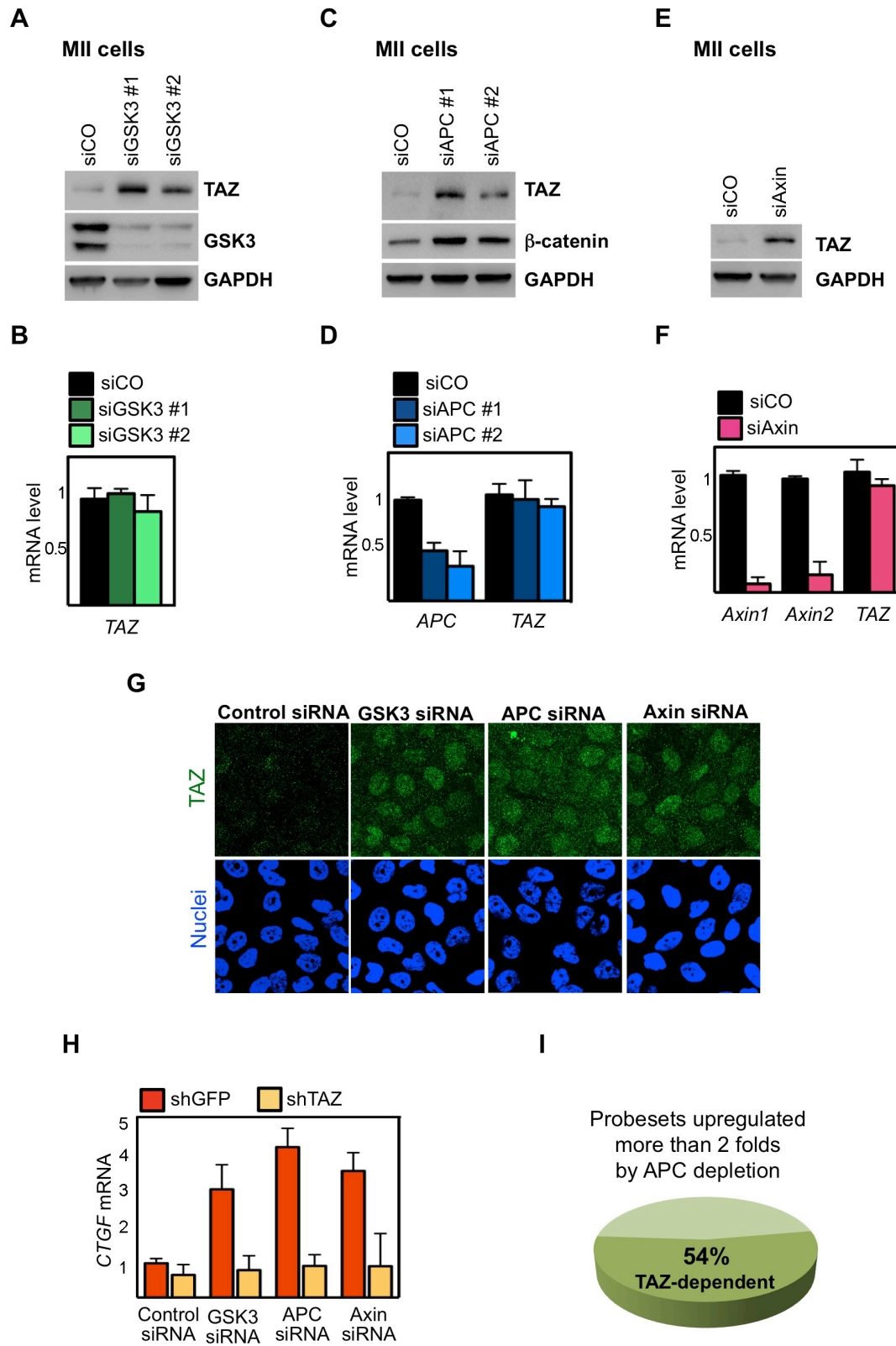


FIGURE 15

- A.** Western blot for TAZ and β -catenin in MII cells transfected with control siRNA (siCO) or with three independent siRNAs targeting β -catenin. GAPDH serves as loading control. β -catenin depletion promotes TAZ stabilization.
- B.** Representative IF pictures for TAZ in MII cells transfected with control or β -catenin siRNAs. Nuclei are stained with Hoechst. TAZ accumulates in the nucleus of β -catenin-depleted cells.
- C.** qRT-PCRs for *CTGF* mRNA (normalized to GAPDH) in MII cells treated as in A. Bars are mean+SD. TAZ target *CTGF* is upregulated in β -catenin-depleted MII cells.
- D.** Pie chart of U133plus2 Affymetrix probe sets upregulated in MII cells transfected with β -catenin siRNA compared to control siRNA-transfected cells. Dark colored area of the graph represents the fraction of probe sets whose upregulation by β -catenin depletion is reverted by co-transfection of TAZ siRNA.
- E.** Quantification of mammospheres formed by MII cells transfected with control siRNA or with a mix of β -catenin siRNAs, alone or in combination with TAZ siRNAs. Data are presented as mean + SEM. Depletion of β -catenin causes a TAZ-dependent increase in mammosphere formation.

