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IDENTIFICATION OF NEW INTERACTORS OF THE TRANSCRIPTIONAL CO-ACTIVATOR TAZ IN HUMAN LUNG CELLS

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INTRODUCTION

Gene expression regulation

Gene expression includes all the events that go from the transcription of a gene to the production of the corresponding protein. All these events are finely regulated. Studying gene expression means to understand in which tissue a gene is expressed, how it is expressed and what causes its expression. In fact, phenotypic differences among different cell types are mainly due to differences in gene expression patterns.

Gene expression can be regulated at different levels: activation of DNA structure, start of transcription, maturation of the transcript, transport into the cytoplasm, translation of mRNA and protein stability. Nevertheless, the most important step is the start of the transcription process which determines whether a gene is transcribed or not. RNA polymerase II, as well as RNA polymerase I and III, needs proteins to find the right transcription start site and these proteins necessary for the start of the transcription process are called general initiation factors. Given that RNA polymerases and general initiation factors are the ultimate targets of regulatory events, during the assemblage of the RNA polymerases complex there are several points for regulation to take place. It is important to note that purified RNA polymerases and corresponding general initiation factors have an intrinsic ability to accurately transcribe DNA templates through core promoter elements, thus allowing the fundamental transcription mechanisms to be elucidated, but these activities are generally suppressed in the cell by the packaging of DNA within chromatin and by negative cofactors that directly interfere with the function of the basal transcription factors. As discussed below, this imposes requirements for transcriptional activators and corresponding cofactors that act in a gene-specific manner both to reverse the repression (antirepression) and to effect a net activation above the intrinsic activity of the basal transcription machinery.

It is possible to subdivide transcriptional factors that cooperates directly with RNA polymerase II in three different categories: a) general transcription factors; b) upstream transcription factors; c) inducible transcriptional factors.

a) General transcription factors are necessary for the start of RNA synthesis in all the genes of class II (genes that encodes for proteins). They form a complex with the RNA

polymerase II close to the transcription start site and this complex is called basal complex of transcription.

b) Upstream transcriptional factors are proteins that recognize short consensus sequence situated upstream the transcription start site (an example is the Sp1 factor that recognizes GC boxes). These factors are ubiquitous and they act increasing the transcription efficiency of all the genes containing a GC box in their promoter.

c) Inducible transcriptional factors work like upstream factors, but they have e regulatory role. They are synthesized or activated in a tissue-specific manner and they recognize sequences called response elements.

In general, transcription factors acts directly recognizing specific and well conserved cis-sequences situated in promoters or enhancers. Some of these factors and of these sequences are common to more than a promoter and they are used in a constitutive manner; some other are specific and their activity is finely regulated. During the assembly process of these factors onto the regulatory sequences there are a lot of check-points and the speed of transcription can be increased or decreased in response to different regulatory signals. Nevertheless, not all the transcription factors bind these specific sequences on the promoter, they can also recognize other proteins already bound to the promoter, and sometimes they can bind directly to the RNA polymerase.

Often DNA-binding factors that regulate the transcription of protein-coding genes act together with an expanding group of cofactors that act either through modifications of the chromatin structure or, more directly, regulating formation or function (transcription initiation or elongation) of the pre-initiation complex. Requirement for cofactors involved more directly in transcription is somewhat surprising in view of the specificity intrinsic to the various DNA-binding regulatory factors, the structural complexity of their ultimate target (the basal transcription machinery), and documented interactions between regulatory factors and components of the basal transcription machinery. Early studies of activator functions in systems reconstituted with DNA templates and purified RNA polymerase II and corresponding general initiation factors revealed that additional ‘‘coactivator’’ or ‘‘mediator’’ activities were required for the activator function but not for basal (activator-independent) transcription.

Coactivators functions in a variety of ways and often contain the enzymatic activities necessary for an alteration in chromatin structure from a quiescent state to one allowing active gene transcription. Broadly speaking, coactivators can be divided in three classes.

One class of proteins modifies histones in ways that allow greater access of other proteins to the DNA. Examples of these are p300 and CBP, powerful histone acetyltransferases (HATs) that interact with a wide variety of transcription factors and other proteins (Hermanson et al., 2002). These proteins support transcription in vitro from chromatinized templates. A second class of coactivators are members of the TRAP/DRIP/Mediator/ARC complex, proteins that bind to transcription factors, recruit RNA polymerase II and interact with the general transcription apparatus. The Mediator complex supports transcription in vitro from DNA templates but does not support efficient transcription from chromatinized templates. Lastly, protein complexes of the yeast SWI/SNF family (or their mammalian homologs BRG1 or BRM) contain ATP-dependent DNA unwinding activities, necessary for efficient gene transcription in vivo. These groups of proteins will not support transcription from naked DNA, but augment transcription from chromatinized templates in vitro (Lemon et al., 2001).

Co-repressors have the opposite effect on chromatin structure, making it unaccessible to the binding of transcription factors or resistant to their actions. These proteins (such as NcoR) are often associated with histone deacetylase (HDAC) activity, though other mechanisms for gene silencing clearly exist (Hermanson et al., 2002). Although coactivators are defined as proteins that increase transcriptional activity without binding to DNA, it is useful to think of those that bind directly to transcription factors and contain relevant enzymatic activities as primary coactivators. Those that dock on transcription factors and serve as scaffolds for the recruitment of other proteins containing these enzymatic activities can be considered secondary co-activators. This distinction rapidly blurs as proteins that can function as primary coactivators on some transcription factors can also be used as enzymatic tools assembled by secondary coactivators in other contexts.

TAZ (Transcriptional co-Activator with PDZ binding motif)

TAZ, also referred to as Wwtr1 (WW-domain containing transcription regulator 1), is a transcriptional co-activator highly expressed in kidney, heart, lung, liver, testis, and placenta. TAZ is characterized by a central WW-domain followed by a highly conserved C-terminal sequence, which contains a coiled-coil domain that recruits core components

of the transcriptional machinery. Both human and mouse TAZ contain a single WW domain that is a very common domain that mediates protein-protein interaction. TAZ also contains a PDZ- binding motif at its C-terminus, that is required for its transcriptional coactivator activity and promotes TAZ nuclear localization particularly to discrete foci, and a 14-3-3 binding motif within the conserved N-terminal portion.

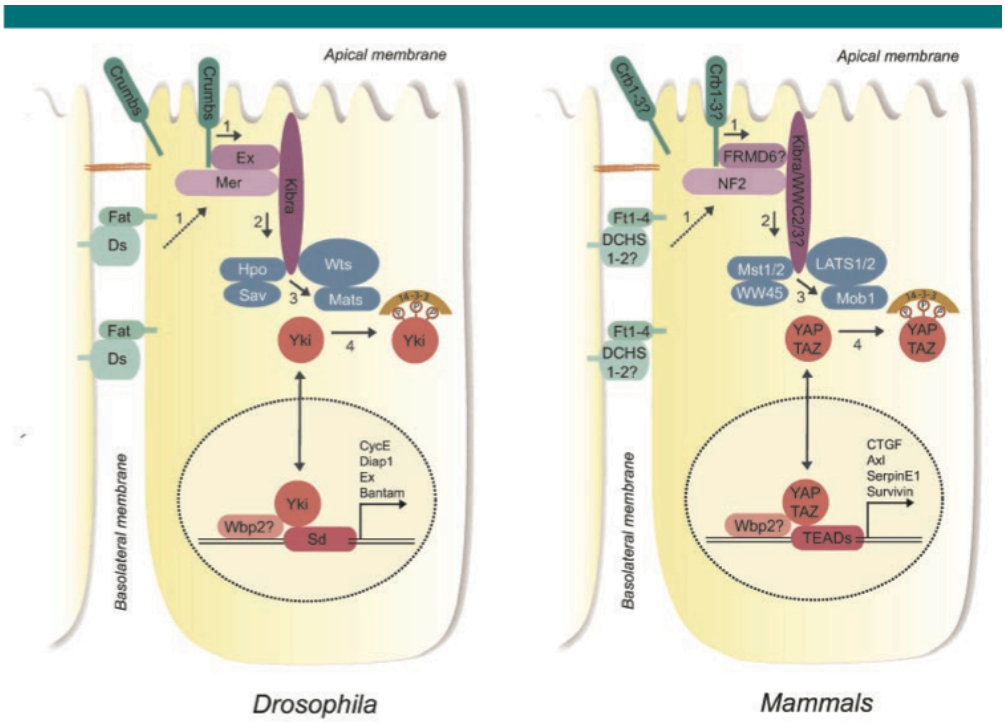
TAZ structure domain. TAZ contains WW domain, a 14-3-3 binding motif, a coiled-coiled motif in the transactivation domain and a PDZ-binding motif in the C-terminal. TB: Tead Binding Domain; TA: Transcriptional Activation Domain.

How extracellular clues are transduced to the nucleus is a fundamental issue in biology. In this context, TAZ constitutes a transducer linking cytoplasmic signaling events to transcriptional regulation in the nucleus and the 14-3-3 binding is the key to this regulation. Interaction of TAZ and YAP (the paralog of TAZ) with the phosphoserine and phosphothreonine-binding proteins 14-3-3 has been known for many years (Kanai et al. 2000; Vassilev et al. 2001; Basu et al. 2003). Hence, an important question is which kinases mediate the phosphorylation and link this event to dynamic signaling networks. Different groups have recently demonstrated that the fly Hippo pathway and the mammalian Hippo-like pathway play a key role in stimulating 14-3-3 interaction with the YAP/TAZ family of transcriptional coregulators.

The fly protein Hippo is a Ste20 protein kinase that interacts with and is activated by the WW-domain protein Salvador. Upstream from Hippo there are different components, including Expanding and Merlyn, an atypical cadherin Fat, which localizes directly to the plasma membrane and the apical-basal polarity proteins (Crumbs complex). This component might link Hippo to extracellular clues. Particularly, the Crumbs complex (PALS1/LIN7C/PATJ) is a tight junction related component which localizes to the

apical domain of polarized epithelial cells in high-density. Genetic studies in *Drosophila* have shown that Crb (Crumb, Crb) influences the Salvador/Warts/Hippo (SWH) pathway and tissue growth, by modulating the expression of Ex (Expanded, Ex) (Grzeschik et al. 2010; Robinson et al. 2010). The mechanism by which the Crumbs complex regulates TAZ activity in mammals is different from that in *Drosophila*. Both the WW-domain and the PDZ-binding motif are required for the interaction with PALS1, therefore, with the Crumbs complex. The knockdown of PALS1 or Crumb3 decreases Ser89 phosphorylation levels and promotes nuclear localization of TAZ in high-density cells (Varelas et al. 2010).

Downstream of Hippo are Warts/Lats and its activator Mats. Lats directly interact and phosphorilates Yorkie (the fly ortholog of YAP) and this phosphorylation stimulates 14-3-3 binding to Yorkie and its nuclear export. A series of regulators form a signaling cascade to promote 14-3-3 binding and cytoplasmic localization of Yorkie, thereby controlling its transcriptional coactivator role. As illustrated in the figure below, these signaling regulators are conserved in mammals. The mammalian counterparts are FERM domain protein (FERM) 6, neurofibromatosis NF2, the Hippo-like kinases MSTs, the regulatory protein WW45, LATS kinases, and Mob1. As shown for Yorkie, LATS kinases phosphorylate YAP and TAZ to promote their 14-3-3 binding and subsequent nuclear export, thereby placing YAP and TAZ downstream of the Hippo-like pathway in mammals. Phosphorylation by LATS is multisite, with five sites on YAP and four sites on TAZ. The four sites on TAZ are analogous to the C-terminal four sites on YAP and three of the analogous sites are conserved in Yorkie but only one is required for 14-3-3 binding, i.e. Ser89, in TAZ.



Adapted from Chan et al. Journal of Cellular Physiology 2011

Comparison between Hippo pathway in fly (Drosophila melanogaster) and mammal.

