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Dissecting the role of zyxin as a mediator of aggressiveness in Ewing sarcoma

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Abstract

Zyxin is a phosphoprotein localized at the focal adhesions and on the actin stress fibres, where it regulates the cytoskeleton organization. In addition, zyxin can shift into the nucleus and modulates the gene expression, affecting key cellular processes. Consequently, zyxin is as a crucial factor in the malignancy of several cancers, like Ewing sarcoma (EWS). EWS is a rare tumour of the bones, affecting children and adolescents. The main features of EWS are the presence of a chimeric transcriptional factor, EWS-FLI1 and the high expression of CD99, a glycoprotein necessary for the maintenance of the malignant phenotype. Triggering of CD99 with specific antibodies causes massive cell death, an effect that requires zyxin presence. In EWS zyxin is repressed by EWS-FLI1 and its forced re-expression counteracts the malignant phenotype.

In this work we decided to deepen our knowledge on how zyxin affects EWS malignancy.

We proved that zyxin is a negative regulator of cell migration, survival and growth in anchorage-independent conditions, confirming the tumour suppressor role of zyxin. Then we focused on the relation between CD99 and zyxin. Loss of function of CD99, by engagement with specific antibodies or use of shRNA, increases zyxin levels and promotes its nuclear translocation. Here, we observed that zyxin impairs the transcriptional activity of the Glioma associated oncogene 1 (Gli1), a member of the Hedgehog signalling pathway, which has a relevant oncogenic function in EWS. To support these evidences, we also reported that the loss of function of CD99 inhibits, trough zyxin mediation, the expression of Gli1 up-regulated target genes, such as NKX2-2, PTCH1 and cyclins, whilst enhances the expression of its down-regulated target GAS1.

In conclusion, we presented a more accurate depiction of zyxin role in EWS, which in the future could be further developed in hope to offer new therapeutic approaches.

1. Introduction

1.1 Zyxin

1.1.1 General features

The interaction between the cell and the extracellular matrix (ECM) is a crucial element for the transmission of signals that regulates several, fundamental processes, such as cell migration, proliferation and survival. The adhesion to the ECM is primary mediated by the focal adhesions (FAs), a group of transmembrane receptors that cluster into multi-molecular structures responsible for the linking between the ECM to the actin cytoskeleton. Among the various proteins that participate to the assembly of FAs emerges the zyxin family, whose members share similar sequences and domain structure; the main components of this family are zyxin, the lipoma preferred partner (LPP), the thyroid receptor-interacting protein 6 (TRIP6) and Ajuba (Gorenne et al., 2003; Sharp et al., 2004; Y. Wang & Gilmore, 2001).

Zyxin, the first member of the family to be identified, was initially described in the avian smooth muscle (Beckerle, 1986; Crawford & Beckerle, 1991) and subsequently was found also in human cells (Reinhard et al., 1995). Zyxin is encoded by the gene ZYX, which is located on the human chromosome 7 (7q34 → q35) (Zumbrunn & Trueb, 1998). The main isoform of the protein consists of 572 aminoacids (amino acids) and by alternative splicing is produced an isoform missing the first 157 amino acids (Ota et al., 2004), whose role remains obscure. Zyxin molecular structure features a N-terminal proline-rich domain and a C-terminal LIM domain (Figure 1). The N-terminal bears the α-actinin binding sites (amino acids 1-50) and four ActA-repeats (amino acids 50-120), which guarantee zyxin interaction with various protein involved in cytoskeleton regulation. Among the others, zyxin can bind the Mena/Vasodilatator-Stimulated Phosphoprotein (VASP), the LIM-Nebulette (LASP-2) and the SH3 domain protein 1 (LASP-1) (Fradelizi et al., 2001; Li et al., 2004). Interaction between zyxin, α-actinin and VASP, as well as others protein, is a crucial factor that governs the assembly and disassembly of the actin cytoskeleton, both at FAs and SFs (Fradelizi et al., 2001; Li et al., 2004). While the N-terminal is primary involved in the regulation of actin cytoskeleton, the C-terminal main role is directing zyxin in the right cell compartment. More

precisely, absence of LIM domains or their alteration inhibits zyxin targeting to the force bearing sites and FAs (Kadrmas & Beckerle, 2004; Velyvis & Qin, 2007).

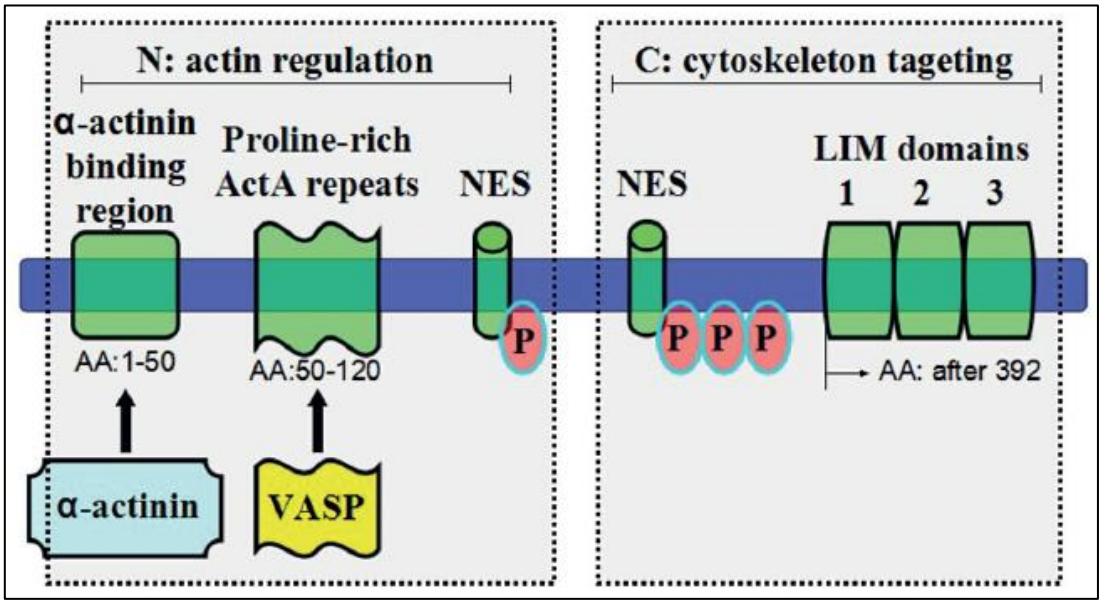


Figure 1. Schematic representation of zyxin domain organization. Reprinted with permission from Wang X, European Review for Medical and Pharmacological Sciences, 2019.

The three LIM domain (amino acids 392 to end), named after lin-11, Isl-1 and mec-3, are dual zinc finger domain, involved in interaction protein-protein and protein-DNA. Interestingly, has been proven that phosphorylation of residues in the N-terminal promotes a head-tail interaction in zyxin, that prevents zyxin binding to several of its partners (Call et al., 2011; Mobley et al., 2006). Finally, in the protein center there are two Nuclear Export Signals (NES), a common sequence in proteins capable of moving between nucleus and cytosol. Accordingly, zyxin nuclear accumulation is observed after treatment with leptomycin B, an inhibitor of Chromosomal Maintenance 1, protein involved in the nuclear trafficking of protein with NES sequences (Hervy et al., 2006; Nix & Beckerle, 1997).

1.1.2. Zyxin role in cytoskeleton organization

Beginning as another protein that localizes at FAs and SFs (Beckerle, 1986; Crawford & Beckerle, 1991), zyxin has dragged more attention once has emerged its high dynamicity in the actin remodeling process. Zyxin primary is involved in the polymerization of actin. When SFs start forming, zyxin

leaves the FAs and is strongly recruited at the formation sites of actin (W. H. Guo & Wang, 2007). Interestingly, this movement requires the correct activity of the myosin and Rho kinase apparatus, which are necessary for the formation of new SFs (W. H. Guo & Wang, 2007), indicating that zyxin presence is mandatory for the aggregation of novel SFs. The tight relation between zyxin and actin organization was also observed on others contest. Actin is strongly recruited in zyxin rich FAs and zyxin fails to reach FAs causes a poorer incorporation of actin (Heinisch et al., 2012); in addition, absence of zyxin inhibits the formation of the shear stress-induced perinuclear actin cap structures (Heinisch et al., 2012).

Zyxin regulation of the cytoskeleton is heavily influenced by mechanical tension. Application of uniaxial cyclic stretch induces zyxin mobilization to the FAs, that are reinforced and reorientated perpendicular to the stretch axis (Ngu et al., 2010). On the contrary, relief of tension, obtained by laser severing, induces zyxin dissociations from the FAs, and consequently their deconstruction (Hansen & Mullins, 2010).

1.1.3. Zyxin role in the nucleus

As said above, zyxin role is not limited to its presence in the cell cytosol. Unlike others FAs proteins, zyxin shares with its family members the capability to shuttle from cytosol to nucleus and back again. This feature is confirmed by both structure and experimental observations. First, zyxin has two NES domains, which permit to the protein to be transported outside the nucleus by the Chromosomal Maintenance 1 (Crm-1), a protein involved in the nuclear trafficking of proteins with NES sequences. Accordingly, zyxin nuclear accumulation is observed after treatment with leptomycin B, an inhibitor of Crm-1 (Hervy et al., 2006; Nix & Beckerle, 1997). Moreover, zyxin persistence in the nucleus is obtained also through mutations or deletions of the NES domains (Huang et al., 2000). The mechanism that allows zyxin transit into the nucleus is still unknown. Zyxin chemical and structural characteristic do not permit its passive diffusion through the nuclear envelope (X. H. Zhao et al., 2007) and to date, no nuclear import signal (NLS) has been found in zyxin structure. These observations suggest that zyxin enters the nucleus in association with other proteins that have a NLS or that zyxin holds a unique import mechanism. Even if the mechanism is still not clear, the nuclear accumulation is evident in various and different physiological and pathological landscape.

Consistently with is role in mechanosensing, application of mechanical stress induces zyxin to transiently shift into the nucleus (Y. X. Wang et al., 2019). Cyclic strain, mimicking physiological and pathological conditions such hypertension, applied *in vitro* to vascular smooth muscle cells

(VSMCs) and endothelial cells induces zyxin to move from FAs to the nucleus, where zyxin affects the expression of gene involved in the regulation of mechanical response (Cattaruzza et al., 2004; Wójtowicz et al., 2010). Stretch forces applied to VSMCs induce release of endothelin-1, a vasoconstrictor, whose autocrine binding to its B-type receptor in turn stimulates the release of the atrial natriuretic peptide (ANP). ANP, also in autocrine manner, activates its receptor, the guanylyl cyclase A (GC-A) which through activation of the protein kinase G pathway causes phosphorylation of zyxin at serine 142. This phosphorylation leads zyxin to shift into the nucleus (Babu et al., 2012). Inhibition of zyxin expression in VMSCs promotes cell growth and migration while hindering apoptosis and cell contraction, a phenotype exasperated by contemporary application of mechanical stress. Remarkably, microarray analysis in the same condition proves that zyxin impacts the expression of 90% of the total stretch response genes (Ghosh et al., 2015). Accordingly, microarray analysis performed in endothelial cells under cyclic stretch again shows that zyxin affects the expression of genes involved in mechanical expression (Wójtowicz et al., 2010). Moreover, Chromatin Immunoprecipitation allowed to see that zyxin can bind directly the promoters of a subset of these gene, such as IL-8, VM-1, Hey-1. Zyxin recognizes, probable through its zinc-finger domains, a pyrimidines sequence (PyPu Box) shared between these promoters (Wójtowicz et al., 2010).

The nuclear shift of zyxin is also been associated to other physiological stimulus, which leads to its interaction to various partner, such Akt, Acinus-S, SIRT-1, HNFB-1, Gli1, allowing the control of more pathway in addition to those strictly mechanical-related.

Zyxin can regulate cell survivals and apoptosis in different manners. Treatment with ANP on cardiomyocytes *in vitro* or of mice myocardium *in vivo* stimulates production of high levels of cGMP that induce nuclear accumulation of both Akt kinase and zyxin (Kato et al., 2005). Once in the nucleus, Akt binds and phosphorylates zyxin at serine 142, allowing it to interact directly with acinus-s, a nuclear protein regulating apoptosis. After cleavage by caspases, acinus-s causes chromatin condensation promoting apoptosis, an effect inhibited by zyxin binding (Chan et al., 2007; Kato et al., 2005). In addition to ANP, other cardioprotective agents depending on PKG signals such as IGF-1, Adrenomedullin and Estradiol, have similar effects on Akt and zyxin accumulation, with slightly differences for kinetic and dose-responding. Confirming the PKG dependency, treatment with the PKG inhibitor KT5823 completely destroy any effects on zyxin sub cellular localization(Chan et al., 2007; Kato et al., 2005).