FIGURE 15

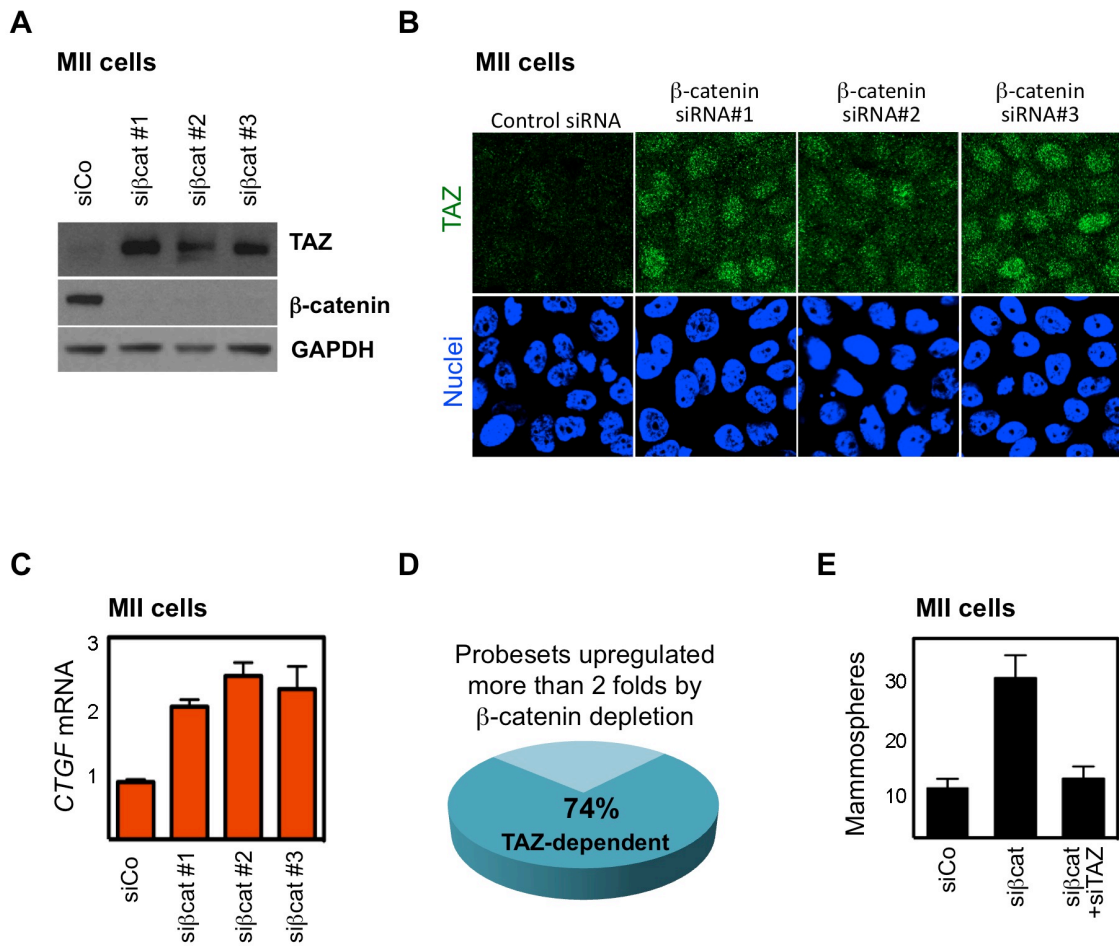


TABLE 1
siRNA SEQUENCES

siRNA/shRNA	Interfering sequence	Notes
Control	UUCUCCGAACGUGUCACGU	
GFP	CAAGCUGACCCUGAAGUUC	
<i>human</i>		
TAZ#1	ACGUUGACUUAGGAACUUU	When the number is not specified, a mix of the three anti-TAZ siRNAs was used.
TAZ#2	AGAGGUACUUCCUCAAUCA	
TAZ#3	AGGUACUUCCUCAAUCACA	
Scribble #1	GUCAUUGGAACAGGACGCU	When the number is not specified, Scribble siRNA#3 was used.
Scribble #2	GGAUGAAGUCAUUGGAACA	
Scribble #3	AGCACUUCAAGAUCUCCAA	
YAP	GACAUCUUCUGGUCAGAGA (YAP #1) CUGGUCAGACAUACUUCUU (YAP #2)	YAP siRNAs were used with TAZ siRNAs #1 and #3.
GSK3#1	CAGGGAACUAGUCGCCAUC (GSK3 α) CCCAAUGUCAAAACUACCA (GSK3 β)	When the number is not specified, GSK3 siRNA#1 was used.
GSK3#2	GAAGGUUCUCCAGGACAAG (GSK3 α) AGUUGGUAGAAAUAAUCAA (GSK3 β)	
APC#1	GACGUUGCGAGAAGUUGGA	When the number is not specified, siRNA#1 was used.
APC#2	UAAUGAACACUACAGAUAG	
Axin	GGGCAUAUCUGGAUACCUG (Axin1) GGAUACCUGCCGACCUUAA (Axin1) GAGUAGCCAAAGCGAUCUA (Axin2) CGAUCCUGUAAUCCUUAU (Axin2)	
β -catenin#1	AGGUGCUAUCUGUCUGCUC	When the number is not specified, a mix of anti- β -catenin siRNAs #3 and #4 was used.
β -catenin#2	AACAGUCUUACCUGGACUC	
β -catenin#3	GUAGCUGAUAUUGAUGGAC	
β -catenin#4	GCUUGGAAUGAGACUGCUG	
<i>mouse</i>		
TAZ	UCAUUGCGAGAUUCGGCUG	

TABLE 2
ANTIBODIES FOR WESTERN BLOT

Primary antibodies		
α - β -catenin	monoclonal	Santa Cruz
α -GSK3	monoclonal	Santa Cruz
α -YAP/TAZ	monoclonal	Santa Cruz
α - β TrCP	polyclonal	Santa Cruz
α -CTGF	polyclonal	Santa Cruz
α -Cyr61	polyclonal	Santa Cruz