Apart from the Hippo-like pathway, growth factors such as bone morphogenetic protein 2 (BMP-2) and fibroblast growth factor 2 (FGF-2) regulate TAZ expression and cell differentiation. In addition, through its WW domain, TAZ interacts with polyomavirus T antigens and this interaction induces nuclear accumulation of TAZ but inhibits its transactivation activity suggesting that nuclear localization of TAZ does not always correlate with its transactivation activity, and may thus have other roles (Tian et al. 2004). TAZ has been defined as a "molecular rheostat" that modulates Mesenchymal stem cells (MSCs) differentiation (Hong et al. 2005). Two key transcription factors, Runx2 (also called Cbfa1 or Pebp2aA) and PPARγ, drive MSCs to differentiate into either osteoblasts or adipocytes, respectively, and the differentiation of each lineage appears to be mutually exclusive and transcriptionally controlled. TAZ interacts both in vitro and in vivo with Runx2 and this interaction leads to Runx2-stimulated osteocalcin gene expression. On the other hand TAZ interacts both in vitro and in vivo also with PPARγ but, in this case, it has been demonstrated that TAZ directly inhibit the ability of PPARγ to stimulate gene expression of the endogenous fatty acid-binding protein aP2, both in the presence or absence of the PPARγ-activating ligand Rosiglitazone. All

together, these are evidence suggesting that TAZ acts as a transcriptional modifier of mesenchymal stem cell differentiation by promoting osteoblast differentiation while simultaneously impairing adipocyte differentiation.

In the last years, a role of TAZ in the migration, invasion, and tumorigenesis of breast cancer cells has been outlined (Chan et al. 2008). In fact, the most highly invasive breast cancer cell lines express TAZ at levels that are approximately four times of those expressed by the majority of the weakly invasive breast cancer cells. The clinical relevance of this observation is supported by the finding that TAZ is overexpressed in a significant fraction of breast cancers (~21.4% of 126 commercially available breast cancer samples examined). Overexpression of TAZ in MCF10A cells (mammary epithelial cells) to a level ~2- to 3-fold of those detected in highly invasive cells caused a morphologic change from an epithelial to a fibroblast-like appearance an dramatically increased the migratory and invasive properties of the cells. Furthermore, shRNA-mediated knockdown of TAZ expression in MCF7 (human breast adenocarcinoma cell line) and Hs578T (non-tumorigenic mammary cells able to form colonies in semisolid medium) cells reduced cell migration and invasion. The epithelial clusters of MCF7 cells became more densely packed with cells when TAZ expression was knocked down. These results indicate that TAZ is a negative regulator of epithelial morphology/architecture, as well as a positive regulator for invasive and migratory behavior. It is conceivable that TAZ overexpression in breast cancer may trigger the loss of epithelial property to promote migration. Moreover, when TAZ expression is knocked down in MCF7 cells, their anchorage-independent growth in soft agar and tumorigenesis in nude mice is retarded, suggesting that TAZ overexpression is an important part of the process involved in breast cancer development and progression. Recently, other two studies have connected TAZ overexpression with the development of the NSCLC (non-small cell lung cancer, NSCLC) (Zhou et al. 2011) and PTC (papillary thyroid carcinoma, PTC) (de Cristofaro et al. 2011). TAZ is overexpressed in the NSCLC cell lines and knockdown TAZ significantly impaired the tumorigenic ability of the NSCLC cells (Zhou et al. 2011), but the NSCLC clinical samples should be explored in the future. The transcripts of TAZ from 61 samples of PTC were examined and found to be overexpressed significantly (de Cristofaro et al. 2011).

Varelas et al. (Nature Cell Biology, 2008) described TAZ like a mediator of Smad nucleocytoplasmic shuttling that is essential for TGFβ signaling. In fact, although

TGFβ-dependent R-Smad phosphorylation and heteromeric complex formation occur in the absence of TAZ, nuclear accumulation of Smad complexes is lost. This results in loss of TGFβ-dependent signaling, which in hESCs (human Embryonic Stem Cells) results in the failure to maintain self-renewal markers. By modulating TAZ localization they demonstrated that TAZ dominantly controls the subcellular localization of the Smads. They further showed that the Mediator complex component ARC105 binds to and can retain TAZ in the nucleus. As TAZ itself shuttles in and out of the nucleus, these results support the existence of a hierarchical system that regulates the localization of Smad2/3 in the cell.

In a recent study (Liu et al. 2010), TAZ was shown to regulate expression of the EMT transcription factor ZEB1 to control retinal pigment epithelial (RPE) cell proliferation and differentiation. ZEB1 expression is increased during RPE dedifferentiation, leading to cell proliferation and EMT. Interestingly, TAZ–TEAD1 translocation to the nucleus coincides with loss of cell–cell contact and onset of ZEB1 expression in the nucleus. Knockdown of TAZ by shRNA prevented the increased expression of ZEB1 and, in turn, prevented proliferation and EMT. TAZ was shown to bind to the ZEB1 promoter in vivo, suggesting that TAZ–TEAD complex may directly induce ZEB1 transcription to promote EMT and cell proliferation. TAZ may also function as a co- activator of MyoD to promote expression of target genes of MyoD to enhance myogenic differentiation (Jeong et al. 2010).

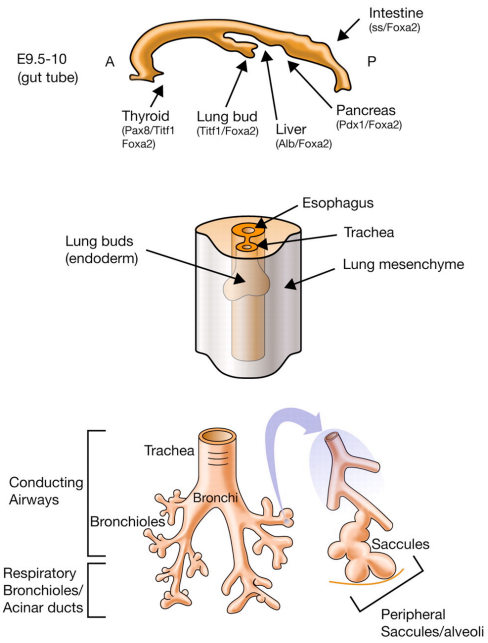
Interestingly, it was recently shown that the Hippo pathway is able to restrict Wnt/beta-Catenin signaling by promoting an interaction between TAZ and disheveled (DVL) in the cytoplasm. TAZ inhibits the CK1delta/epsilon-mediated phosphorylation of DVL, thereby inhibiting Wnt/beta-Catenin signaling. This study suggests that the cytoplasmic pool of TAZ may function to regulate Wnt signaling (Varelas et al. 2010).

To date TAZ knock-out mice have been described in four papers (Hossain et al. 2006; Tian et al. 2007; Lei et al. 2008; Mitani et al. 2009). As mentioned before, TAZ was reported as a modulator of mesenchimal stem cell differentiation. Despite this, the staining of embryonic day 17.5 (E17.5) TAZ-/- mice embryos with Alcian blue and Alizarin red to reveal cartilage and bone tissue respectively, showed only minor skeletal abnormalities and knock-out mice present just a slightly smaller stature than wild type. Overall, the most relevant phenotypes described in TAZ null mice are severe polycystic kidney disease and lung emphysema. Signs of histological anomalies in TAZ-/- kidneys

were first apparent around E15.5 as dilations of the Bowman's space between visceral podocytes and the parietal cell layer of the Bowman's capsule. Pathological changes in TAZ-/- kidneys included parietal and tubular basement membrane thickening, thinning, and folding, and interstitial fibrosis and inflammation as evidenced by mononuclear leukocyte infiltration. TAZ-deficient mice have also emphysema-like features including enlarged air space and low elastance. The lungs of the $Taz^{+/+}$ littermates developed normally from E16.5 to P14. At E18.5, the terminal bronchioles opened into a smooth-walled channel dividing into several saccules. After the formation of the secondary septa, the saccules transformed into alveolar ducts and alveolar sacs lined with alveoli by the end of the alveolar stage. Once fully developed, $Taz^{+/+}$ lungs showed a slight increase in alveolar space. Interestingly, most $Taz^{-/-}$ lungs grew normally until birth, whereas some embryos had smaller and immature lungs than their wild-type littermates. After birth, the terminal airways were in close proximity to the pleural surface with larger airspace by P5. Since then, although the number of saccules increased in proportion to the whole lung size, enlarged airspace in $Taz^{-/-}$ lungs showed less alveolar compartmentalization than wild-type lungs, and the histological difference between $Taz^{-/-}$ and wild-type lungs became much clearer by 3 months. The alveoli of $Taz^{-/-}$ lungs showed a further small increase in size by 9 months. These mice exhibit abundant inflammatory cells in bronchoalveolar lavage fluid, evidence of chronic inflammation in lungs, and some TAZ-/- lungs have small focal areas with inflammatory changes (Mitani et al. 2009). Therefore, it looks like TAZ plays an important role in lung development, especially during the saccular and alveolar stages.

The Lung

The major function of the lung is to excrete carbon dioxide from blood and replenish oxygen. The structure of the lung varies in complexity among vertebrate species but is highly conserved in its position along the foregut, ventral to the esophagus, between the thyroid and stomach.



Patterning of the foregut endoderm: lung bud formation and branching morphogenesis.

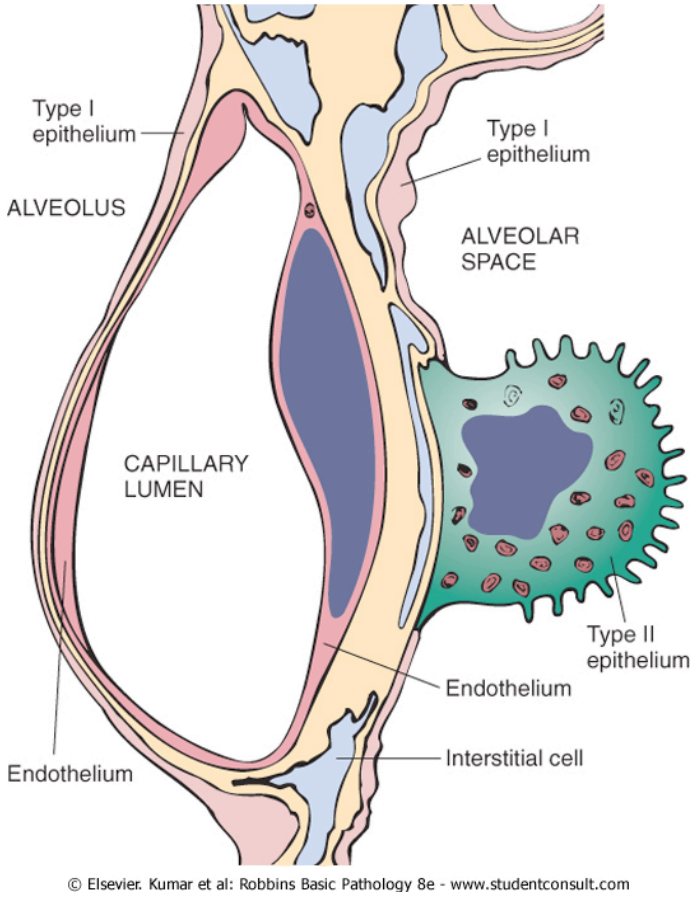
Adapted from Maeda et al. Physiol Rev 2007

Lungs are spatially organized along both cephalo-caudal (from the conducting airways to the peripheral saccules) and dorsal-ventral axes. The midline trachea develops two lateral outpocketings, the lung buds. Expression of transcription factors or markers characteristic of specific organs are observed along the anterior-posterior axis of the foregut tube before formation of each organ (Serls et al. 2005, Zorn A. M and Wells J. M.). TTF-1, an Nkx2 homeodomain-containing transcription factor, marks the region from which lung buds arise. As a general theme, many of the transcription factors required for development of the early foregut are reutilized later in lung morphogenesis (Zaret KS, 2002). For example, deletion of the *Foxa2*, *Catnb*, *Sox17*, *Gata-6*, *Stat3* genes, and other transcription factors expressed in endodermally derived cells along the foregut, results in failure of normal embryonic patterning well before the formation of the lung. Mammalian lungs are usually asymmetrically lobulated; for example, the mouse lung consists of four right and left main lobes. Pulmonary situs is determined by genes regulating left-right asymmetry (Raya et al. 2006). Formation of the lung also requires information that regulates right and left asymmetry and the gradual tapering of the conducting airways that lead to ever smaller tubes that lead to the alveoli where gas exchange occurs. The right lung bud eventually divides into three main bronchi, and the left into two main bronchi, thus giving rise to three lobes on the right and two on the left.