Contrary at its pro-survival role, zyxin act also as an important regulator of UV-induced apoptosis (Hervy et al., 2010). UV irradiation on fibroblast induces zyxin phosphorylation and its nuclear accumulation. In the nucleus zyxin binds the Cell Cycle and Apoptosis Regulator Protein-1 (CARP-1), promoting apoptosis (Hervy et al., 2010). Accordingly, zyxin-null fibroblasts are less responsive to the UV-induced apoptosis.

As said above, treatment with leptomycin B blocks zyxin in the nucleus (REF). In COS-7 cells, zyxin nuclear accumulation leads to interaction between zyxin and SIRT1 (Fujita et al., 2009), a deacetylase targeting various protein involved in the regulation of several cell processes, such as cell survival and differentiation (Choupani et al., 2018). SIRT1 actively deacetylase nuclear zyxin, an event suggesting that SIRT1 modulates EMT through zyxin alteration (Fujita et al., 2009).

Apart of these partner, zyxin can also interact with the transcriptional factors Zinc Finger protein 384 (ZNF384), Hepatocyte nuclear factor β1 (HNF-β1) and Glioma associated oncogene 1 (Gli1), modifying their activity (Y. H. Choi et al., 2013; Janssen & Marynen, 2006; N. Y. Martynova et al., 2018).

ZNF384, similar to zyxin, is a focal adhesion protein that can translocate into the nucleus, involved in the modulation of osteoblast differentiation (Yamamoto et al., 2019). By yeast two-hybrid technology zyxin was identified as a partner of ZNF384. Because in rat ZNF384 also interacts with p130Cas, a feature not proved in human, while zyxin can bind it, it has been suggested that zyxin acts as a mediator between ZNF384 and p130Cas interaction (Janssen & Marynen, 2006).

In renal epithelial cells treatment with Epidermal Growth Factor (EGF) induces zyxin nuclear accumulation through Akt activation. Luciferase assays indicated that in this condition zyxin strongly up-regulates HNF-β1 activity by direct binding to this transcriptional factor on his target promoters, as confirmed by immunoprecipitation and re-Chip. EGF stimulation ultimately promotes cell migration, but this effect is completely lost with HNF-β1 or zyxin knockdown, confirming the importance of the interaction (Y. H. Choi et al., 2013).

In *Xenopus laevis* embryogenesis zyxin modulates the signaling of Sonic Hedgehog signaling cascade (Shh) to allow the correct development of the central neural system. Zyxin regulates Shh through interaction with one of its most important effectors, the transcriptional factor Gli1. In *X. Laevis* cells zyxin colocalizes and co-immunoprecipitates with Gli1. The interaction causes a reduction of activity of Gli1, as confirmed both by luciferase assay and by expression of its target genes such as FOXOA, PTCH2 and NKX2-2. Accordingly, loss of function of zyxin increases Gli1 activity, while zyxin overexpression inhibits it (Natalia Y. Martynova et al., 2013).

1.1.4. Zyxin in the regulation of malignancies

All the previous information describes zyxin as a key protein in the regulation of fundamental cellular processes, which are extremely important not only in physiological but also in pathological conditions, as example in cancer development. Consequently, it is not surprising that zyxin is involved in the tumorigenesis of various types of cancer.

Immunohistochemical analysis of 84 tumor samples of different grades gliomas found out a net increase of zyxin expression in Glioblastoma multiforme (GMB), the most aggressive stage (Wen et al., 2020). In addition, studies conducted on different database prove that glioma patients with higher zyxin expression have a shorter overall survival when confronted to patients with low zyxin expression (Wen et al., 2020). Zyxin knockdown altered the expression of 708 genes, that by Gene Ontology analysis were enriched in gene cluster involved in cell invasion and migration. Accordingly, zyxin knockdown inhibits GMB migration and invasion both *in vivo* and in *vitro* (Wen et al., 2020). Zyxin is probably the downstream effector of well-known protein involved in GMB malignancy (Semir et al., 2020; Zhao et al., 2016). PI3K/Akt and JNK pathway are hyperactivated in GMB, promoting cell migration, survival and invasion. The blockage of both pathway has synergistic inhibitory effects on survival and migration, an action that pass through phosphitylation of zyxin (H. F. Zhao et al., 2016). Moreover, downregulation of PHIP causes a dramatic decrease in cell migration and growth, but the effect is partially reversed by zyxin overexpression (Semir et al., 2020).

In colorectal cancer (CRC) zyxin higher expression is associated with metastatic disease and shorter relapse free survival (Zhong et al., 2019). Experiments in vivo and in vitro shown that downregulation of zyxin impairs proliferation and metastasis capability, while its over-expression had opposite effects (Zhong et al., 2019). Explanations of the zyxin role come from different work. During mitosis in CRC cells CDK1 phosphorylates zyxin, activating it. With a still unknow mechanism, this event cause phosphorylation of CDK8 which in turn phosphorylates YAP1. Phosphorylated YAP1 transit in the nucleus promoting cell growth and invasion (Zhou et al., 2018).

Analyzing 52 cases of Hepatocellular carcinoma and the related non-malignant tissue, zyxin expression was found particularly high in 33% of the tumors. Zyxin mRNA levels were also higher in multifocal disease compared to solitary lesions. Finally, patients with higher zyxin expression are more likely to have recurrence after resection. Silencing zyxin in Hep38 cell line decrease cell migration and invasion *in vitro*, confirming the tumor samples data (Sy et al., 2006).

LIM and SH3 protein 1 (LASP-1) promotes cell migration and survival in ovarian and breast cancer. Silencing of LASP1 in ovarian and breast cancer cell lines inhibits zyxin binding to focal adhesion,

suggesting that LASP1 absolves to its oncogenic role by influencing zyxin sub cellular localization (T. G.P. Grunewald et al., 2007; Thomas G.P. Grunewald et al., 2006). Analysis of tissue microarray shows that zyxin is highly or moderated expressed in 70.7% of the examined tumors sample and only in 5.9% of normal tissue, indicating a correlation between zyxin expression and tumor development in breast cancer (B. Ma et al., 2016). Moreover, zyxin immunohistochemical intensity correlates with histological stage and lymph node metastasis (B. Ma et al., 2016). In breast cancer zyxin is at the center of a crosstalk between TGFβ pathway and the Hyppo pathway, ultimately promoting cell growth, migration and epithelial mesenchymal transition (EMT). Hypoxia or TGFβ stimulus induce zyxin to create a ternary complex with Lats2 and Siah2, which causes rapid ubiquitination and degradation of Lats2. Consequently, YAP1 is dephosphorylated and shift into the nucleus, where by interaction with Teads transcriptional factor promotes tumor progression through transcriptional regulation (B. Ma et al., 2016). Interestingly, zyxin is also an upregulated target of Tead2 (Diepenbruck et al., 2014). Accordingly, zyxin is required for the oncogenic phenotype promoted by TGFβ stimulus, hypoxia or Tead2 nuclear activity, and zyxin silencing severely impairs cell survival cell migration, proliferation and tumorigenesis in a xenograft model (Diepenbruck et al., 2014; B. Ma et al., 2016).

Zyxin is over-expressed in melanoma cell lines in confront of melanocytes cell lines (Van der Gaag et al., 2002). Silencing of Wilms' tumor suppressor WT1 causes a reduction of zyxin expression and a decrease in tumor growth (Wagner et al., 2008). Peroxisome proliferator-activated receptor beta (PPARβ) is a ligand-activated transcriptional factor that can repress WT1 transcription by direct binding to its promoter. Pharmacological activation of PPARβ decrease WT1 expression and consequently zyxin expression (Michiels et al., 2010). Zyxin expression directly correlates with cell growth and spreading, while inversely correlates with cell differentiation (Van der Gaag et al., 2002).

Analysis by tissue microarray on 173 patients affected by bladder transitional cell carcinoma demonstrated that zyxin is scarcely expressed in more advanced tumor grade and stage.

Finally, in Ewing sarcoma (EWS) zyxin acts as a tumor suppressor; EWS-FLI1, the main oncogenic driver of EWS, upregulates the transcriptional factor NKX2-2 that, in turn, repress zyxin expression (Amsellem et al., 2005; Chaturvedi et al., 2014; Fadul et al., 2015). Consequently, zyxin in EWS cell lines is scarcely expressed and is distributed diffusively in the cytosol, rather than localizes at the FAs and SFs (Amsellem et al., 2005). Zyxin forced re-expression induces novel organization of the actin cytoskeleton, decreases cell motility and anchorage-independent growth and impairs tumor formation in athymic mice (Amsellem et al., 2005; Chaturvedi et al., 2014; Fadul et al., 2015). Moreover, zyxin

is up-regulated after the engagement with monoclonal antibodies of CD99, another oncogenic protein in EWS, and its presence is required for the massive cell death caused by the this treatment (Cerisano et al., 2004).

1.2. Ewing sarcoma

1.2.1. Classification

Ewing sarcoma (EWS) is an aggressive tumor of the bone (Grünewald et al., 2018), first identified by James Ewing as a "diffuse endothelioma of bone" (Ewing, 1921). EWS features a poorly differentiated phenotype with small round cells, a trait shared with other childhood tumors that had challenged the differential diagnosis until the discovery of the specific genetic aberration of EWS.

Before 2013 the World Health Organization (WHO) classified EWS as a member of the Ewing's sarcoma family tumors (ESFT), along with Askin tumor, peripheral primitive neuroectodermal tumor (PNET), osseus and extra-skeletal Ewing sarcoma (De Alava & Gerald, 2000; Kovar, 1998). Afterward, all these neoplasms have been grouped under the name "Ewing sarcoma", which main feature is the presence of a pathognomonic FET-ETS chimeric gene fusion (Doyle, 2014; Savita Sankar & Lessnick, 2011; S. Watson et al., 2018). The WHO also indicated a new category, "Ewing-like tumors", comprehending small round cell sarcomas having similar morphological phenotype to EWS but different chromosomic aberration, such as BCOR-rearranged sarcomas and CIC-fused sarcomas (Sbaraglia et al., 2020).

1.2.2. Epidemiology

After osteosarcoma, EWS is the second most frequent bone tumor in children, adolescents and young adults, with a peak of incidence at the age of 15 years. Nevertheless, EWS is a rare tumor with an incidence rates of 3.3 cases per million every year (Cotterill et al., 2000; Herzog, 2005). To this date is still not clear the cause for the imbalance of incidence between boys and girls, that stands at a ratio of 3:2 (Jawad et al., 2009).

Ethnicity plays a crucial role in the risk of being affected by EWS. Children and young adults of Africa and Asian origins have an incidence rate of 0.8 and 0.2 per million respectively, much lower than the 1.5 in their Caucasian counterpart. These well-established epidemiologically data indicate that people with European origin probably have a genetic predisposition to develop the EWS (Jawad et al., 2009; Worch et al., 2011).

Extensive researches have been conducted on environmental and familiar context but, apart of ethnicity and sex, no other risk factors have been identified (Ludwig, 2008). Limited studies seem

indicate that EWS patients present above average congenital mesenchymal defects (Nakissa et al., 1985) and in relatives of EWS patients there is a slightly higher risk to develop stomach cancer and neuroectodermal tumor (Novakovic et al., 1994).

1.2.3. Localization and histopathology

EWS mainly affected the diaphysis of the long bones, particularly femur and tibia, and pelvis, as reported in Figure 2; less frequently can origin in soft tissues such as kidney, bladder and prostate (Paulussen et al., 2001; Rushing et al., 2012). Lungs (50%), bones (25%) and bone marrow (20%) are usually the site of primary metastasis development (Bernstein et al., 2006).