α -HA tag	polyclonal	Santa Cruz
α -Scribble	polyclonal	Santa Cruz
α -Survivin	polyclonal	Santa Cruz
α -E-cadherin	monoclonal	BD Biosciences
α -PAI-1	monoclonal	BD Biosciences
α -TAZ	monoclonal	BD Biosciences
α -MST2	rabbit monoclonal	Epitomics
α -LATS1	polyclonal	Cell Signaling Technologies
α -Ph-LATS1 (Ser 909)	polyclonal	Cell Signaling Technologies
α -Ph-YAP(Ser127)/ Ph-TAZ(Ser89)	polyclonal	Cell Signaling Technologies
α -TAZ	polyclonal	Sigma Prestige
Secondary antibodies		
α -mouse		GE Healthcare
α -rabbit		Sigma
α -goat		Santa Cruz

TABLE 3
PCR PRIMER SEQUENCES

PCR primers for human cDNA		
GAPDH	forward	AGCCACATCGCTCAGACAC
	reverse	GCCCAATACGACCAAATCC
TAZ	forward	GGCTGGGAGATGACCTTCAC
	reverse	CTGAGTGGGGTGGTTCTGCT
CTGF	forward	CTGCGAGGAGTGGGTGTGT
	reverse	AGGAGGCGTTGTCATTGGTAA
APC	forward	GCCCCTGACCAAAAAGGAAC
	reverse	TGGCAGCAACAGTCCCCTA
Axin1	forward	AGCCGTGTCGGACATGGA
	reverse	AAGTAGTACGCCACAACGATGCT
Axin2	forward	TGTGAGGTCCACGGAAACTG
	reverse	CGTCAGCGCATCACTGGATA
Cyclin-D1	forward	TCAAATGTGTGCAGAAGGAGGT
	reverse	GACAGGAAGCGGTCCAGGTA
PCR primers for mouse cDNA		
GAPDH	forward	ATCCTGCACCACCAACTGCT
	reverse	GGGCCATCCACAGTCTTCTG
TAZ	forward	ATGAATCCGTCCTCGGTGC
	reverse	GAGTTGAAGAGGGCTTCGAG
PCR primers for human microRNAs		
Universal reverse primer		GCGAGCACAGAATTAATACGACTCAC
hsa-miR-200a	forward	TAACACTGTCTGGTAACGATGT
hsa-miR-200b	forward	TAATACTGCCTGGTAATGTGA
hsa-miR-200c	forward	TAATACTGCCGGGTAATGATGGA
hsa-miR-141	forward	TAACACTGTCTGGTAAAGATGG
hsa-miR-429	forward	TAATACTGTCTGGTAAAACCGT