The main right and left bronchi branch dichotomously, giving rise to progressively smaller airways, termed *bronchioles,* which are distinguished from bronchi by the lack of cartilage and submucosal glands within their walls. The bronchiolar epithelium is a simplified columnar epithelium made up of ciliated cells, a few basal cells and non-ciliated secretory cells called Clara cells (Boers et al. 1999). More distally, the respiratory bronchioles are lined by a cuboidal epithelium comprised entirely of ciliated and Clara cells. Clara cells produce Clara cell secretory protein (also known as CCSP, CC10, CC16, Clara cell antigen, secretoglobin and uteroglobin), which is the most abundant secretory protein found in the airway surface fluid. Studies from CCSP knock-out mice have shown that changes in CCSP levels have a profound impact on not only the composition of airway surface fluid but also the airway epithelial response to environmental stimuli (Stripp et al. 2002). Additional branching of bronchioles leads to *terminal bronchioles;* the part of the lung distal to the terminal bronchiole is called an *acinus.* Pulmonary acini are composed of *respiratory bronchioles* (emanating from the terminal bronchiole) that proceed into *alveolar ducts,* which immediately branch into *alveolar sacs,* the blind ends of the respiratory passages, whose walls are formed entirely of *alveoli,* the ultimate site of gas exchange. The microscopic structure of the alveolar walls (or alveolar septa) consists, from blood to air, of the following:

- the capillary endothelium basement membrane and surrounding interstitial tissue separating the endothelium from the alveolar lining epithelium;
- the pulmonary interstitium, composed of fine elastic fibers, small bundles of collagen, a few fibroblast-like cells, smooth muscle cells, mast cells, and rare mononuclear cells, is most prominent in thicker portions of the alveolar septum;
- alveolar epithelium, which contains a continuous layer of two principal cell types: type II and type I alveolar epithelial cells that are in direct contact with respiratory gases, creating collapsing forces at the air-liquid interface. To maintain inflation, these surface forces are mitigated by the presence of pulmonary surfactant (a mixture of proteins and lipids) that is synthesized and secreted onto the alveolar surface by type II epithelial cells. Because pulmonary surfactant reduces surface tension, it is critical for the maintenance of lung volumes during the respiratory cycle. Lack of pulmonary surfactant in preterm infants with respiratory distress syndrome or adults with acute respiratory distress syndrome causes atelectasis leading to respiratory failure. The type II cell plays a critical role in surfactant production and in repair of the lung following injury, and it is

the progenitor cell for type I epithelial cells, which comprise the majority of the gas-exchange region of the alveolus. It is increasingly clear that alterations in genes and processes affecting both type II cell homeostasis and surfactant function underlie the pathogenesis of a number of severe pulmonary diseases affecting infants, children, and adults, which, until very recently, were considered idiopathic.



Microscopic structure of the alveolar wall. The basement membrane *(yellow)* is thin on one side and widened where it is continuous with the interstitial space. Portions of interstitial cells are shown.

The complexity of gene transcription during lung morphogenesis is immediately evident from the multiple cell types that comprise the lung, being derived from ectodermal, mesenchymal, and endodermal compartments, all present in appropriate numbers and sites to support respiration. Transcription factors uniquely specifying lung formation have not been identified to date. Although a number of transcription factors and their binding sites have been characterized and associated with the regulation of lung specific

genes, the principles that govern the design and evolution of transcriptional networks operating during lung formation and function have just begun to be understood. Not surprisingly, some of these genes encode proteins that are unique to the lung, namely, proteins critical for pulmonary surfactant homeostasis, which are required for reduction of surface tension at the air-liquid interface and without which gas exchange would not be possible.

Study of lung-selective gene expression is relatively recent, beginning with the recognition that regulatory regions of all the surfactant protein genes, including *Sftpa*, *Sftpb*, *Sftpc*, and *Sftpd*, are controlled by the homeodomain containing protein thyroid transcription factor-1 (TTF-1; Nkx2.1)

Surfactant proteins

Pulmonary surfactant is a complex mixture of lipids, mostly phosphatidylcholine (PC), and associated proteins. Four of these surfactant proteins, designated SP-A, SP-B, SP-C, and SP-D, play critical roles in various aspects of surfactant structure, function, and metabolism (Perez-Gil et al. 2008). All four are expressed at relatively high levels in type II cells and have distinct structures and functions. Alveolar surfactant takes on discrete physical forms, including the abundant, highly ordered material called tubular myelin as well as multilamellated and smaller, vesicular, protein-lipid structures that can be isolated from the lung. Surfactant forms are determined by the presence or absence of surfactant proteins and lipids, by the physical forces generated during the respiratory cycle, and by selective processes mediating uptake or degradation. Surfactant multilayers, derived from highly lamellated and tubular myelin forms, spread over the surface of the alveolus and reduce surface tension. Surfactant components are recycled by type II cells or catabolized by alveolar macrophages in a highly regulated system that maintains precise levels of pulmonary surfactant throughout life. Both surfactant lipids and proteins are synthesized primarily by type II cells. Surfactant lipids are stored in large, lipid-rich, intracellular organelles, termed lamellar bodies. Lamellar bodies are rich in the surfactant-associated phospholipids, PC and phosphatidylglycerol (PG), and in two low-molecular-weight, hydrophobic surfactant proteins, SP-B and SP-C, that are cosecreted with the surfactant lipids and interact closely with them (Whitsett et al.

2002).

SP-B and SP-C alter lipid packing and spreading and enhance the surface tension–lowering activity of the lipids, as well as stabilizing the lipid layers during the respiratory cycle. The surfactant proteins SP-A and SP-D are larger, relatively abundant, oligomeric proteins that are also synthesized and secreted by type II cells. SP-D and SP-A are structurally related members of the collectin family of C-type mammalian lectins that share distinct collagen-like and globular, carbohydrate-binding domains. SP-A is required for the formation of tubular myelin and plays diverse roles in host-defense functions of the lung (Kingma et al. 2006; Wright 2005). SP-A binds lipopolysaccharides and various microbial pathogens, enhancing their clearance from the lung. Unlike SP-B and SP-C, SP-A does not play a critical role in surface functions, metabolism, or pulmonary surfactant under normal conditions. SP-D, however, influences the structural forms of pulmonary surfactant and is important in the regulation of alveolar surfactant pool sizes and reuptake (Korfhagen et al. 1998; Ikegami et al. 2005). SP-D is also necessary in the suppression of pulmonary inflammation and in host defense against viral, fungal, and bacterial pathogens. Taken together, surfactant lipids and proteins play critical roles in (a) reducing surface tension in the alveolus, as required for ventilation, and (b) modulating various aspects of innate host defense of the lung against diverse pulmonary pathogens.

TTF-1

Thyroid transcription factor-1 (TTF-1, also termed T-EBP/Nkx2.1) is a member of the Nkx-2 class of homeodomain-containing transcription factors (Civitareale et al. 1989, Guazzi et al. 1990) that is selectively expressed in the developing thyroid, respiratory epithelium, and restricted areas of the developing brain (Lazzaro et al. 1991 – Minoo et al 1999). TTF-1 expression begins at the onset of thyroid and lung formation, and its expression is maintained in a highly regulated pattern in the thyroid follicular cells and subsets of respiratory epithelial cells (Boshart et al. 1992 - Zhou et al. 1996). In the thyroid, TTF-1 regulates thyroid-specific genes including thyroglobulin, thyroperoxidase, and the sodium-iodide symporter (Civitareale et al. 1989, Endo et al. 1997, Francis-Lang et al. 1992). In the lung, TTF-1 activates the expression of surfactant

proteins A, B, C and D (SP-A, SP-B, SP-C and SP-D) and Clara cell secretory protein (Bruno et al. 1995 - de Felice et al. 2003). The critical role of TTF-1 for lung morphogenesis was shown in the study of TTF-1-null mice that lacked lung parenchyma, thyroid, and pituitary glands. TTF-null mice also have defects in the ventral area of the forebrain (Kimura et al. 1996, Minoo et al. 1999). Clinically, mutations in the human *Ttf-1* gene result in central nervous system, thyroid, and respiratory disorders. Human TTF-1 haploinsufficiency has been linked to choreoathetosis, hypothyroidism, and various pulmonary disorders including neonatal respiratory distress and pulmonary infections (Krude et al. 2002). Transgenic mice in which TTF-1 is overexpressed in alveolar type II cells caused dose-dependent epithelial cell hyperplasia, emphysema, and pulmonary inflammation (Wert et al. 2002). Taken together, the temporal, spatial, and quantitative regulation of TTF-1 activity is critical for normal lung morphogenesis and physiology. TTF-1 binds an element termed the NKX binding element(s), containing 5′-TNAAGTG-3′, found in regulatory regions of target genes (Damante et al. 1994). Multiple NKX binding element(s) have been identified in the promoters of surfactant protein and Clara cell secretory protein genes (Bohinski et al. 1994, de Felice et al. 1995). These elements are required for the activity of the surfactant promoters. TTF-1 directly activated the promoters of target genes when expressed in non-respiratory epithelial cells. However, the mechanisms by which TTF-1 regulates transcriptional activity on target promoters remain to be defined. TTF-1 contains three distinct domains: an NH_2-terminal transactivation domain, a DNA-binding homeodomain, and a COOH-terminal activation domain. Structural and functional studies of TTF-1 NH_2-terminal domain have suggested that the NH_2-terminal domain has functional properties similar to the typical acidic activation domain in VP16 (de Felice et al. 1995, Tell et al. 1998). The NH_2-terminal domain was shown to mediate the primary transactivation activity because a TTF-1 mutant in which the NH_2-terminal domain was deleted lost its transcriptional activity, whereas a mutant in which the COOH terminus of TTF-1 was deleted partially reduced activity (Ghaffari et al. 1997). Differences between the NH_2-terminal and COOH-terminal activation domains of TTF-1 were not observed when tested in the thyroglobulin promoter (de Felice et al. 1995, Di Palma et al. 2003). Thus, the NH_2- and COOH-terminal domains may mediate interactions of TTF-1 with other proteins to regulate target gene expression in a gene-

specific manner. Consistent with this concept, it is increasingly clear that TTF-1 functions cooperatively with a number of other transcription factors, forming complexes on regulatory regions of target genes. TTF-1 interacts with Pax8, GATA6, STAT3, retinoic acid receptor and associated cofactors, nuclear factor-I (NFI-B), AP1 family members, and BR22 (Di Palma et al. 2003 – Yang et al. 2001). Recently it was been published that TTF-1 interacts with the transcriptional coactivator TAZ in the respiratory epithelial cells and this interaction brings to a more potent transcriptional activation of the SP-C promoter by TTF-1 (Park et al. 2004).

Aim of the project

Transcriptional regulation is a fundamental process for cellular differentiation and development. In an adult organism differentiated cells are characterized by the expression of a specific subset of genes and this expression is regulated mainly at the transcriptional level. Study of lung-selective gene expression is relatively recent, beginning with the recognition that regulatory regions of all the surfactant protein genes, including *Sftpa*, *Sftpb*, *Sftpc*, and *Sftpd*, are controlled by the homeodomain containing protein thyroid transcription factor-1 (TTF-1; Nkx2.1). In recent years, different laboratories demonstrated that the Transcriptional co-Activator with PDZ-binding motif (TAZ) is involved in several pathways: from cell migration to proliferation, from molecular "rheostat" in Mesenchimal Stem Cell to promoter of invasion in breast cancer cells. Interestingly, it was been published that TTF-1 interacts with the transcriptional coactivator TAZ in respiratory epithelial cells and that this interaction brings to a more potent transcriptional activation of the SP-C promoter by TTF-1.

An important contribution to the understanding of the physiological role of TAZ was given by several papers recently published in which TAZ-knockout mice were analyzed. The mice have major alterations in kidney and lung; in particular the kidney shows multicystic formations in the corticomedullary region similar to those found in cases of polycystic kidney syndrome in humans, while the lungs are greatly enlarged with impaired alveolarization, fibrosis and emphysema.

As the transcriptional mechanisms mediated by TAZ are not yet clear, the aim of my study was to identify new partners of TAZ possibly involved in these phenotypes, using human lung cells.

The identification and the characterization of such new partners might represent the basis for the elucidation of the molecular mechanisms underlying normal and abnormal lung development and to shed light on the pathogenesis of common human diseases.

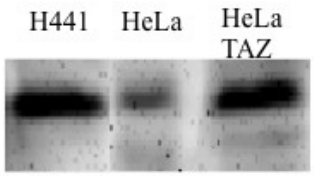
RESULTS

Identification of the AMOTL2 protein as a TAZ-associated protein

Preliminary, TAZ expression was examined by Western blot analysis of protein extracts prepared from H441 cells, HeLa cells and HeLa cells transiently transfected with an expression vector encoding TAZ, used as positive control. As shown in Fig. 1A, the polyclonal antibody that specifically recognizes TAZ reveals the presence of a protein of the predicted relative molecular mass in all the extracts analyzed.