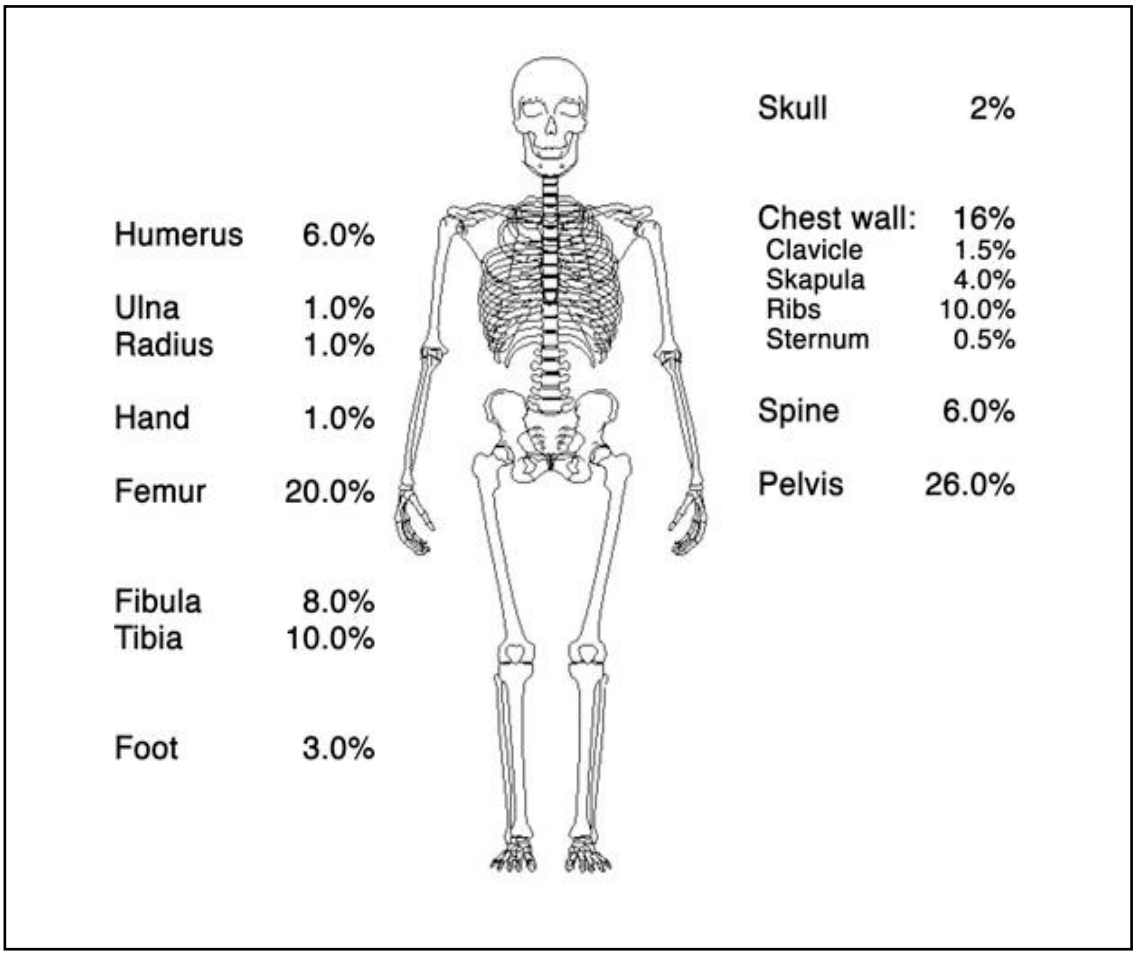


Figure 2. Primary tumor sites in EWS. Data based on 1,426 patients from European Intergroup Cooperative Ewing Sarcoma Studies trials. Abbreviation: BM, bone marrow**.** Adapted from Bernstein M et al, the Oncologist, 2006.

Local pain at the tumor site, often intermittent and with variable intensity, is the prominent symptom of EWS. Sometimes the patients display unspecific symptoms, like asthenia and fever. Moreover,

site-peculiar symptoms may be present. Considering the challenge of distinguishing EWS from other pathologies, the diagnosis requires a radiography of the suspect region followed by computerized tomography or scintigraphy and finally a biopsy for the immunohistochemistry validation (Bernstein et al., 2006; Paulussen et al., 2001).

Histologically, EWS is a monomorphic tumor, with little round cells scarcely differentiated. The cells, organized in monolayer, have few cytoplasm and blue nucleus at hematoxylin-eosin staining (Figure 3).

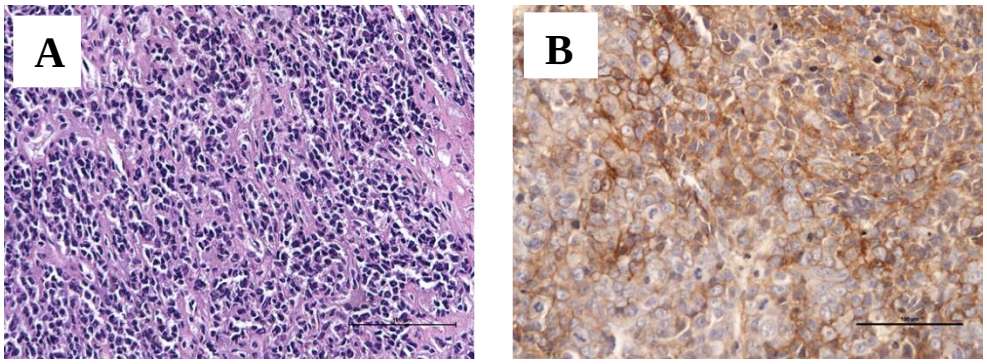


Figure 3. Histologic appearance of EWS. (**A**) hematoxylin-eosin stained section. Lobular appearance with small undifferentiated cells; (**B**) immunohistochemistry analysis of the antigen CD99. (magnification 10x)

The immunohistochemistry analysis of EWS-specific antigens on the biopsy is strictly required for the differential diagnosis of EWS from other small cell tumors like rhabdomyosarcoma and lymphoma. The routinely evaluated diagnostic markers of EWS are the antigen CD99, which is expressed at high levels in more than 90% of EWS cases (Figure 3B) (Shibuya et al., 2014), Caveolin 1, extremely useful in CD99 negative EWS, NKX2-2 and FLI1, whose reliability is conditioned by the type of chromosomal rearrangement (Zambo & Veselý, 2014).

The diagnosis must be confirmed by molecular analysis, which relies on Reverse Transcription PCR (RT-PCR), Fluorescent in situ hybridization (FISH) or cytogenic analysis of the karyotype, to identify the typical chromosomal translocation of EWS (Sorensen et al., 1993).

After the diagnosis, the assessment of the tumor stage and grade is crucial for the prognosis, and consequently for the application of the correct therapeutic protocol.

1.2.4. Course and therapy

Unfortunately, EWS is a highly aggressive tumor that can easily develop metastasis, factors that make this tumor extremely dangerous and hardly manageable. Patients with localized disease have an overall survival at 5 years of 70-80%, which is below 30% in patients with metastasis (Gaspar et al., 2015).The following are the most relevant of several elements capable of influencing the prognosis:

- **Tumor location**: Patients with primary tumor in the distal extremities have a better prognosis than patients with primary tumor at pelvis (Gaspar et al., 2015)
- **Tumor dimension**: Tumors of greater dimension are associated with a lower overall survival rate (Paulussen et al., 2009)
- **Age**: Younger patients have a better prognosis (Paulussen et al., 2009)
- **Metastasis**: presence of metastasis is the main negative prognostic factor, but in addition there are significative differences related to their localization: patients with lung metastasis have a better prognosis in confront of patients with metastasis in other sites (Gaspar et al., 2015)
- **Sex**: female patients have a more favorable prognosis (Ferrari et al., 2007)
- **Lymph nodes**: the involvement of lymph nodes is associated with a poor prognosis (Bernstein et al., 2006)
- **Status of p53**: Loss of function mutations in *TP53* are a negative prognostic factor (López-Guerrero et al., 2001)
- **Lactate dehydrogenase (LDH) level**: High concentration of LDH in the serum is correlated with a negative prognosis (Bacci et al., 1999)

Thanks to the evolution of EWS therapy in the last decades, patients affected by this disease now have significantly better chances of survival and enhanced quality of life.

The standard approach of new diagnosed EWS is a combination of chemotherapy and surgery or, when the latter is not available, radiotherapy. Cycles of systemic chemotherapy are administered before and after the surgery. Usually, in localized disease, neo-adjuvant chemotherapy is dispensed to arrest tumor growth and consequently simplify and facilitates the following surgery/radiotherapy. During the treatment the patient reaction to chemotherapy is strictly observed for the correct administration of adjuvant chemotherapy and evaluation of the clinical response. The standard chemotherapy applied in EWS cases are: vincristine, actinomycin D, doxorubicin, cyclophosphamide, ifosfamide and etoposide. Interestingly, the combination of actinomycin D, doxorubicin, vincristine and cyclophosphamide has higher therapeutic efficacy than the combination of three drugs or the use of the single drug alone (Bernstein et al., 2006; Meyers & Levy, 2000).

Patients treated with chemotherapy plus radiotherapy have a significantly higher (20%) probability of tumor recurrence in comparison to patients treated with chemotherapy plus surgery. For the treatment of patients with metastasis, whose prognosis remains poor, recent studies have suggested the treatment with chemotherapy at high dose and more frequent cycles, and transplantation of autologous stem cells. Patients with metastatic disease, classified as at elevated risk, treated with high dose chemotherapy plus surgery/radiotherapy have an overall survival of 30% (Ladenstein et al., 2010). The transplantation of autologous stem cells in patients with lung and bones metastasis has an overall survival rate of 50% (Luksch et al., 2012). Despite the promising results, these therapeutic strategies cause intense side effects, requiring further advancement for the treatment of patient affected by recurrence or metastatic disease.

1.2.5. Genomic and molecular characteristics

Historically sarcoma have been categorized in two major group according to cytogenetical features: one group comprehends tumors with complex karyotype and many genomic alterations and the other group tumors with simple karyotype and few chromosomal aberrations (Grünewald et al., 2018; Helman & Meltzer, 2003). EWS belongs to this second group, due to the presence of a specific and balanced chromosome translocation involving the *EWSR1* gene, on chromosome 22, and a member of the ETS transcription factor family (Helman & Meltzer, 2003; Turc-Carel et al., 1983). The genic product of *EWSR1* is EWS, a protein of the FET family whose members shared a common structure: the N-terminal includes a transcriptional domain while the C-terminal bears a RNA-binding domain. The ETS family member have a transcriptional domain at the N terminal and a DNA-binding domain at C-terminal. The translocation in EWS generates an aberrant transcriptional factor with the transactivation domain of EWS and the DNA-binding domain of an ETS family member (Ross et al., 2013; Sharrocks, 2001). By far, the most common translocation is t(11;22)(q24;q12), which originates the protein EWS-FLI1, accounting for 85% of all EWS cases. Another fairly frequent chimeric product is EWS-ERG, expressed in 10-15% of cases and generated by the translocation t(21;12)(22;12). The remain cases present other translocations involving the *EWSR1* gene and one of the ETS transcription factors, such as FEV, ETV1, ETV4 or, in extremely rare cases, *EWSR1* is replaced by *FUS,* another member of the FET family (Ross et al., 2013; Savita Sankar & Lessnick, 2011) (Table 1).

Translocation	Fusion gene	Frequency (%)
t(11;22)(q24;q12)	*EWSR1-FLI1*	≈ 85
t(21;22)(q22;q12)	*EWSR1-ERG*	≈ 10
t(7;22)(p22;q12)	*EWSR1-ETV1*	<1
t(17;22)(q21;q12)	*EWSR1-ETV4*	<1
t(2;22)(q33;q12)	*EWSR1-FEV*	<1
t(16;21)(p11;q22)	*FUS–ERG*	<1
t(2;16)(q35;p11)	*FUS–FEV*	<1

Table 1. Reported translocations between FET and ETS in Ewing sarcoma

EWS-FLI1 is a crucial element int tumor development of EWS, a role discovered since its first cloning experiment (Delattre et al., 1992; Jedlicka, 2010; Lessnick & Ladanyi, 2012; Toomey et al., 2010). Accordingly, its forced expression induces transformation of the murine fibroblast cell lines C3H10T1/2 (González et al., 2007) and NIH3T3 (May et al., 1993), it is required for cell growth and tumor progression of EWS cells (Delattre et al., 1992; Jedlicka, 2010; Lessnick & Ladanyi, 2012; Toomey et al., 2010), and the impairment of EWS-FLI1 promotes apoptosis and inhibits tumor growth both *in vitro* and *in vivo* (Kovar et al., 1996; Lambert et al., 2000).