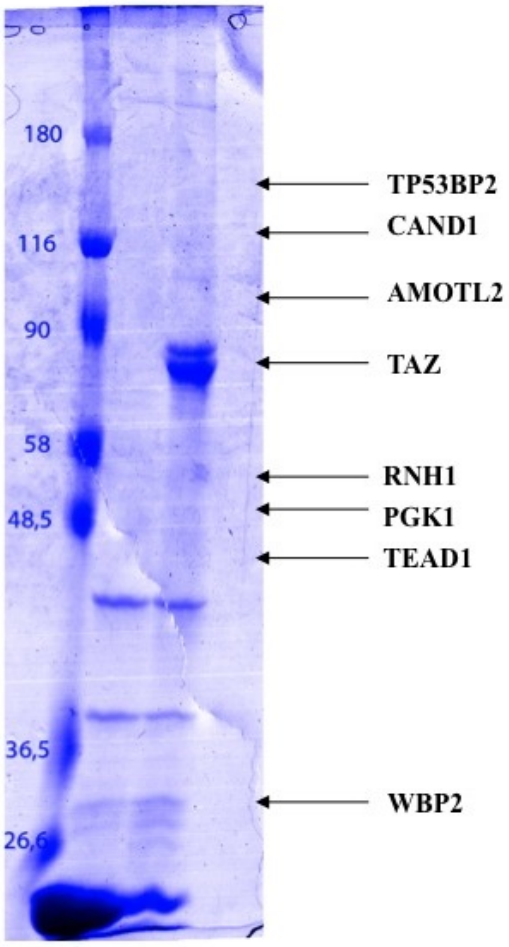
To identify proteins able to interact with the transcriptional coactivator TAZ in the lung environment, we challenged the GST–TAZ fusion protein, expressed in bacteria and affinity purified, with a total protein extract prepared from H441 human lung cells in a GST-pull down assay. To perform the GST-pull down in large scale, 200 mg of protein extract were pre-cleared onto GST-loaded beads and then divided into two tubes and incubated with GST and GST–TAZ loaded beads. Bound proteins were eluted, analyzed by SDS-PAGE and visualized by Coomassie blue staining. Several proteins bound specifically to the bait (Fig. 1B) and peptide mass fingerprint analysis, performed in collaboration with Dr. Andrea Scaloni of the ISPAAM-CNR of Naples, allowed us to obtain a list of possible interactors (Fig. 1C). Some of the identified proteins were already described as TAZ partners; interestingly, among the novel TAZ-interacting proteins there was the Angiomotin-like 2 protein (AMOTL2), which belongs to the Motin protein family (Bratt et al. 2002). Notably, AMOTL2 is on the top of the peptide hit list suggesting a strong interaction between AMOTL2 and TAZ. Angiomotin (AMOT), the founding member of the Motin family, is a vascular angiogenesis-related protein, which was initially identified as an angiogenesis inhibitor angiostatin-binding protein through a yeast two-hybrid screen (Troyanovsky et al 2001, Bratt et al. 2002). AMOT can induce endothelial cell migration and tubule formation and therefore promotes angiogenesis (Bratt et al. 2005). There are two angiomotin-like proteins, AMOTL1 and AMOTL2. Just like AMOT, AMOTL1 and AMOTL2 also play important roles in cell migration and angiogenesis (Gagnè et al. 2009, Huang et al. 2007), suggesting that this family of proteins may share similar functions *in vivo*.

A



W.B: anti-TAZ

B



C

H441	
Bait: TAZ	
AMOTL2	**58**
INADL	45
TP53BP2	16
LAMB3	11
LGALS3	10
ATAD3C	9
RNH1	9
PGK1	8
TEAD1	8
WARS	8
HADHB	7
SQRDL	7
CAND1	7
AIFM1	7
MAGUK p55	6
WBP2	5

Fig. 1: (A) TAZ is expressed in H441 human lung cells. Total protein extracts prepared from H441, HeLa and HeLa cells transiently transfected with CMV-TAZ were separated on SDS-PAGE and subjected to Western blot analysis with a specific anti-TAZ antibody. (B) To isolate cellular proteins interacting with GST–TAZ, glutathione-sepharose beads loaded with GST–TAZ or GST were incubated with total cell lysate obtained from H441, as described under Materials and Methods. After binding, the eluted proteins were separated on 8–15% SDS-PAGE and visualized by Coomassie blue staining. Lane 1, proteins eluted from GST beads. Lane 2, proteins eluted from GST–TAZ beads. Molecular weight markers are indicated on the left. (C) The table shows the number of peptide hits for AMOTL2 and several other TAZ-interacting proteins.

To confirm AMOTL2 as a TAZ-interacting protein, recombinant GST-TAZ protein was used in small scale GST pull-down experiments with total protein extracts prepared from lung and non-lung cells. In particular, we used the H441 cells as a source of lung-specific protein extract and HeLa cells transiently transfected with an expression vector encoding for Amotl2 as a source of non-lung protein extract containing exogenous AMOTL2. Results of the binding reactions show that the AMOTL2 protein is specifically bound by GST-TAZ but not by the unfused GST protein (Fig. 2A).

We then investigated whether the interaction described above could also be observed *in vivo*. To this end, 2 mg of total protein extract prepared from H441 cells were immunoprecipitated with the anti-TAZ antibody. The bound proteins were separated on 10% SDS-PAGE and then analyzed by western blot with a polyclonal antibody recognizing AMOTL2. As shown in Fig. 2B, AMOTL2 was specifically co-immunoprecipitated by the anti-TAZ antibody demonstrating that TAZ and AMOTL2 co-exist in a complex in vivo.

To obtain information on the subcellular distribution of TAZ and AMOTL2 in H441 lung cells, we transiently transfected H441 with an expression vector encoding HA-AMOTL2, and analysed their localization by indirect immunofluorescence. Confocal microscopy examination of dual-labelled samples confirmed that the anti-HA antibody stained the cytoplasm, and did not give any staining in the nucleus. At the same time, TAZ was detected both in the nuclear and in the cytoplasmic compartments; only in the latter it co-localized with AMOTL2. (Fig. 2C).

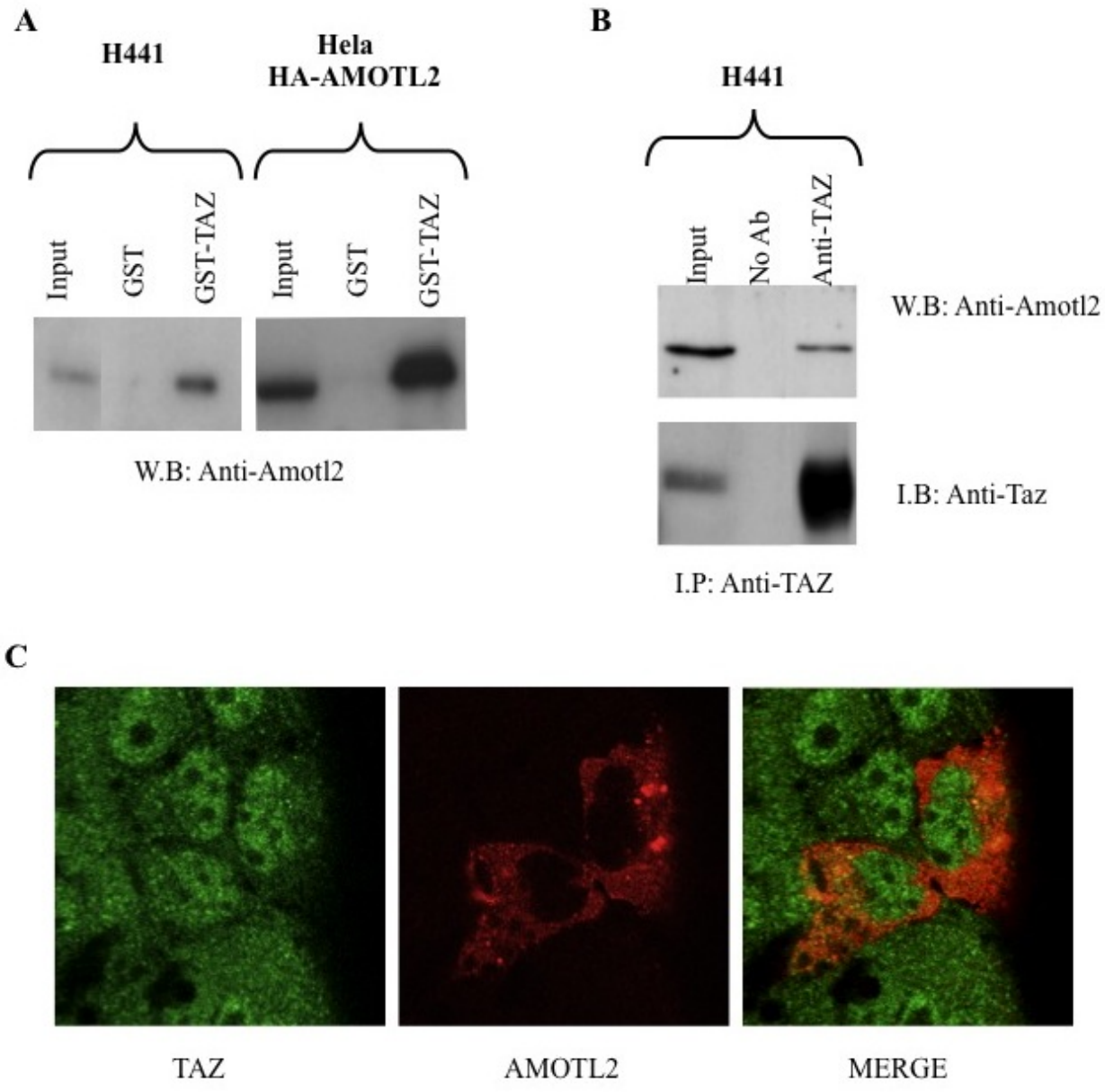


Fig. 2: (A) the GST-TAZ fusion protein immobilized on sepharose beads was incubated with total protein extracts prepareted from H441 human lung cells and from HeLa cells transiently transfected with an expression vector encoding AMOTL2. (B) in vivo interaction of TAZ with AMOTL2 revealed by co-immunoprecipitation in H441 cells. Immunoblotting was conducted using antibodies as indicated. (C) H441 cells were plated on glass coverslips and subsequently transfected with an expression vector encoding HA-tagged AMOTL2. At 48 h after transfection, cells were double-stained by immunofluorescence with a monoclonal anti-HA antibody and with a polyclonal anti-TAZ antibody. Fluorescein isothiocyanate and TRITC signals were acquired together at a confocal microscope, by line-wise scanning. The staining for AMOTL2 was detected only in the cytosol of the cells. TAZ staining was detected both in the nucleus and in the cytosol of the cells. The overlay of the signals is also shown.

The WW domain of TAZ and the PPXY motif of AMOTL2 mediate the protein-protein interaction

We next mapped the region responsible for TAZ and AMOTL2 interaction. It is reported in the literature that TAZ interacts with proteins containing a L/PPXY motif by means of its WW domain (Kanai et al. 2000), but also that it is able to interact with proteins lacking the above mentioned motif (Murakami et al. 2005). Therefore, to understand whether the WW domain of TAZ was the region of the protein responsible for the interaction with AMOTL2, we analyzed more in details the domains of TAZ involved in the binding with the protein. To this aim, we generated three GST-TAZ deletion mutants designated NW-TAZ, W-TAZ and C-TAZ, which preserve the N-terminal region and the WW domain (NW-TAZ) , only the WW domain (W-TAZ) or the C-terminal domain (C-TAZ) (Fig. 3A). Pull down experiments were performed using the fusion proteins GST-TAZ, GST-NW-TAZ, GST-W-TAZ and GST-C-TAZ, produced in bacteria and affinity purified, that were challenged with a protein extract prepared from HeLa cells transiently transfected with a vector encoding HA-AMOTL2. As shown in Fig. 3B, AMOTL2 is able to interact with full-length TAZ and also with NW-TAZ and W-TAZ deletion mutants but not with C-TAZ. The same experiment was performed also using a protein extract prepared from H441 cells in which the AMOTL2 is endogenously expressed at physiological levels (Fig. 3C). Bound proteins were detected by Western blot using a specific antibody against AMOTL2 while the hybridization with anti-GST antibody assessed the validity of the experiment and the protein uniform loading.

To determine whether the interaction of AMOTL2 with TAZ occurs through the L/PPXY motif that is present in the N-terminal region of the protein, we used an expression vector encoding for the AMOTL2 mutated in the PPXY motif (Tyr^{213} to Ala). In addition, to investigate whether AMOTL2 contained other important regions involved in the binding with TAZ beyond the LPPY motif, we also analyzed a AMOTL2 deletion mutant which is deleted of the PDZ binding domain present in the C-terminal region (Fig. 4A).

To test whether the two mutants were still able to associate with TAZ, we performed pull down experiments with recombinant GST-TAZ and total extracts prepared from HeLa cells transiently transfected with the two mutants and the wild-type AMOTL2, separately.

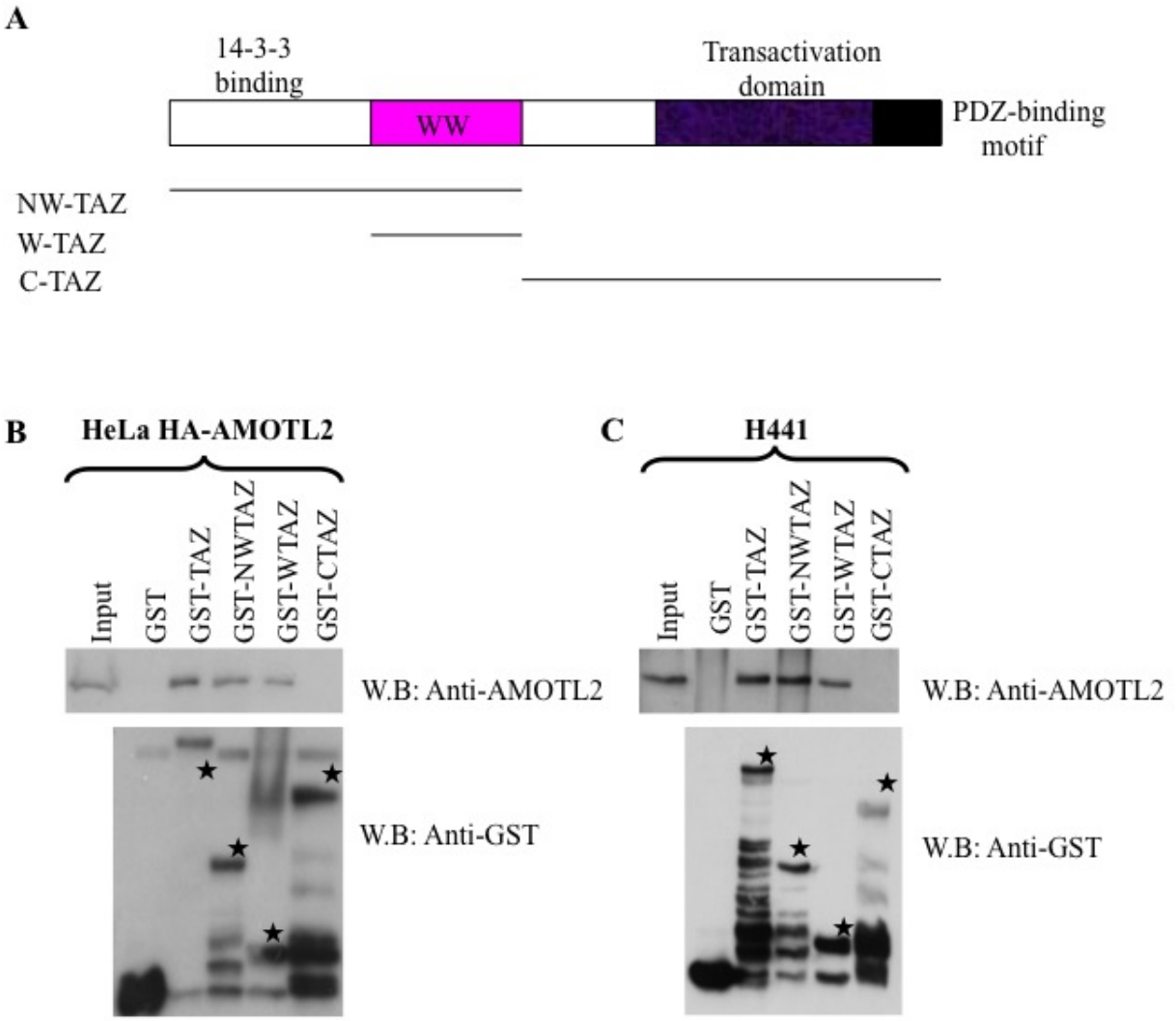


Fig. 3: (A) Schematic representation of TAZ structure and of its deletion mutants used in GST-pull down assays. The NW-TAZ deletion mutant contains the entire N-terminal region and the WW domain; W-TAZ contains only the WW domain; C-TAZ contains the C-terminal region downstream the WW domain. (B, C) GST-Pull down assays were performed with GST and GST-TAZ proteins immobilized on glutathione-sepharose beads and protein extract from HeLa transiently transfected with HA-AMOTL2 (B) and from H441 cells (C). Bound proteins were detected by Western blot using a specific antibody against AMOTL2 as indicated. The hybridization with anti-GST antibody assessed the validity of the experiment and the protein uniform loading. The asterisk indicates the full-length form of the fusion proteins.

The results of the GST-pull down experiments demonstrated that the mutation of the tyrosine residue in the PPXY motif disrupts the interaction of AMOTL2 with TAZ. On the other hand, deletion of the PDZ-binding motif does not affect AMOTL2 interaction with TAZ (Fig. 4B).

These results were also confirmed *in vivo* by co-immunoprecipitation assays. HeLa cells were transiently transfected with expression vectors encoding HA-AMOTL2 full-lenght, HA-AMOTL2-(ΔPDZ) and MYC-AMOTL2(Y213A) alone or in combination with 3xFLAG-TAZ. As shown in Fig. 4C, HA-AMOTL2 and HA-AMOTL2-(ΔPDZ) were co-immunoprecipitated with 3xFLAG-TAZ while MYC-AMOTL2(Y213A) was not.

All together the above results clearly demonstrate that the WW domain of TAZ and the PPXY motif of AMOTL2 play a major role in TAZ-AMOTL2 interaction.

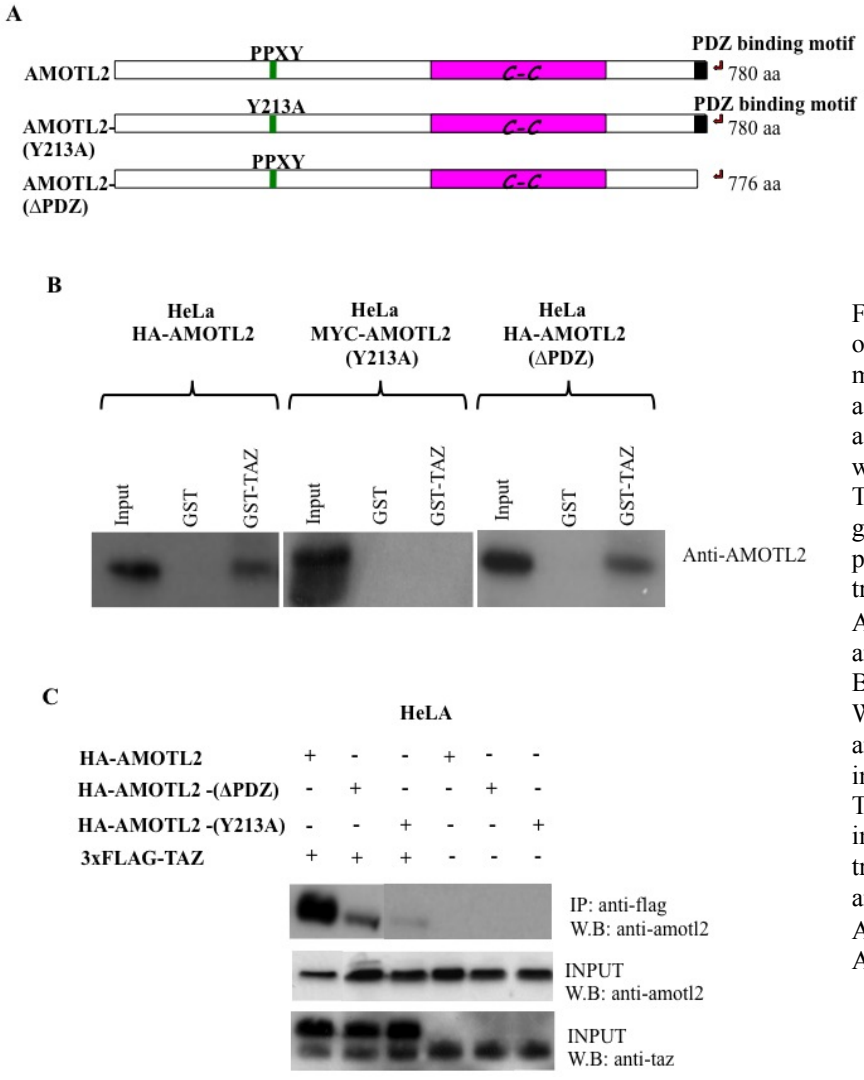


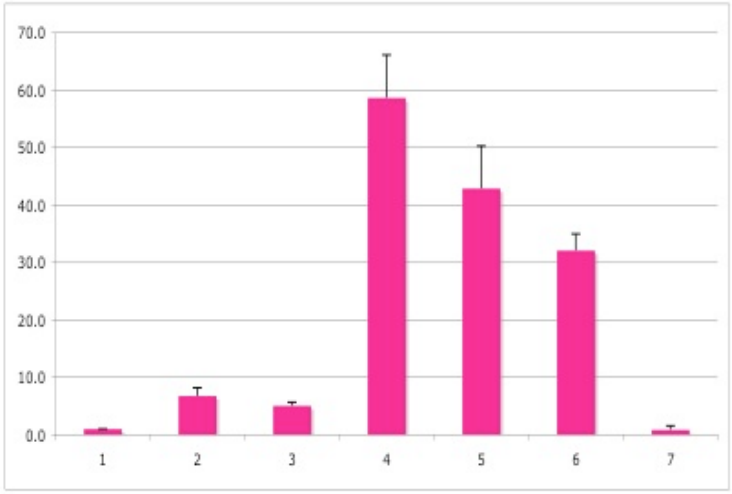
Fig. 4: (A) Schematic representation of AMOTL2 structure and of its mutants used in GST-pull down assays and in co-immuprecipitation assays. (B) GST-Pull down assays were performed with GST and GST-TAZ proteins immobilized on glutathione-sepharose beads and protein extract from HeLa cells transiently transfected with HA-AMOTL2, HA-AMOTL2(ΔPDZ) and MYC-AMOTL2(Y213A).
Bound proteins were detected by Western blot using a specific antibody against AMOTL2, as indicated. (C) In vivo interaction of TAZ with AMOTL2 revealed by co-immunoprecipitation in HeLa cells transiently transfected with TAZ and HA-AMOTL2, HA-AMOTL2(ΔPDZ) or MYC-AMOTL2(Y213A).

AMOTL2 inhibits TAZ transactivation properties

It has been published that TTF-1 interacts with the transcriptional coactivator TAZ in respiratory epithelial cells and that such interaction brings to a more potent transcriptional activation of the SP-C promoter by TTF-1 (Park et al. 2004).

Hence, as next step we asked whether the interaction of TAZ with AMOTL2 could somehow affect the ability of TAZ to function together with TTF-1 in the modulation of SP-C gene expression. To investigate the functional outcome of the biochemical interaction between TAZ and AMOTL2, we performed luciferase assays using a reporter construct containing the TTF-1 responsive element identified in the promoter of the SP-C gene (0.32 mSP-C-luc). HeLa cells transfected with the reporter construct and TTF-1 showed a ~sixfold increase over the basal level in the transcriptional activity of the SP-C promoter and co-transfection with TAZ resulted in a strong increase of TTF-1 transcriptional activity on this promoter. Interestingly, when AMOTL2 was also co-expressed in the same cells the ability of TAZ to enhance TTF-1 transcriptional activity on the SP-C promoter was significantly reduced (Fig. 5A). At the same time, the capability of TAZ to synergize with TTF-1 on the SP-C promoter was not affected by the co-expression with AMOTL2 mutated in the PPXY domain (Fig. 5B). Thus, our luciferase functional assays suggest that the biochemical interaction of AMOTL2 with TAZ inhibits the capability of TAZ to function as a transcripotional co-activator.