EWS-FLI have more than one thousand target genes both up-regulated and down-regulated, with the latter that are around 80% of the total (Toomey et al., 2010). In addition, several studies indicate that EWS-FLI1 modulates the expression of a great variety of micro-RNAs and long non condign RNAs. Interestingly, the chimera is able to modify gene expression at (i) epigenetic, (ii) transcriptional and (iii) post-transcriptional level (Sand et al., 2015; Toomey et al., 2010).

(i) EWS-FLI1 interacts with several proteins involved in the remodeling of the chromatin, such as CBP/p300, a histone acetylase, the Lysine Specific Demethylase 1 (LSD1) and the Nucleosome Remodeling Deacetylase (NuRD) complex. Through this partners, EWS-FLI1 modulates the accessibility of specific chromatin regions to others transcriptional factors, promoting or repressing their transcriptional activity and, consequently, inducing or inhibiting the expression of precise target genes. This aberrant action is probably due to the capability of the chimeric protein EWS-FLI1 to bind DNA sequences that are not target of FLI1, like the GGAA sequence (Guillon et al., 2009). Indeed, the presence of two GGAA sequences nearby recruits two molecules of EWS-FLI1, that in these status bind LSD1 and CBP/p300. Then, the histone tails are demethylated and acetylated by

LSD1 and CBP/p300, facilitating chromatin accessibility. In the repressions mode, the target DNA region display only one GGAA site that attracts just one molecule of EWS-FLI1, which is insufficient to activate LSD1 and CBP/p300 but recruits the NuRD complex; the latter contains the enzymes Histone Deacetylase 1 and 2 (HDAC1/2) that promote the deacetylation of histones leading to chromatin condensation (Ramakrishnan et al., 2004; Riggi et al., 2014; Sand et al., 2015; S. Sankar et al., 2013).

(ii) The normal EWS protein has a stable interaction with RNA polymerase II and with the transcription Factor II D (TFIID), both members of the basal transcription complex (Tan & Manley, 2009). Instead, with the chimeric product EWS-FLI1, these relations are lost, but the capability of transcriptional modulation are maintained because EWS-FLI1 is able to bind, even if weakly, the sub unit RPB7 of the RNA polymerase II and the RNA helicase (Bertolotti et al., 1998; Petermann et al., 1998; Toretsky et al., 2006). Moreover, EWS-FLI1 interacts directly with several transcriptional factors, such as E2F, the Specificity Protein 1 (SP-1), NRoB1, and the dimer Jun-Fos (Fuchs et al., 2004; Kim et al., 2015; Kinsey et al., 2009; Schwentner et al., 2015).

(iii) EWS-FLI1 is able to join the spliceosome complex and consequently regulates the post-transcriptional activity. Indeed, EWS-FLI1 can bind directly the splicing factor Small Nuclear Ribonucleoprotein-Specific U1C (snRNP U1C) and indirectly TLS-Associated SR and YBOX, all members of the spliceosome (Chansky et al., 2001; Erkizan et al., 2010; Knoop & Baker, 2001; Yang et al., 2000).

Even if EWS-FLI1 is the main oncogenic driver of EWS, numerous studies indicate that its presence is essential but not sufficient for the malignant phenotype. Forced expression of EWS-FLI1 in mesenchymal stem cell, putative cell of origin of EWS, does not cause transformation (Jedlicka, 2010; Toomey et al., 2010) and it is impossible to obtain a transgenic mouse model of EWS with only EWS-FLI1 (Jacques et al., 2018; Jedlicka, 2010).

These observations demonstrate that EWS-FLI1 is able to induce transformation only in a specific biological context, which is sustained by the involvement of other factors. Particularly, is well established the requirement of CD99, a 32 KDa glycoprotein, and the activity of the Insulin-like growth factor (IGF) system. CD99 is highly expressed in EWS and it is essential for the maintenance of malignity by promoting cell growth, migration and inhibiting cell; as already said, CD99 is an important diagnostic biomarker of EWS (Manara et al., 2018; Pasello et al., 2018). For the IGF1 system, EWS-FLI1 promotes the transcription of several member of the pathway resulting in high expression of IGF1 and its receptor IGF1-R, leading to an autocrine and paracrine effect which

promotes the malignant phenotype (Mancarella & Scotlandi, 2018). Interestingly, EWS-FLI1 increases IGF1 expression by direct binding to its promoter (Herrero-Martín et al., 2009), and by sustaining the expression of pappalysin-1 (PAPPA), a protease that degrades the IGF binding protein, and consequently promotes IGF1 bioavailability (Jayabal et al., 2017).

1.3. CD99

1.3.1. General features

CD99 is a 32 kDa protein of the membrane encoded by *MIC2*, a gene localized on the pseudoautosomal regions of human chromosomes X and Y (Banting et al., 1989; Goodfellow et al., 1986).

Until know three CD99-related genes have been identified in the human genome, whose similarity to CD99 sequence is due to multiple duplications of a common ancestral gene: PDBX, encoding for the antigen of erythrocytes Xga (Ellis et al., 1994), CD99 antigen-like 1 and CD99 antigen-like 2 (Suh et al., 2003).

Through alternative splicing two isoforms of CD99 are originated (Figure 4): the full-length wild type (CD99 type 1 or CD99wt) and the truncated form (CD99 type 2 or CD99sh). CD99wt (32 kDa), accounting for 185 aminoacids, contains an extracellular domain, a transmembrane domain and a cytosolic domain. Because of the insertion of in-frame stop codon between exons 8 and 9, CD99sh (28 kDa) lacks the cytosolic portion (J. H. Hahn et al., 1997).

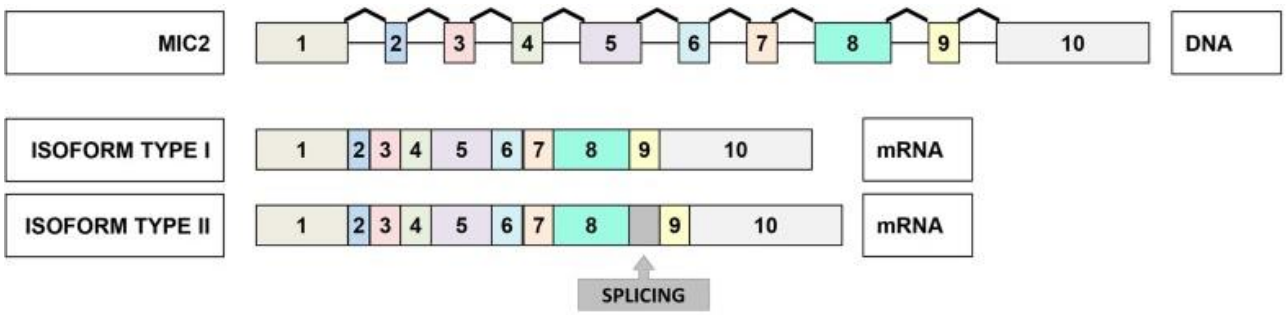


Figure 4. Molecular structure of the MIC2 gene and of its two alternative transcripts. Reprinted with permission from Pasello et al., 2018.

CD99 is heavily glycosylated, with the carbohydrate element making up to 14 kDa of the total molecular weight. Interestingly, all the glycosylation sites are O-linked and there are no evidences, nor prediction, of the presence of N-links (Aubrit et al., 1989; Gelin et al., 1989).

CD99wt and CD99sh are able to dimerize already in the Golgi apparatus then, after exportation to the cell surfaces, the complex act as receptor (M. K. Lee et al., 2008). Depending on the cell type, the

two isoforms are differently expressed and their functions are distinct (Alberti et al., 2002; Byun et al., 2006; Scotlandi et al., 2007). In T cells the simultaneous expression of the two isoforms is necessary to start T cell death, but the forced expression of CD99wt is sufficient to induce cell adhesion (G Bernard et al., 1997). In B cells their presence have opposite effects: CD99wt promotes cell-cell adhesion, while CD99sh impairs homotypic adhesion (J. H. Hahn et al., 1997).

1.3.2 CD99 in cell biology

CD99 expression is extremely various and its higher levels can be found in pancreatic islet cells, keratinocytes, cortical thymocytes, Sertoli cells, endothelial cells, ovarian granulosa cells, ependymal cells, stromal lymphocytes, hemopoietic cells, particularly in the cells of the immature thymic T-lineage and in the progenitors cells of tonsillar lymphoid (Banting et al., 1989; G. Choi et al., 2016; Dworzak et al., 1994; Edlund et al., 2012; Gelin et al., 1989; Levy et al., 1979).

CD99 is involved in the regulation of several processes: apoptosis and cell adhesion (G. Bernard et al., 1995; G Bernard et al., 1997; Cerisano et al., 2004; Husak et al., 2010), lymphocytes diapedesis (Dufour et al., 2008; R. L. Watson et al., 2015), modulation of intracellular membrane protein trafficking (Brémond et al., 2009; Yoon et al., 2003) and T cell differentiation (G. Bernard et al., 1995; G Bernard et al., 1997). Therefore, is evident the relevance of CD99 in the setting of neural and hemopoietic progenitor cells differentiation and in the peripheral immune response.

Even if is established that the two CD99 isoforms have different functions, still there are few information about how they regulate the CD99-related signaling pathway.

T cells express on the cell surface CD99wt alone or the heterodimer containing both the two isoforms (J. H. Hahn et al., 1997). Interestingly, peripheral T cells and single-positive thymocytes express only CD99wt, while double-positive thymocytes, as well immature T cell, express the heterodimer (Alberti et al., 2002)

In Jurkat T cells the presence of the two isoforms is mandatory to achieve apoptosis, while they can individually influence adhesion, even if CD99wt only can modulate the actin cytoskeleton (Alberti et al., 2002). When CD99wt and CD99sh are simultaneously expressed, they are organized in heterodimer localized inside glycosphingolipid rafts, which promotes sphingomyelin degradation; this sub-cellular distribution is necessary for the induction of apoptosis, as proved by forced cholesterol depletion (Alberti et al., 2002). In B cells the two isoforms oppositely modulate the

expression of the cell adhesion molecule LFA-1, promoting or decreasing homotypic aggregation (J. H. Hahn et al., 1997).

Despite this works, the major part or our knowledge is related to CD99wt. Interestingly, still have not been identified any natural circulating ligands of CD99, suggesting that CD99 creates homophylic interactions with other CD99 molecules on the surface of different cells (Schenkel et al., 2002). Consequently, almost all the information about its signaling pathway in literature is based on the use of murine monoclonal antibodies (DN16, 12E7, O13, F21, 0662) that engage CD99 (Jung et al., 2003).

1.3.3 CD99 in lymphocytes development

CD99 is extremely relevant in lymphocytes development. Its role in the normal thymus ontogeny is suggested by the scarceness of the thymic development in CD99-deificient fetuses (Shin et al., 1999). Indeed, when positive selection of thymocytes happens, CD99 triggers homotypic aggregation and induces cell death (G. Bernard et al., 1995; G Bernard et al., 1997; Pettersen et al., 2001). Precisely, CD99 causes apoptosis of immature CD4+ CD8+ thymocytes holding a median level of CD3, without affecting others thymocytes or T cells (G Bernard et al., 1997). Interestingly, CD99 acts independently of the main signaling pathway involved in modulation of peripheral T cells and thymocytes. CD99 triggering leads to exposure of phosphatidylserine on the cell surface of immature thymocytes (Aussel et al., 1993; E. Y. Choi et al., 1998), but the following apoptosis can be classical or non-classical, and the determination of which depends on the CD99 domain activated by the different mAbs used in a still unexplained manner (Aussel et al., 1993; Pettersen et al., 2001). Moreover, in thymocytes CD99 up-regulates the mobilization of MHC class I and II and TCR on the cell membrane from the cytosol, enhancing the chances of positive selection (E. Y. Choi et al., 1998; M. J. Hahn et al., 2000; Sohn et al., 2001), in apparent contradiction with its role in the stimulation of cell death.

CD99 seems also involved in the activation of T cell: triggering of CD99 with mAbs stimulates the expression of T cell activation marker, increases intracellular levels of CA2+ and promotes tyrosine phosphorylation, inducing the activation of various mitogen-activated protein kinases (MAPK) (M. J. Hahn et al., 2000; H. J. Lee et al., 2002).

CD99wt is up-regulated during the maturation of normal B cell precursors, particularly in the most immature grade. On the opposite, CD99sh expression is low or absent (Husak et al., 2010). In

addition, in these cells the long duration (7 days) of the engagement of CD99 with mAbs promotes cell death, indicating that the protein plays a major role in B cell selection (Husak et al., 2010).