A



0.32 SpC-luc	+	+	+	+	+	+	+
TTF-1	-	+	-	+	+	+	-
TAZ	-	-	+	+	+	+	-
AMOTL2	-	-	-	-	+	+	+

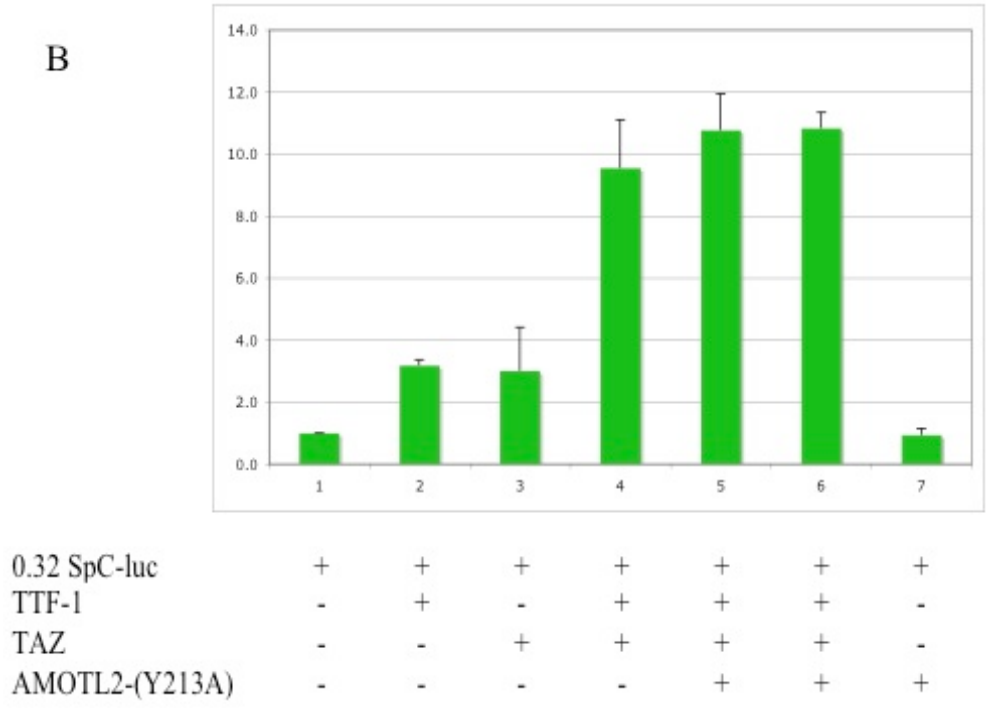
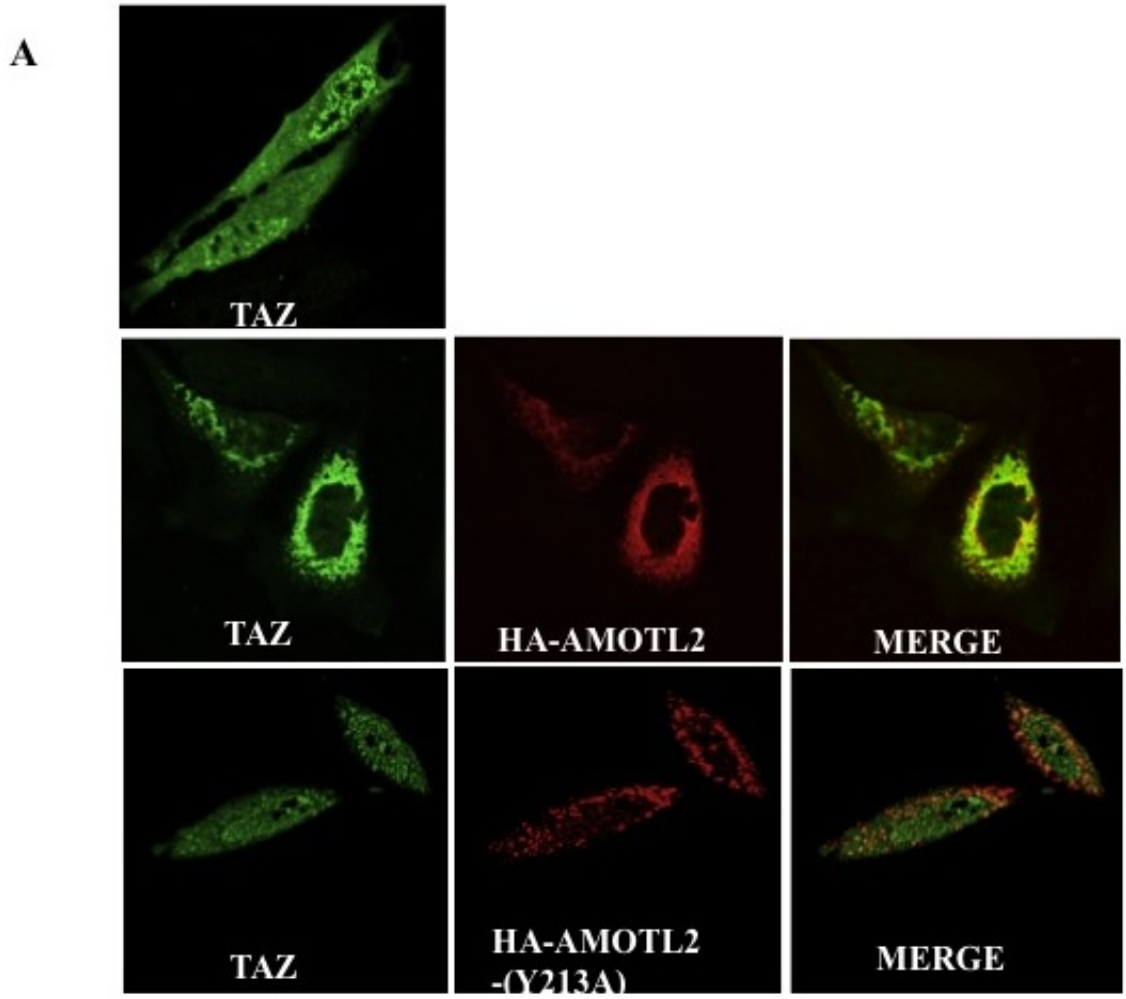


Fig. 5: (A-B) HeLa cells were transfected with 500 ng of a luciferase reporter construct carrying the TTF-1 responsive element of the SP-C promoter together with expression vectors encoding TTF-1, TAZ and AMOTL2 separately or in different combinations. Cells were also transfected with an expression vector for Renilla as an internal control.
Cells were harvested after 48 h, and luciferase and renilla activities were measured. The results are presented as fold increases in activity as compared with the control promoter alone. Values are mean of three independent experiments.

Because AMOTL2 is a cytoplasmatic protein, whereas TAZ can shuttle between nucleus and cytoplasm, we investigated whether the observed inhibitory effect could be due to AMOTL2 sequestration of TAZ in the cytoplasm. To address this issue, HeLa cells were grown directly on glass coverslips and transiently transfected with TAZ and HA-AMOTL2 or HA-AMOTL2-(Y213A). After 48 hours, cells were processed for double immunofluorescence analysis with a polyclonal anti-TAZ antibody and a monoclonal anti-HA antibody to follow the intracellular localization of the two proteins by confocal fluorescence microscopy. The signals from the two immunostained proteins were acquired together, at high resolution, by line-wise scanning. It is well-known that in low-density HeLa cell cultures, TAZ is localized mainly in the nucleus. Interestingly, when we co-transfected TAZ together with AMOTL2, it was predominantly localized in the cytoplasm indicating that indeed TAZ subcellular localization is affected by the

interaction with AMOTL2. In agreement with our previous data, TAZ cellular distribution was not affected by the co-transfection with AMOTL2 mutated in the PPXY domain (Fig. 6A), supporting the relevance of the biochemical interaction between the two proteins for the modulation of TAZ cellular localization. To further strenghten these data, we co-transfected again HeLa cells plated on glass coverslips with the mutant TAZ-Ser^{89}, in which the Ser^{89} phosphorylation site is mutated to Ala, together with AMOTL2 and we analyzed TAZ cellular distribution. Additionally, we were able to demonstrate that in such context AMOTL2 was still able to promote the cytoplasmic localization of TAZ-Ser^{89} suggesting that the mechanism by which AMOTL2 regulates the subcellular localization of TAZ is mediated by a direct protein-protein interaction and does not require TAZ phosphorylation at Ser^{89} site (Fig. 6B).



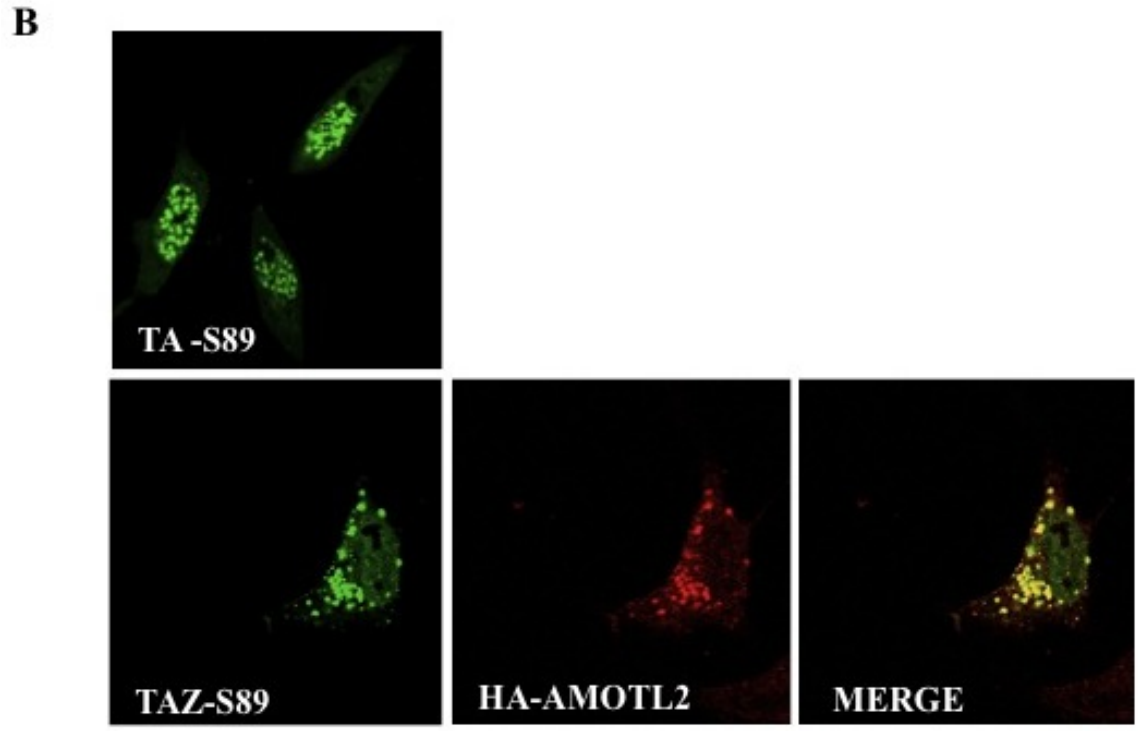


Fig 6: (A) TAZ was transfected in HeLa cells with or without co-transfection of HA-AMOTL2 or MYC-AMOTL2-(Y213A). 48h after transfection, cells were processed for double immunofluorescence analysis with polyclonal anti-TAZ antibody and monoclonal anti-HA antibody. (B) HeLa cells were co-transfected with GFP-TAZ-S89A and HA-AMOTL2. 48h after transfection, cells were processed for immunofluorescence analysis with the monoclonal anti-HA antibody. In both experiments, the signals from the two immunostained proteins were acquired together, at high resolution, by line-wise scanning.

Subsequently, we were interested in the assessment of the role of AMOTL2 in the regulation of TAZ subcellular localization in a more physiological context. To this aim, H441 lung cells were transfected with 2 μg of the expression vector HA-AMOTL2 and 48 hours after the transfection, selection with the specific antibiotic was started and ~ forty independent clones were isolated. The over-expression of HA-AMOTL2 in the stable clones was examined by Western Blot analysis of protein extracts preparated from each clone. As shown in Fig 7A, the monoclonal antibody that specifically recognizes the HA tag revealed the presence of HA-AMOTL2 in three clones. We further analyzed the subcellular localization of TAZ and AMOTL2 in the positive clones by indirect immunofluorescence followed by confocal fluorescence microscopy. Also in these experiments, the signals from the two immunostained proteins were acquired together, at high resolution, by line-wise scanning. As shown in figure 7B, the stable overexpression

of AMOTL2 in H441 cells induces a massive translocation of TAZ from the nucleus to the cytoplasm, as already observed in HeLa cells.

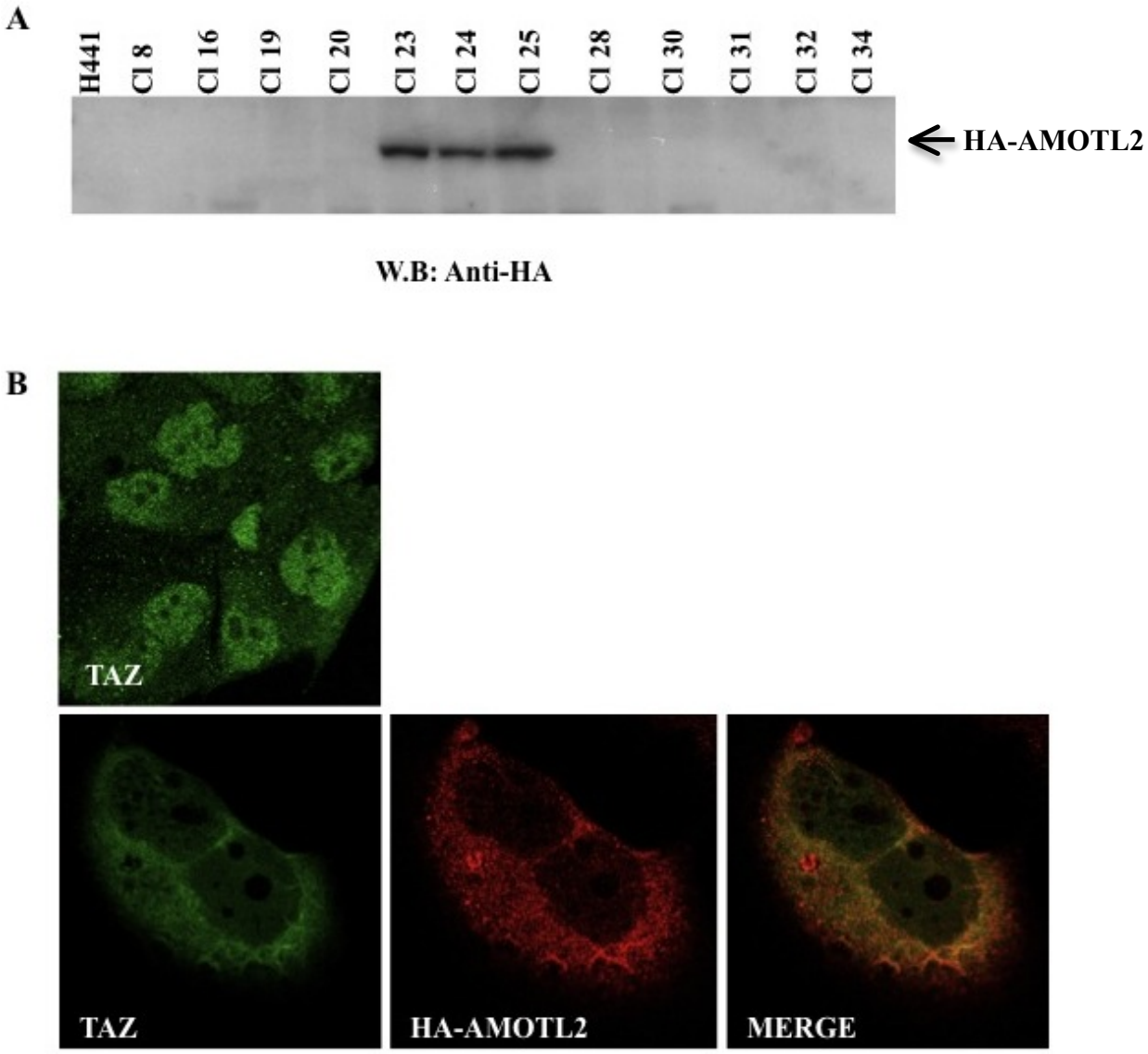


Fig 7: (A) Total protein extracts prepared from each stable clones were separated on SDS-PAGE and subjected to Western blot analysis with a specific anti-HA antibody. (B) One representative clone overexpressing HA-AMOTL2 was processed for double immunofluorescence analysis with polyclonal anti-TAZ antibody and monoclonal anti-HA antibody. The signals from the two immunostained proteins were acquired together, at high resolution, by line-wise scanning.

DISCUSSION

Transcriptional regulation is the most important way of modulating gene expression both in eukaryotes and in bacteria because it contributes to define the proteins expressed by a cell and, as a consequence, the functions characteristic of a cell. Specifically, this mechanism of regulation is more relevant in pluricellular organism where it allows a gene to be expressed in the right cell and at the right moment during development and differentiation.

A pivotal role in transcriptional regulation is hold by transcription factors. We can define transcription factor any protein other than RNA Polymerase that is required for transcription. Transcription factors often don't act alone but they are part of protein heterocomplexes in which they interact with different transcription factors, with accessories proteins and with transcriptional co-activators/repressors.

TAZ (Transcriptional co-Activator with PDZ binding motif) is a transcriptional coactivator that, in these last years, has been described to interact with, and to regulate, several transcription factors (Murakami, et al. 2005; Murakami, et al. 2006; Mahoney, et al. 2005; Hong, et al. 2005; Park, et al. 2004). Moreover, it was suggested also a role of TAZ in migration, invasion and tumorigenesis of breast cancer cells and it has been demonstrated that TAZ is overexpressed in a significant fraction of breast cancers (Siew Wee Chan, et al. 2008). Recently, other two studies have connected TAZ overexpression with the development of NSCLC (non-small cell lung cancer, NSCLC) (Zhou et al. 2011) and PTC (papillary thyroid carcinoma, PTC) (de Cristofaro et al. 2011). TAZ is also a component of Hippo pathway in which the activation of TAZ leads to migration and cell proliferation (Zhang, et al. 2009). Studies from three independent laboratories have also shown that knockout of the TAZ gene in the mouse results in major defects in kidney and lung. In particular, the kidney shows multicystic formations in the corticomedullary region similar to those found in cases of polycystic kidney syndrome in humans, while the lungs are greatly enlarged with impaired alveolarization, fibrosis and emphysema.

Summing up all the data present to date in the literature, TAZ turnes out to be a potent regulator of organ structure negatively regulated by the Hippo pathway. Hence, during

my Ph.D. I have been interested in identifying new partners of TAZ in human lung cells possibly involved in the lung phenotype observed in the knockout mouse.

The lung is the essential respiratorian organ in many air-breathing animals. Its principal function is to transport oxygen from the atmosphere into the bloodstream, and to release carbon dioxide from the bloodstream into the atmosphere. Cellular differentiation in the lung is complex, and several morphologically distinct cell types comprise the airway epithelium. In particular, alveolar epithelium contains a continuous layer of two principal cell types: type II and type I alveolar epithelial cells. Preliminary data on the temporal/spazial expression pattern of TAZ in the developing mouse lung, demonstrated that TAZ mRNA is detectable from the embryonic stage E13.5 and at relatively high levels in purified mouse alveolar type II cells, which are known to express the transcription factor TTF-1 and the surfactan proteins (Stahlman et al. 1996, Zhou et al. 1996, Liu et al. 2002). In this study, we identified through a GST-pull down assay followed by mass spectrometry analysis the Angiomotin-like 2 protein (AMOTL2) as a novel regulator of TAZ. AMOTL2 belongs to the Motin protein family that includes also Angiomotin (AMOT) and Angiomotin-like 1 (AMOTL1). Motin proteins have two coiled-coil domains and the C-terminal PDZ-binding motif in common and otherwise distinct domains, like a different number of PPXY motif. Angiomotin, the founding member of the Motin family, is a vascular angiogenesis-related protein, which was initially identified as a binding protein of angiostatin through a yeast two-hybrid screen, and it is able to regulate endothelial cell migration (Troyanovsky et al 2001, Bratt et al. 2002). Accordingly, also AMOTL1 is able to promote angiogenesis by controlling endothelial polarity and junction stability (Zheng et al. 2009).

AMOTL2 has been studied in zebrafish embryos where it is expressed in blood vessels (Huang et al. 2007) and is required for angiogenesis. *AMOTL2* is expressed maternally and in a restricted manner as soon as the zygotic genome begins to be expressed. Inhibition of zebrafish *amotl2* expression by antisense morpholinos causes epiboly arrest and aberrant convergent extension in zebrafish embryos, which coincides with disruption of juxtamembrane actin fibers and formation of membrane protrusion. In vitro analyses reveal that zebrafish *amotl2* regulates cell migration by binding to and promoting peripheral membrane translocation of the nonreceptor tyrosine kinase c-Src. In conclusion, these data suggest that *amotl2* is essential for cell movement in vertebrate embryos, which might be associated with c-Src translocation. So, all three members of

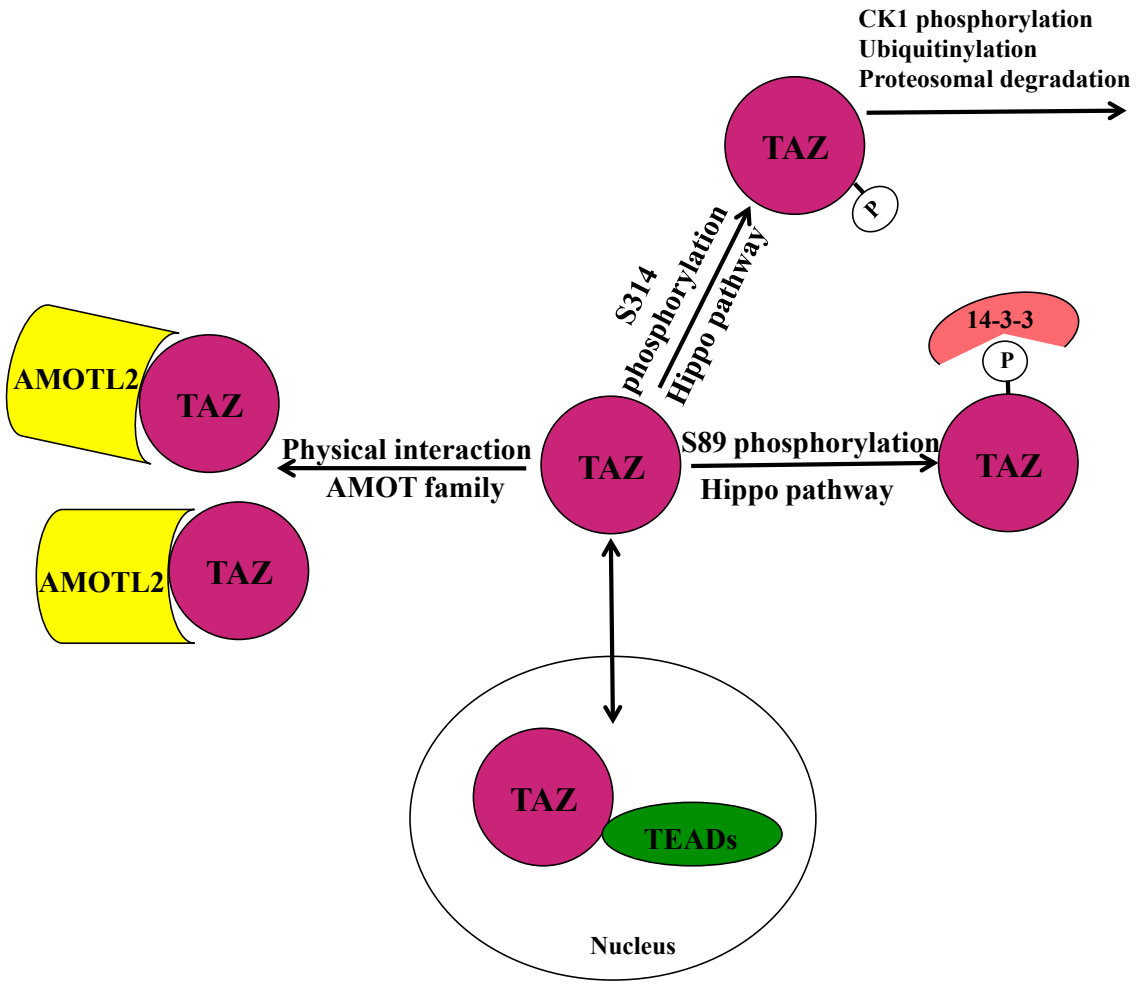
Motin family are implicated in blood vessel formation, implying that they all have inherited this function from their common ancestor. However, they may have distinct functions in other cellular and developmental processes.