1.3.4 CD99 in cell migration and leukocyte diapedesis

Triggering of CD99 promotes the up-regulation of various adhesion molecules, such as α4β1, ELAM-1, ICAM-1, VCAM-1 (Alberti et al., 2002; Ghislaine Bernard et al., 2000; J. H. Hahn et al., 1997), that are involved in leukocyte adhesion and trans endothelial migration (TEM), a crucial element of inflammation. *In vivo* and *in vitro* studies report that the presence of CD99 is mandatory for TEM of lymphocytes, monocytes, neutrophils and CD43+ cells (Goswami et al., 2017; R. L. Watson et al., 2015; Winger et al., 2016). In this context CD99 of endothelial cells establishes homophilic interaction with CD99 on leukocytes, promoting the latter TEM via activation of protein kinase A (PKA). More precisely, PKA is recruited in complexes formed by the cytoplasmic domain of CD99, soluble adenylyl cyclase (sAC) and the A-Kinase anchoring protein ezrin, allowing PKA to shift the membrane trafficking to the sites of TEM from the compartment of lateral border recycling, and consequently favoring the diapedesis of leukocytes (R. L. Watson et al., 2015).

Moreover, the impairment of CD99 activity reduce the migration of neutrophils and monocytes into the peritoneal cavity (Dufour et al., 2008) and of T cells into the skin (Bixel et al., 2004; Goswami et al., 2017). At the cell surface CD99 is also cleaved by meprin β, a metalloprotease that starts the regulated intramembrane proteolysis of adhesion molecules, modulating the permeability of the endothelial cells (Bedau et al., 2017).

1.3.5 CD99 as modulator of osteoblastogenesis and mesenchymal differentiation

CD99 expression among mesenchymal stem cells is highly variable (Elsafadi et al., 2016; Rocchi et al., 2010; Sciandra et al., 2014). In physiological osteoblastogenesis and during osteoblast maturation CD99 expression increases, while, on the opposite, decrease in mesenchymal stem cells that differentiate toward a neural phenotype (Rocchi et al., 2010). *In vitro* CD99 is localized at the adhesion structures of osteoblastic cell cultures, while *in vivo* is along the bone surface of tissue samples (Manara et al., 2006). Another evidence that supports CD99 involvement in osteoblastogenesis came from the observation that during osteoblast differentiation CD99 levels increase (Oranger et al., 2015). Finally, the engagement of CD99 with mAbs stimulates the activation

and differentiation of osteoblasts through up-regulation of several other effectors, such as RUNX2, JUND, Collagen I and Alkaline phosphatase.

1.3.6 CD99 in Ewing sarcoma

CD99 is highly expressed in EWS and it is commonly used for its differential diagnosis from other pediatric small round cell tumors (Ambros et al., 1991; Fellinger et al., 1991). Mice transplanted with EWS cells silenced for CD99 have decreased tumor growth and bone metastasis, and the tumoral mass displays terminal neural differentiation (Kreppel et al., 2006; Rocchi et al., 2010). Several evidences support the presence of a cross-talk between the chimera EWS-FLI1 and CD99. The EWS-FLI1-induced transformation requires the presence of high levels of CD99 (Miyagawa et al., 2008; Rocchi et al., 2010), which in turn is up-regulated by EWS-FLI1; this modulation happens both directly, by binding to its promoter (Amaral et al., 2014; Rocchi et al., 2010), and indirectly, through downregulation of miRNA 30a-5p, which binds the 3'UTR of MIC2 mRNA, impairing its translation (Franzetti et al., 2013). Interestingly, knockdown of CD99 in EWS cell lines promotes phosphorylation of nuclear ERK1/2 and reduces NF-kβ and AKT activity, a series of effects that promotes neural differentiation, even in presence of EWS-FLI1 (Cheng et al., 2013; Rocchi et al., 2010; Ventura et al., 2016). These results suggest that EWS cell lines express both EWS-FLI1 and CD99 to develop neural features without losing the malignant phenotype.

The use of mAbs against CD99 in *vitro* induces elevated homotypic aggregation and massive cell death, which is independent from caspases and Fas/CD95 pathway (Cerisano et al., 2004; Scotlandi et al., 2000) while *in vivo* causes a mightily reduction of tumor growth and bone metastasis (Nanni et al., 2019; Rocchi et al., 2010).

Interestingly, the engagement of CD99 produces a decrease of the mitochondrial membrane potential, a necessary event for apoptosis; however, the use of inhibitors of calcineurin has no effects on the mAbs-induced cell death, proving that there is no involvement of the CA2+/calmodulin signaling pathway (Sohn et al., 1998).

More recently, has been observed that the treatment with mAbs against CD99 promotes p53 activity by causing Mdm2 ubiquitination; consequently, EWS cell lines with wild type p53 are more sensible to the triggering of CD99 (Guerzoni et al., 2015).

Finally, the efficiency of the engagement of CD99 in EWS cell lines relies on the rapid formation of actins stress fibers and focal adhesion; indeed, treatment with cytochalasin-D, an inhibitor of

cytoskeletal assembly, or silencing of zyxin, an important mediator of actin polymerization, completely abrogate the effects of mAbs against CD99 (Cerisano et al., 2004).

2. Aim of the study

EWS is characterized by a stable genome with hardly any alterations, relying almost exclusively on EWS-FLI1 activity to support its malignant phenotype. Among the few others crucial proteins CD99 stands out, due to its high expression and to the necessity of its presence for the EWS-FLI1-induced transformation. In this context, several researches have focused their attention on zyxin, because of its involvement in cellular processes of extremely significance for EWS. Indeed, zyxin is a major regulator of actin polymerization and cytoskeletal organization, both profoundly manipulated by EWS-FLI1, through also inhibition of zyxin. Another reason for zyxin relevance is its requirement for the apoptosis induced by CD99 triggering with specific antibodies.

Despite the attention received, zyxin mechanism of suppressing the tumorigenesis in EWS is for large part undiscovered. In fact, all the previous works offer information only about zyxin as a mere cytoskeletal regulator, without taking in account all the other roles that in recent years have been attributed to zyxin, not last its capacity to alter the gene expression by interaction with various transcription factors.

Therefore, the PhD project was built on the intention of providing an extensive and accurate knowledge of zyxin role in EWS. More precisely, the first part of the project aimed to produce a large and clear report of the functional effects of zyxin, amplifying the results of the works previously cited. With these stronger foundations, in the second part of the study we wanted to offer a mechanistic explanation for these functional effects, taking in consideration the multiple possibility of zyxin to affect various cellular processes and focusing on its relation with CD99.

3. Results

3.1. Functional role of zyxin in EWS

Previous researches have presented zyxin as a potential tumor suppressor in EWS, reporting its down-regulation by EWS-FLI1 and its involvement in some aspects of tumor progression (Amsellem et al., 2005; Chaturvedi et al., 2012, 2014; Fadul et al., 2015). Still, there are too few observations to ascribe to zyxin a well-established role in the processes that drive malignancy.

Consequently, in this first section we focused more deeply on zyxin as a sole factor in EWS, studying its function in EWS cell lines.

3.1.1. Zyxin is down-regulated in EWS cell lines

Zyxin expression was evaluated in a panel of EWS patients derived cell lines, including lines obtained from Patients-Derived Xenograft (PDX) (Nanni et al., 2019) by RT-qPCR (Figure 5A) and western blot analysis (Figure 5B). The EWS cell lines showed a wide range of zyxin levels, but their expression is severely lower when confronted to the mesenchymal cell lines (Figure 5), which are the putative cell of origin of EWS.

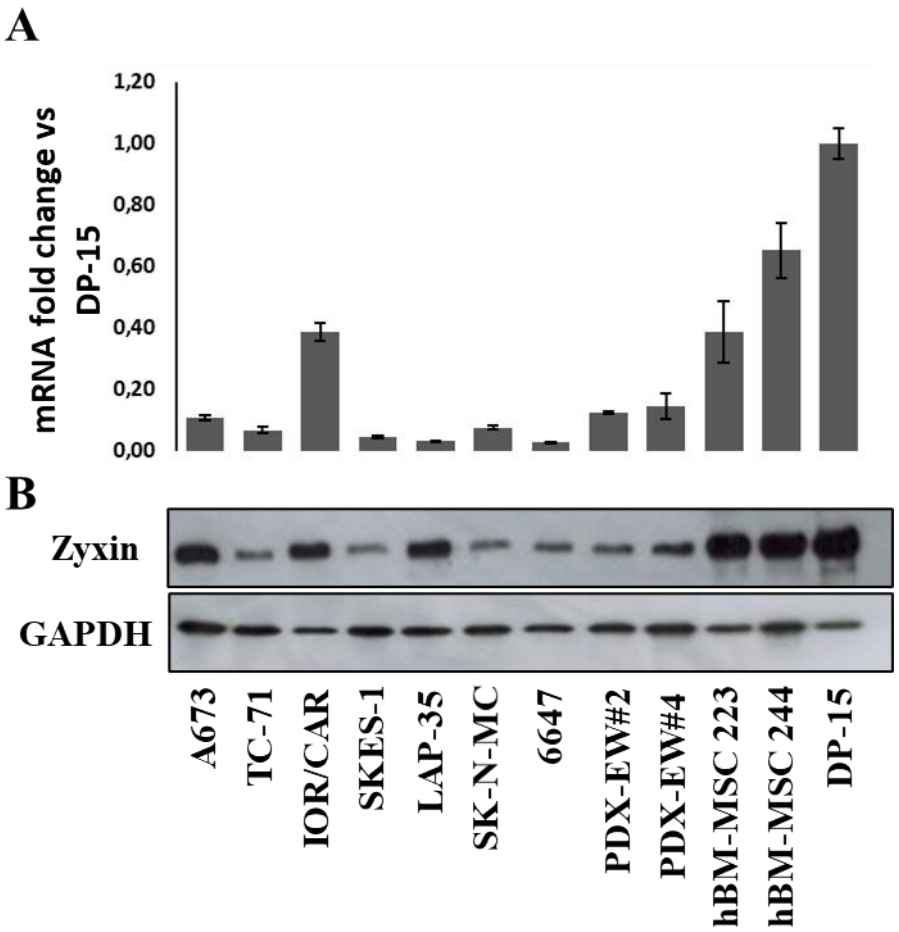


Figure 5. Zyxin is poorly expressed in EWS cell lines. Panel of 9 EWS cell lines and 3 mesenchymal cell lines1 (**A**) Zyxin gene expression was evaluated by RT-qPCR using GAPDH as housekeeping gene; the bars represent mean ± SE from at least three independent experiments (**B**) Representative western blot of zyxin, equal sample loading was monitored by blotting for GAPDH;

3.1.2. Zyxin acts as a tumor suppressor in EWS

Given the variability of zyxin expression in EWS cell lines, we searched for potential association involving zyxin and parameters of malignancy. We find a strong inverse correlation (r = -0.85, *p*= 0.0061, Spearman's test) between zyxin protein expression and migration capability, evaluated by motility assay (Table 2).

Cell lines	Relative Densitometric Unit	Migrated cells
PDX-EWS#2	**0,245±0,02**	**1453±133**
6647	**0,277±0,01**	**573±54**
TC-71	**0,294±0,02**	**75±7**
PDX-EWS#4	**0,459±0,03**	**177±8**
SK-N-MC	**0,510±0,04**	**53±7**
SK-ES-1	**0,878±0,2**	**85±10**
LAP-35	**0,936±0,08**	**23±3**
A673	**1,000±0**	**11±3**
IOR/CAR	**3,049±0,3**	**37±4**

Table 2. Zyxin expression is inversely correlated with cell migration. Zyxin expression is reported as densitometric units relative to A673 of western blot experiments. Migration was evaluated by Boyden chamber assay. The values are means of at least three independent experiments ± Standard error.

To deepen our knowledge of zyxin influence on the tumor progression we performed loss and gain of function experiments. siRNAs against zyxin were used transiently in IOR/CAR, a cell line with relatively high zyxin expression (Figure 6), and stable over-expression of zyxin was forced in the TC-71 and 6647, the cell lines with the lowest zyxin level (Figure 6). The efficacy of the silencing and over-expression was confirmed by RT-qPCR (Figure 6D) and western blot (Figure 6E). Accordingly with the correlation data, the decrease of zyxin promotes cell motility, which, on the opposite, is impaired by zyxin over-expression (Figure 6A). Augmented expression of zyxin impairs also cell survival and growth in anchorage-independent condition, assessed by agar assay; on the contrary, zyxin silencing promotes both the processes (Figure 6B, C)

These results unequivocally present zyxin as a tumor suppressor agent in ES, where it inhibits crucial processes for the tumor progression.

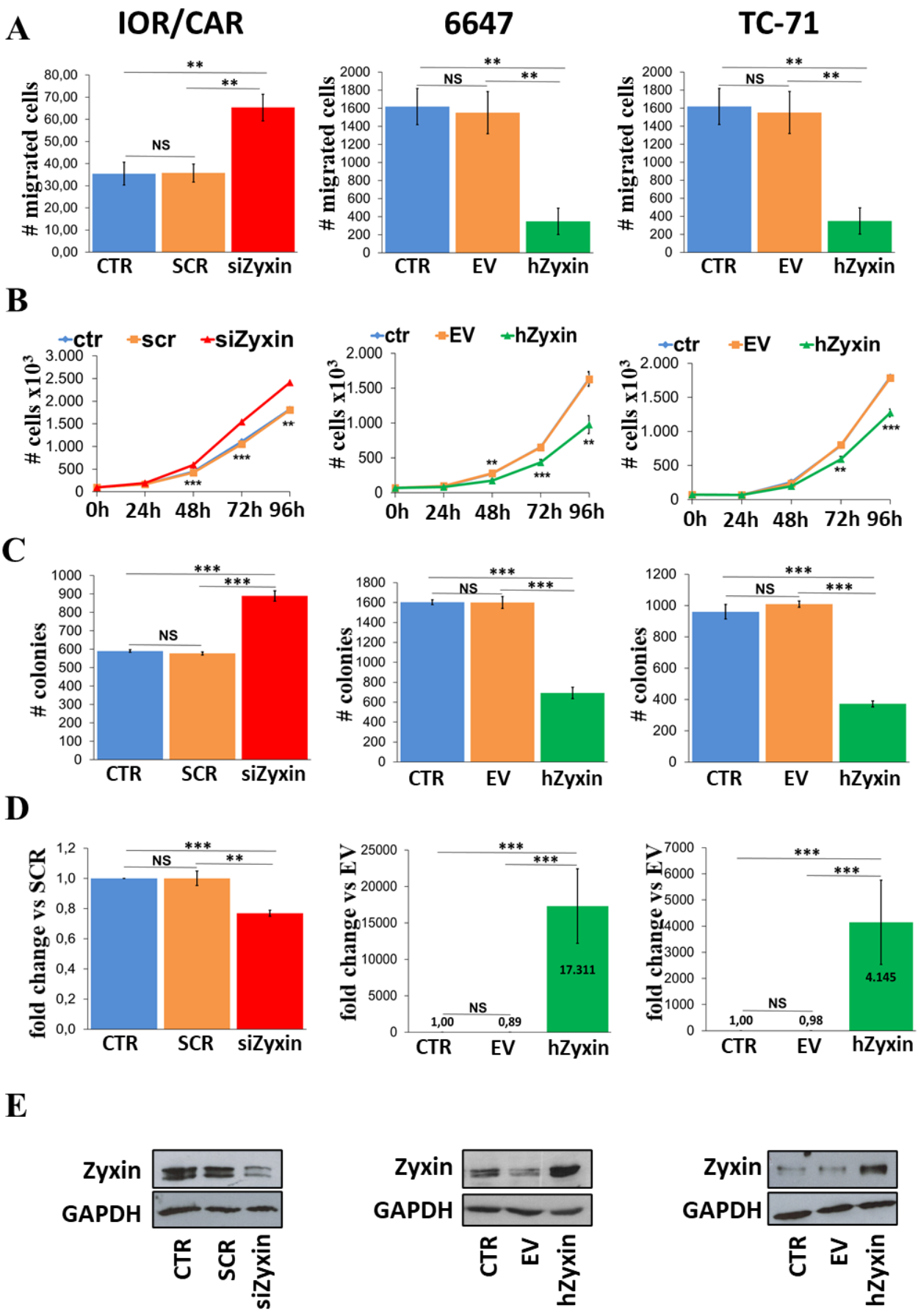

A
IOR/CAR
6647
TC-71
migrated cells
CTR
SCR
siZyxin
EV
hZyxin
NS
B
ctr
scr
siZyxin
EV
hZyxin
cells x10³
0h
24h
48h
72h
96h
C
colonies
D
fold change vs SCR
fold change vs EV
17.311
4.145
1,00
0,89
0,98
E
Zyxin
GAPDH

Figure 6. Zyxin acts a tumor suppressor in EWS. Experiments of gain and loss of function of zyxin to evaluate (**A**) Migration by Boyden Chamber assay, (**B**) Cell growth, (**C**) Number of colonies in soft agar. Zyxin silencing or over-expression was assessed by (**D**) mRNA and (**E**) protein level. Gene expression was evaluated by RT-qPCR using GAPDH as housekeeping gene. Protein expression was evaluated by western blotting; equal sample loading was monitored by blotting for GAPDH; The values are means of at least three independent experiments; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, One –way ANOVA

3.2. Zyxin as mediator of CD99

In EWS cells engagement of CD99 with the murine monoclonal antibody (mAb) 0662 causes homotypic aggregation, actin polymerization and massive cell death (Cerisano et al., 2004; Guerzoni et al., 2015; Manara et al., 2016). Zyxin is required for these effects, and its silencing severely reduces the efficacy of mAb 0662 (Cerisano et al., 2004). The role of zyxin in this cascade of signals is not clear, and we don't know if others anti-CD99 antibodies, such as the human bivalent single-chain fragment variable diabody (dAbd) C7 (Moricoli et al., 2016) required zyxin presence.

In this second section we present, through the use of several models of CD99 loss of function, an accurate examination of zyxin as mediator of CD99.

3.2.1. CD99 loss of function induces an increase of zyxin protein level

We performed a time course experiment treating the cell line 6647 with the antiCD99 mAb 0662 (3μg/mL) and afterward we evaluated the effects on zyxin mRNA (Figure 7A) and protein levels (Figure 7B).

The treatment induces a net increase of zyxin protein that reaches a peak after 3 hours and returns to normal levels, or below, after 6 hours (Figure 7B). Interestingly, no significative changes are observable on zyxin mRNA expression (Figure 7A). To validate that this modulation is related to the engaging of CD99, rather than to a mAb 0662-specific effect, we performed an analogue experiment using the dAbd C7 (200μg/mL). We obtained similar results at the same time points and again there are no significative effects on mRNA (Figure 7)

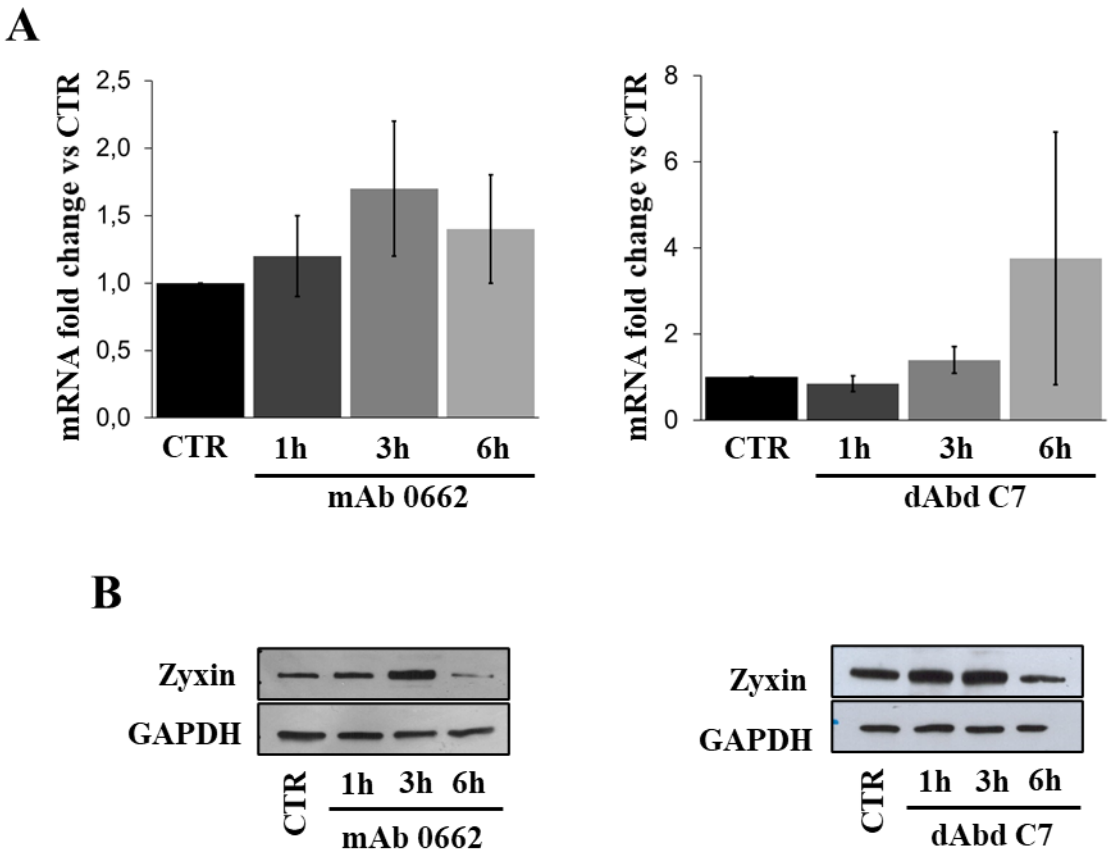


Figure 7. Engagement of CD99 with specific antibodies causes increase of zyxin protein. 6647 cell line was treated with mAb 0662 (3μg/mL) or dAbd C7 (200μg/mL) at the indicated time. (**A**) Zyxin gene expression was evaluated by RT-qPCR using GAPDH as housekeeping gene; the bars represent mean ± SE from at least three independent experiments (**B**) Representative western blot of zyxin, equal sample loading was monitored by blotting for GAPDH;

Interestingly, after 6 hours of treatment with both the anti-CD99 antibodies, the expression of zyxin is lower in confront to the untreated cells (Figure 7B); because at the time the majority of cells are necrotic (Guerzoni et al., 2015) and knowing that zyxin is required for the anti-CD99 antibodies induced apoptosis, we speculated that the 6647 cell population has slightly variable zyxin expression. Hence the triggering of CD99 causes massive death in all 6647 cells but the sub-population with lower expression of zyxin, that totally or partially survive the treatment, becoming the representative population.

In recent years is emerged that the use of specific antibodies to trigger CD99 causes an internationalization of the protein and its degradation (Manara et al., 2016). Consequently, CD99 is absent on the cell membrane, a situation that can be mimicked by its silencing. Therefore, we evaluated the effects of stable silencing of CD99 on zyxin expression. While in the parental cell lines

IOR/CAR and TC-71 zyxin expression is scarce or absent, the loss of CD99 promotes a several increase of the protein in both the employed models (Figure 8B); instead, no coherent variations of mRNA expression are reported (Figure 8A).