It is reported in the literature that TAZ interacts with proteins containing a L/PPXY motif by means of its WW domain (Kanai et al. 2000), but also that it is able to interact with proteins lacking the above mentioned motif (Murakami et al. 2005). So in a first time, we mapped the regions responsible for TAZ and AMOTL2 interaction and demonstrated *in vitro* and *in vivo* that such interaction is mediated by the PPXY motif present in AMOTL2 protein and the WW domain present in the N-terminal region of TAZ.

The numerous functions of TAZ described in the last years suggest that its availability and subcellular localization are tightly controlled to govern its functionality within the cell. The major known regulatory mechanism for TAZ is via the recently defined Hippo pathway. The Hippo pathway negatively regulates TAZ by two different mechanisms through cytoplasmic sequestration and proteasomal degradation. For these reasons, we investigated the effect of TAZ interaction with AMOTL2 on the transcriptional activity of TAZ. To this end, we used a luciferase report construct containing TTF-1 responsive elements identified in the SP-C promoter since the expression of surfactant protein C is dependent on the cooperation of the transcription factor TTF-1 and TAZ. The results of the luciferase assays suggested an inhibitory role of AMOTL2 on TAZ ability to co-activate transcription. We further demonstrated that such inhibition is due to the sequestration of TAZ in the cytoplasm induced by the intercation with AMOTL2. In fact, our immunofluorescence experiments clearly demonstrate that TAZ is detectable exclusively in the cytoplasm of the cells, both HeLa and H441, when co-transfected together with AMOTL2. In addition, our data also indicate that the delocalization of TAZ in the cytoplasm does not depend on its phosphorylation status, while is strictly correlated to the capability of TAZ to physically bind to AMOTL2.

All together the results presented in this study offer an additional novel mechanism of regulation of the transcriptional co-activator TAZ which could take place through direct interaction with the AMOTL2 protein leading to the cytoplasmic sequestration of TAZ independently from the Hippo pathway-regulated interaction with 14-3-3 proteins. The general working model derived from our experiments is that AMOTL2 is primary cytosolic protein that is able to interact with TAZ through its PP*X*Y motif and such

interaction causes the cytoplasmic sequestration of TAZ that consequently becomes incapable to exert its functions in the nucleus.



MATERIALS AND METHODS

Plasmid constructs

The plasmids used have been previously described and were as follows: GST-TAZ, CMV-TAZ and 0.32 mSP-C-luc (Park et al. 2004), GFP-TAZ-S89A (Kanai et al. 2000) and CMV-TTF-1 (Bohinski et al. 1994). The GST-TAZ-6HIS was generated by PCR amplification of TAZ coding region from pEF1A-TAZ-MYC-6HIS producted in our laboratory before and subsequent subcloning in the EcoRI-XhoI sites of the pGEX-4T3 vector. The GST-NW-TAZ, GST-W-TAZ and GST-C-TAZ fusion proteins were generated by PCR amplification of the different portions of TAZ coding region and subsequent subcloning in the EcoRI-XhoI sites of the pGEX-4T3 vector. The 3xFLAG-TAZ was generated by PCR amplification of mouse TAZ coding region and subcloned in the NotI-XbaI sites of the p3xFLAG-CMV10. HA-AMOTL2 full length and HA-AMOTL2-(ΔPDZ) were kindly provided by S. sukita and MYC-AMOTL2-(Y213A) was kindly provided by J. Chen.

Cell culture, transfections and reporter assays

H441 cells were grown in RPMI medium (Euroclone) supplemented with 10% fetal bovine serum. For stable transfection of pCAGGS-HA-AMOTL2, H441 cells were cultured in 100mm plates and transfected with 2μg of DNA with the addition of 200ng of pBABE-puro. After 48h from transfection selection with the specific antibiotic was started. 0.4 μg/ml puromycin (Sigma) was used to select clones. HeLa cells were grown in Dulbecco's modified Eagle's medium (Euroclone) supplemented with 10% fetal calf serum (Hyclone). For transient transfection experiments, cells were plated at 3×105 cells/60-mm tissue culture dish 5 to 8 h prior to transfection. Transfections were carried out with the FuGENE6 reagent (Roche Diagnostics) according to the manufacturer's directions. The DNA/FuGENE ratio was 1:2 in all the experiments. The reporter construct 0.32 mSP-C-luc was co-transfected with expression vectors encoding transactivators TTF-1 and TAZ and HA-AMOTL2 or HA-AMOTL2-(Y213A). Firefly Luciferase activity was normalized on the activity of pRL-Renilla vector in order to correct each sample for transfection efficiency. The total amount of transfected DNA

was kept constant with an empty expression vector in all the transfection assays. Luciferase assay and Renilla assay were performed using the Luciferase Assay System (Promega) and the Renilla Luciferase Assay System (Promega) following the instruction of the manufacturer. Protein extraction was performed using the Passive lysis buffer (Promega) supplemented of protease inhibitors (100X protease inhibitor cocktail, SIGMA). Luminescence was measured with LUMAT LB 9507 luminometer (Berthold technologies).Transfection experiments were done in duplicate and repeated at least three times. Statistical analysis has been performed by means of an unpaired two-tailed Student's t test to obtain the P value associated with the observed fold of activation differences.

Protein extracts and immunoblotting

For Western blot, cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in JS buffer containing 50 mM Hepes pH 7.5, 150 mM NaCl, 5 mM EGTA pH 7.8, 10% glycerol, 1% Triton, 1.5mM $MgCl_2$, 1 mM dithiothreitol (DTT), 1 mM phenylmetilsulfonil fluoride (PMSF). The protein concentration was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Inc., Hercules, CA). For Western blot analysis, proteins were separated by SDS-10% PAGE, gels were blotted onto Immobilon P (Millipore, Bredford, MA, USA) for 2 h and the membranes were blocked in 5% nonfat dry milk in Tris-buffered saline for 1 h or overnight before the addition of the antibody for 1 h. The primary antibodies used were anti-TAZ (kindly provided by M. Sudol), anti-AMOTL2 (kindly provided by), anti-Myc (Santa Cruz, CA), anti-HA (Covance), anti-FLAG (Sigma), anti-GST (kindly provided by P. Di Fiore). The filters were washed three times in Tris-buffered saline before the addition of horseradish peroxidase-conjugated secondary antibodies for 45 min. Horseradish peroxidase was detected with ECL (GE Healthcare).

Pull down assay and co-immunoprecipitation

GST–TAZ and deletion mutants proteins were purified from BL21 (DE) LysS bacterial cells transformed with pGEX-TAZ, pGEX-NWTAZ, pGEX-WTAZ and pGEX-CTAZ. At *D*600 = 0.6, isopropyl β-D- thiogalactoside (0.1 mM final) was added to the culture to induce the expression of the fusion protein, and cells were harvested 4 h later. Cells were resuspended in lysis buffer (1 × PBS, 0.5 mM EDTA, 1 mg/ml lysozyme, 0.5 mM

dithiothreitol, 1 mM PMSF and protease inhibitors diluted 1:1000), and sonicated. Triton X-100 (1 %, v/v) was then added, and the cell extract was centrifuged at 200 000 ***g*** for 40 min at 4 ◦ C. The supernatant was subjected to affinity chromatography using glutathione–agarose beads (Amersham-Pharmacia). After binding, beads were washed three times with washing buffer (10 mM Tris/HCl, pH 8.0, 100 mM NaCl, 5 mM EDTA, 5 mM dithiothreitol). GST–TAZ and deletion mutant proteins were eluted with a buffer containing 10 mM glutathione, 50 mM Tris/ HCl, pH 8.0, and 50 mM NaCl for 10 min at 4 ◦C. The eluted proteins were stored at − 80 ◦C. Pull-down assays were performed by challenging 4 μg of GST or GST–TAZ, GST–NWTAZ , GST–WTAZ or GST–CTAZ purified proteins bound previously to glutathione–agarose beads with proteic extract from H441 or HeLa cells transiently transfected with HA-AMOTL2, HA-AMOTL2-(ΔPDZ) or MYC-AMOTL2-(Y213A). The binding reactions were carried out for 90 min at 4 ◦C on a rotating wheel, and then the beads were washed several times with JS buffer. The bound proteins were eluted by resuspending the beads directly in 2 × SDS/PAGE sample buffer and heating at 95 ◦C for 3–5 min before loading on the gel.

To analyze the interaction of TAZ with AMOTL2, a construct expressing 3xFLAG-TAZ was transfected into HeLa cells alone or with vectors encoding HA-AMOTL2, HA-AMOTL2-(ΔPDZ) or MYC-AMOTL2-(Y213A). About 48 h after transfection, cells were washed twice with PBS and lysed in JS buffer. The co-immunoprecipitation experiment was performed by incubating 2 mg of total protein extract with 20 μl of anti-FLAG agarose affinity gel (Sigma) overnight at 4 ◦C on a rotating wheel. The samples were then centrifuged and the agarose gel-bound proteins were washed several times with JS buffer, resuspended in 2 × SDS/PAGE sample buffer and heated at 95 ◦C for 3–5 min before loading on the gel. The same conditions were used when the coimmunoprecipitation experiment was performed using H441 cells.

Protein identification by mass spectrometry

GST and GST–TAZ-6HIS proteins, purified from BL21 (DE) LysS bacterial cells as described before, were loaded onto glutathione–Sepharose beads (GE Healthcare, Waukesha, WI, USA) at a concentration of 1 μg/μl packed beads for 20 min at 4 °C. A total of 5×10^8 H441 cells were lysed; after a pre-clearing step on GST-coated beads, the total extract was adsorbed with GST and GST–TAZ-6HIS beads. After washing, the

bound proteins were resolved by SDS-PAGE, and visualized by comassie blue staining. For identification, protein bands were excised from the gel, triturated, *in-gel S*-alkylated and digested with trypsin as previously reported (Caratu *et al*. 2007, Vascotto *et al*. 2007). Gel particles were extracted with 25 mM NH_4HCO_3/acetonitrile (1:1 v/v) by sonication. Samples were desalted using microZipTipC18 pipette tips (Millipore, Bredford, MA, USA) before MALDI-TOF-MS analysis. Peptide mixtures were loaded on the MALDI target together with a-cyano-4-hydroxycinnamic acid as matrix, using the dried droplet technique. Samples were analyzed with a Voyager-DE PRO spectrometer (Applera, Foster City, CA, USA; Caratu *et al*. 2007, Vascotto *et al*. 2007). Mass spectra were acquired in reflectron mode; internal mass calibration was performed with peptides from trypsin autoproteolysis. Data were elaborated using the DataExplorer 5.1 software (Applera, USA). ProFound software (Zhang & Chait 2000) was used to identify protein bands from an NCBI non-redundant database using peptide mass fingerprint data. Candidates with program Est'd Z scores more than 2 were further evaluated by comparison with Mr experimental values obtained from SDS-PAGE. Eventual occurrence of protein mixtures was ascertained by sequential searches for additional protein components using unmatched peptide masses.

Indirect immunofluorescence and confocal scanning laser microscopy

HeLa and H441 cells were plated and cultured on 12 mm diameter glass coverslips 24 to 48 h prior to transfection with FuGENE6 reagent. Transfections were performed with 500 ng of each plasmid used and DNA/FuGENE ratio was 1:2 in all the experiments. Twenty-four hours after transfection, cells were fixed for 20 min at room temperature with a 4% solution of paraformaldehyde in PBS, treated for 20 min with 50 mM solution of NH4Cl in PBS, permeabilized for 5 min with a 0.5% solution of Triton X-100 in PBS and incubated for 60 min in 1% BSA in PBS. The coverslips were subsequently incubated for 60 min at room temperature with primary antibodies diluted in 0.5% BSA in PBS. After PBS washings, the coverslips were incubated for 30 min with Alexa 594-tagged goat anti-rabbit, and Alexa 488-tagged goat anti-mouse secondary antibodies diluted 1:70 in 0.5% BSA in PBS. After final washings with PBS, the coverslips were mounted on a microscope slide using a 50% solution of glycerol in PBS.

Primary antibodies were rabbit polyclonal anti-TAZ (kindly provided by M. Sudol), anti-c-Myc (9E10) monoclonal antibody from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.) and anti-HA from COVANCE. Confocal scanning laser microscopy images were collected with a Zeiss LSM 510 confocal laser scanning microscope, equipped with a 543 nm HeNe laser, a 488 nm argon ion laser and a Plan-Apochromat 63×/1.4 oil-immersion objective. Emitted fluorescence was detected using a BP 505–530 bandpass filter for Alexa Fluor 488 and a LP 560 long pass filter for Alexa Fluor 594. Pairs of images were collected simultaneously in the green and red channels. High magnification images were collected as 1024×1024×32 voxel images.

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