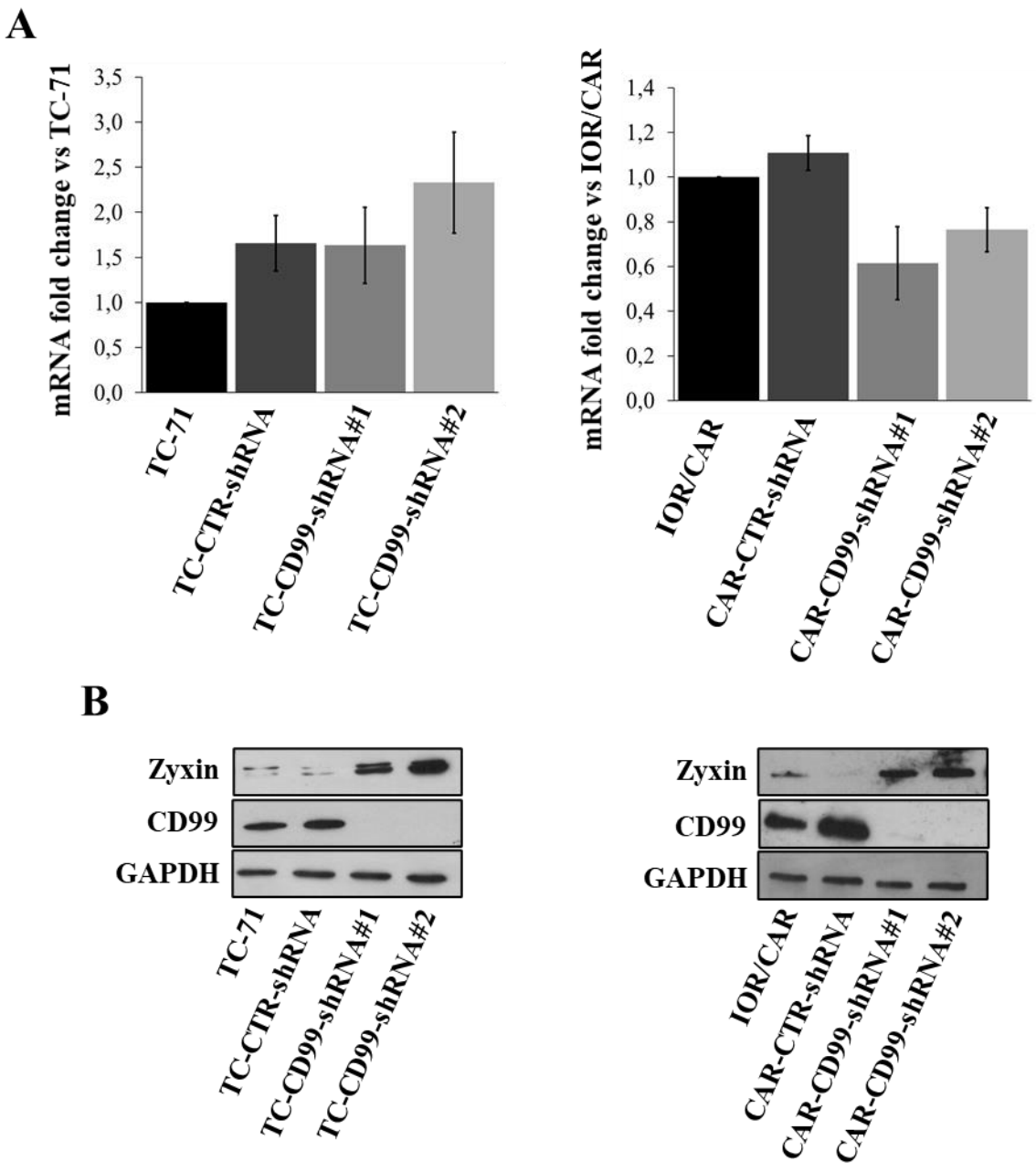


Figure 8. Silencing of CD99 causes increase of zyxin protein. Evaluation of zyxin expression in the models of CD99 stable silencing derived from the cell lines TC-71 and IOR/CAR (**A**) Zyxin gene expression was evaluated by RT-qPCR using GAPDH as housekeeping gene; the bars represent mean ± SE from at least three independent experiments (**B**) Representative western blot of zyxin, equal sample loading was monitored by blotting for GAPDH.

The relationship between CD99 presence and zyxin expression was also dynamically confirmed by using the TET ON inducible silencing of CD99 in the A673p6TR/shCD99 cell line (Figure 9). The cells were incubated with tetracycline (TET) for 48 hours to induce CD99 silencing and subsequently tetracycline was removed (wash-out) to guarantee the rescue of CD99 expression, which occurred in a time-dependent manner (Figure 9B). Accordingly to the previous data, zyxin increases with absence of CD99; moreover, after wash-out zyxin quantity decreases proportionally to CD99 expression recovery, until after 7 days the status of both the proteins is back to the basal condition (Figure 9B). No significant effects are visible on zyxin mRNA (Figure 9A)

This result proves that loss of function of CD99, both by triggering with specific antibodies or by use of shRNA, cause a rapid increase in zyxin protein level, which happens in a quantity dependent manner. No remarkable effects on zyxin mRNA have been reported, indicating that CD99 regulates directly the quantity of zyxin protein rather that influencing the transcription of its gene.

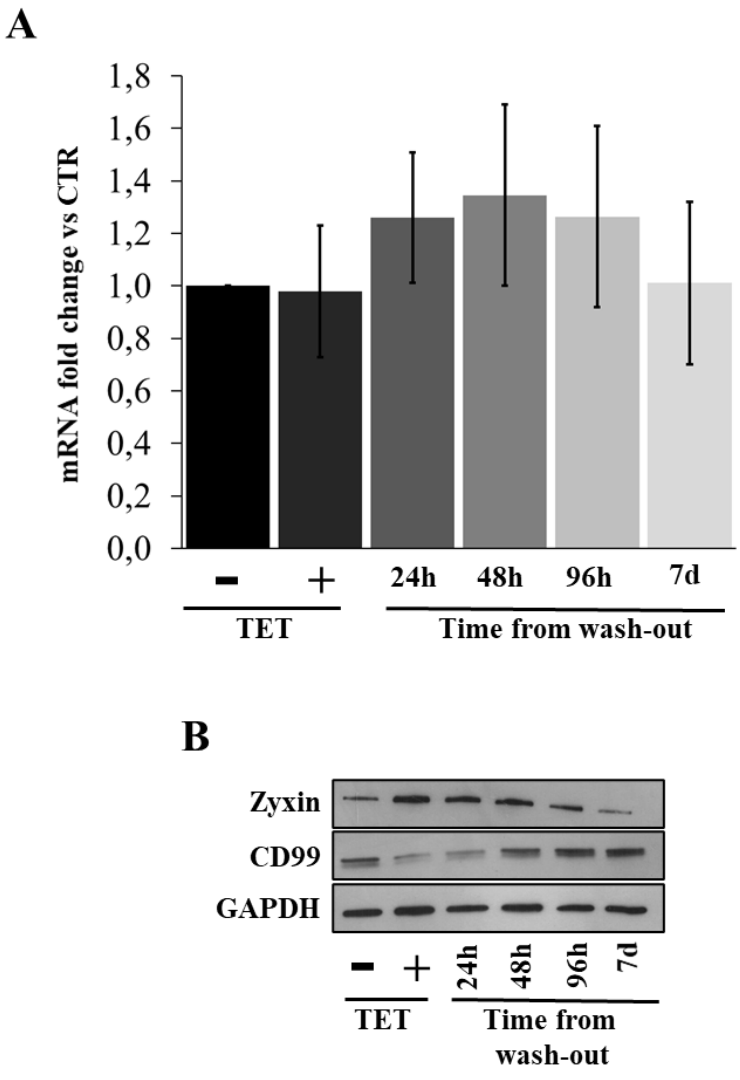


Figure 9. Zyxin levels are dependent from CD99 quantity. A673p6TR/shCD99 cell line was treated with tetracycline (TET) (50 μg/mL) for 48 hours then the drug was removed (wash out) and the tetracycline treated cells harvested at the indicated time points (**A**) Zyxin gene expression was evaluated by RT-qPCR using GAPDH as housekeeping gene; the bars represent mean ± SE from at least three independent experiments (**B**) Representative western blot of zyxin and CD99, equal sample loading was monitored by blotting for GAPDH.

3.2.2. CD99 loss of function induces nuclear accumulation of zyxin

Zyxin role is strictly related to its location in the cell, which can be changed by several physiological and non-physiological stimuli (Hervy et al., 2006; Kotb et al., 2018; Y. X. Wang et al., 2019) . Therefore, we focused our attention on the sub-cellular localization of zyxin in our models of CD99 loss of function.

We performed immunofluorescent staining of zyxin in 6647 cells treated with mAb 0662 and dAbd C7 at various timepoints reporting, beyond the already described protein increase, a drastic shift in zyxin localization; while in untreated cells zyxin is almost exclusively in the cytosol with few cells showing a faint nuclear staining, after 1 hour of treatment we observed the beginning of a nuclear accumulation of zyxin which reaches a maximum at 3 hours; after 6 hours zyxin quantity is similar at the untreated cells (Figure 10A). In the same experimental conditions, we confirmed the nuclear accumulation of zyxin through nucleus-cytosol separation followed by western blot (Figure 10B).

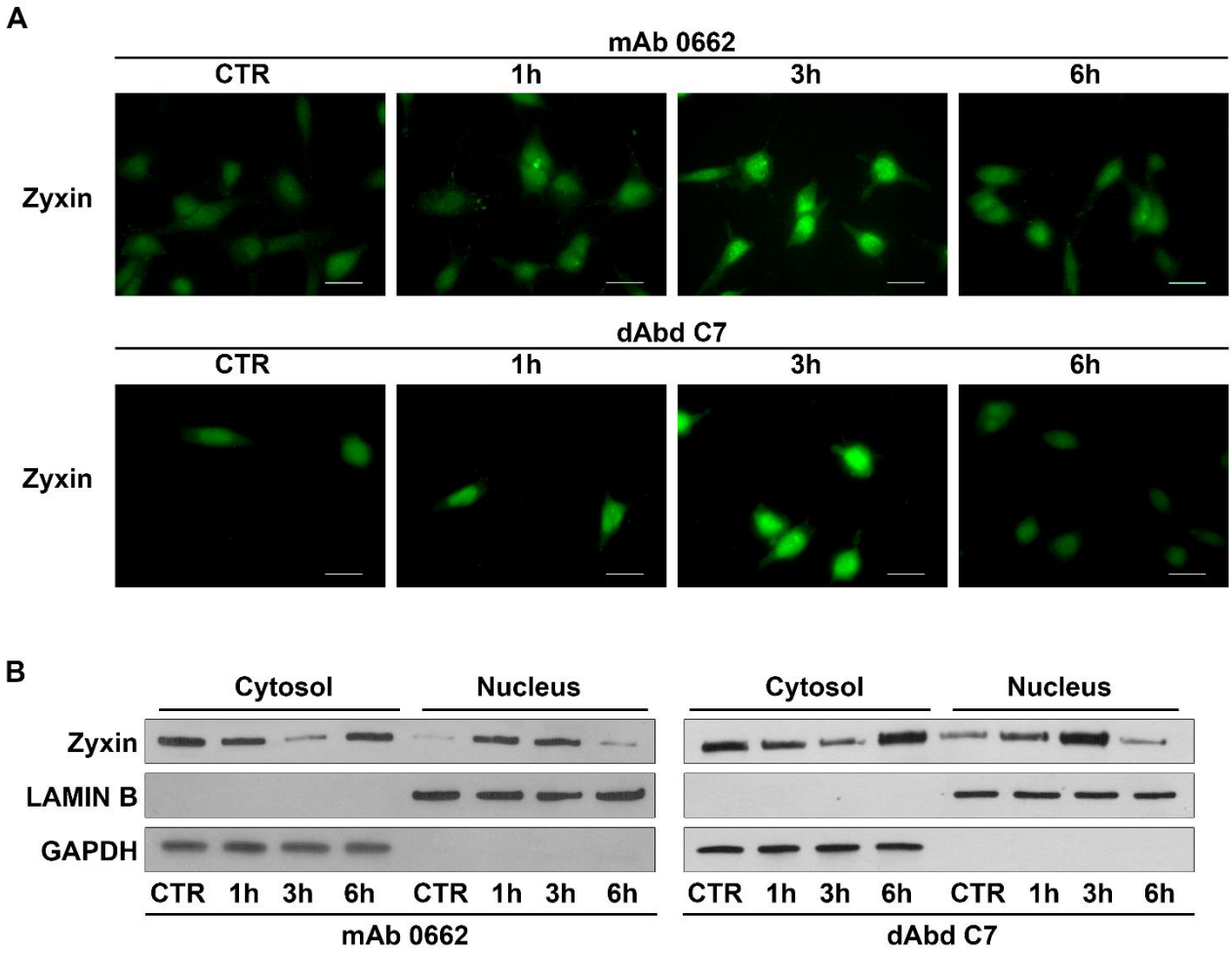


Figure 10. Triggering of CD99 with specific antibodies promotes nuclear accumulation of zyxin. 6647 cell lines was treated with mAb 0662 (3µg/mL) or dAbd C7 (200µg/mL) for the indicated times to evaluate zyxin location (**A**) immunofluorescence staining of zyxin, images were obtained through confocal microscopy Nikon A1 (x600 magnification) (**B**) Representative western blot of zyxin after nucleus-cytosol separation, equal sample loading was monitored by blotting for GAPDH (cytosol) and Lamin B (nucleus).

Finally, we evaluated zyxin location in our models of stable silencing of CD99, both by immunofluorescence staining (Figure 11A) and nucleus-cytosol separation (Figure 11B). Confirming the parallelism that we have already seen between the triggering of CD99 and its silencing, we observed a severe and continuous localization of zyxin in the nucleus, absent or extremely low in the basal cells (Figure 11)

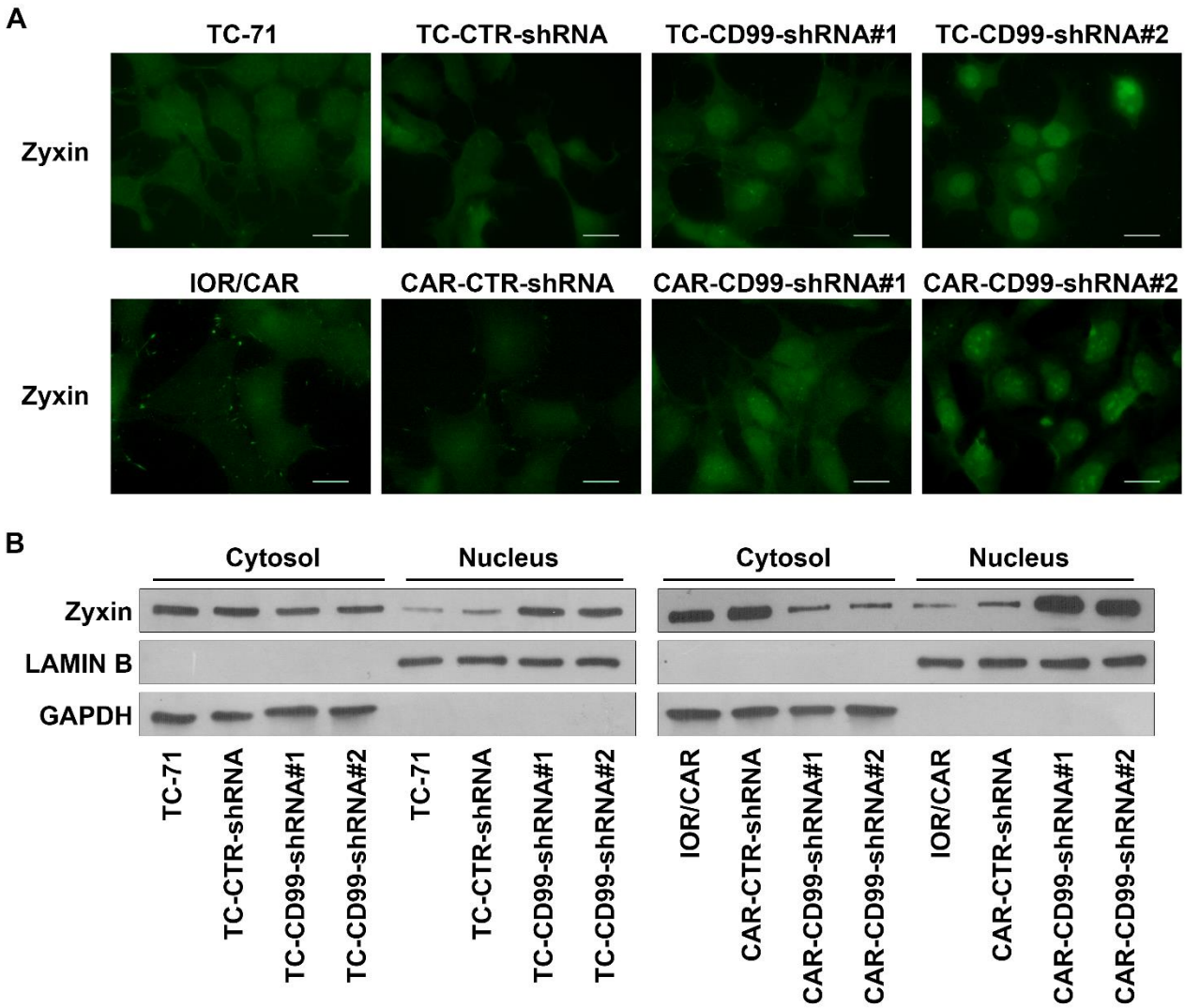


Figure 11. Silencing of CD99 causes nuclear accumulation of zyxin. In the stable silencing of CD99 models derived from the cell lines TC-71 and IOR/CAR was evaluated zyxin location (**A**) immunofluorescence staining, images were obtained through confocal microscopy Nikon A1 (x600 magnification) (**B**) Representative western blot of zyxin after nucleus-cytosol separation, equal sample loading was monitored by blotting for GAPDH (cytosol) and Lamin B (nucleus).

Overall, we have proved that CD99 and zyxin have a close relationship, were CD99 controls extremely carefully zyxin amount and sub-cellular position. Given the crucial role of CD99 in the maintenance of EWS malignancy, the necessity to keeping zyxin at low levels and outside the nucleus indicates that zyxin activity can probably counteract deeply the CD99-driven tumor progression.

3.3. Zyxin nuclear shift: implications on EWS tumorigenesis

The nuclear shift of zyxin induced by CD99 loss of function has deeply intrigued us, because from several researches it is known that zyxin is able to interact with numerous nuclear factors, modifying their activity and consequently modulating processes fundamental for tumor progression such as cell growth, apoptosis and migration (Kotb et al., 2018; Y. X. Wang et al., 2019).

In an effort to better understand the effects of nuclear zyxin on EWS cells malignancy, we focused our attention on zyxin possible interactions with three leading actors in this tumor: the main oncogenic driver EWS-FLI1, YAP1, a crucial effector of the Hippo signaling pathway and Gli1, member of the Hedgehog signaling pathway.

3.3.1. Triggering of CD99 inhibit EWS-FLI1 activity, an effect for which zyxin is dispensable

EWS-FLI1 is the most common chimeric product of EWS, accounted in 85% of all cases (Ross et al., 2013; Savita Sankar & Lessnick, 2011) and its presence is mandatory for the malignant transformation (Delattre et al., 1992; Jedlicka, 2010; Lessnick & Ladanyi, 2012; Toomey et al., 2010).

There are no precedent evidences that zyxin interacts with EWS-FLI1, but we decided to investigate this possibility on the foundation of these observation:

- EWS-FLI1 maintains scarce zyxin expression by promoting NKX2-2 activity, which in turn inhibits zyxin transcription (Fadul et al., 2015) and by up-regulation of CD99 (Hu-Lieskovan et al., 2005; Rorie et al., 2004), which we have just proved down-regulates zyxin at protein level. These multiple ways of keeping zyxin low by EWS-FLI1 could indicate that zyxin presence inhibits its activity.
- EWS-FLI1 is not sufficient to induce the malignant phenotype of EWS, which requires also a high expression of CD99 (Manara et al., 2018; Pasello et al., 2018), suggesting a cross talk between the two proteins in which zyxin, being repressed by CD99, may has a significant role
- The lipoma preferred partner (LPP), a member of zyxin family which shares similar structure and functions with zyxin, is able to modify the activity of ETS transcription factors, to whom also belongs FLI1 (B. Guo et al., 2006).

To test our hypothesis, first we induced zyxin nuclear accumulation in the 6647 cell line through triggering of CD99 with mAb 0662 and performed Chromatin Immunoprecipitation (ChIP) to evaluate EWS-FLI1 binding to the promoters of its well-established target genes: NR0B1, EZH2, and

LOX. In all the cases, after 3 hours of treatment EWS-FLI1 promoter occupation is severely impaired, indicating that absence of CD99 disrupts EWS-FLI1 activity (Figure 12). Unfortunately, zyxin silencing is not able to recovery EWS-FLI1 binding to the promoters after CD99 triggering, proving that the observed displacement is not mediated by zyxin (Figure 12).

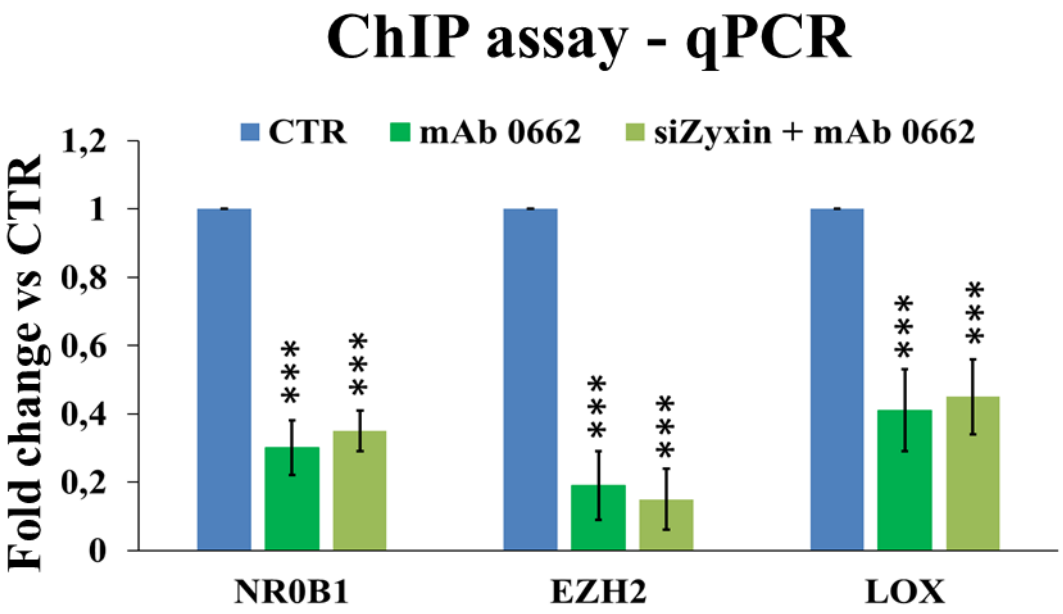


Figure 12. CD99 engagement with mAb 0662 displaces EWS-FLI1 from its target promoters. Anti-FLI1 chromatin immunoprecipitation (ChIP) was performed on 6647 cells untreated (CTR), treated with mAb 0662 (3µg/mL) or silenced for zyxin and treated with mAb 0662 (3µg/mL). The results were obtained by qPCR. The data represent the recovery of each DNA fragment relative to the total input DNA; the bars represent mean ± SE from at least three independent experiments; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Student's t test vs CTR

3.3.2. Triggering of CD99 with specific antibodies promotes YAP1 nuclear translocation, an effect for which zyxin is dispensable

In the last decade the Hippo pathway had become one of the most deeply studied pathway in cancer development, in an effort to clarify its involvement in several tumorigenic effects and in the hope to find new available target for therapy (Calses et al., 2019; Han, 2019). The final effectors of the pathway in mammals are YAP1 and TAZ, two transcriptional co-activators that promote cell proliferation, survival and migration (S. Ma et al., 2019; B. Zhao et al., 2011). In EWS YAP1/TAZ expression is associated with tumor progression and poorer prognosis and their down-regulation *in vitro* decreases cell invasion and growth (Kovar et al., 2020; Rodríguez-Núñez et al., 2020). In addition TRIP6, a member of zyxin family with oncogenic role in EWS (Thomas G.P. Grunewald et

al., 2013), inhibits YAP1 phosphorylation by LATS1/2, promoting YAP1 nuclear translocations and activity (Dutta et al., 2018). Zyxin role in Hippo pathway is almost completely undiscovered, but it is reported to favors YAP1 nuclear shift in the epithelial-mesenchymal transition (EMT) by inducing ubiquitination of LATS1 in breast cancer (B. Ma et al., 2016) and to enhance YAP activity through CDK8 in colon cancer (Zhou et al., 2018). However, zyxin can act as tumor suppressor or oncogene depending on the tumor context, therefore is modulation of YAP1/TAZ in EWS could be different from the previous examples.

First, we decided to evaluate YAP1 status and sub-cellular distribution in EWS cells. In the 6647 cell line we observed that YAP1 is located in the nucleus and cytosol (Figure 13A), where it is partially sequestered, probably because of its phosphorylation (Figure 13B). These data indicate that a considerable fraction of total YAP1 is active in the nucleus, in accord to its reported oncogenic role. The incubation with dAbd C7 leads to complete loss of nuclear YAP1 (and a partial decrease of cytosolic YAP1) already after 3 hours of treatment, a condition maintained until 6 hours (Figure 13A). In a seeming opposite manner, treatment with dAbd C7 also induces a time-dependent strong dephosphorylation of YAP1, an event that should promote its nuclear translocations (Figure 13B). However, the lack of nuclear YAP1 is unequivocally an evidence of its inactivity, which is consistent with the massive cell death induced by dAbd C7. To test if zyxin could have a potential role on this modulation, we perform the same experiment in presence of zyxin silencing, but the absence of zyxin do not causes any difference in the dAbd C7 induced phosphorylation and translocation of YAP1 (Data not shown).

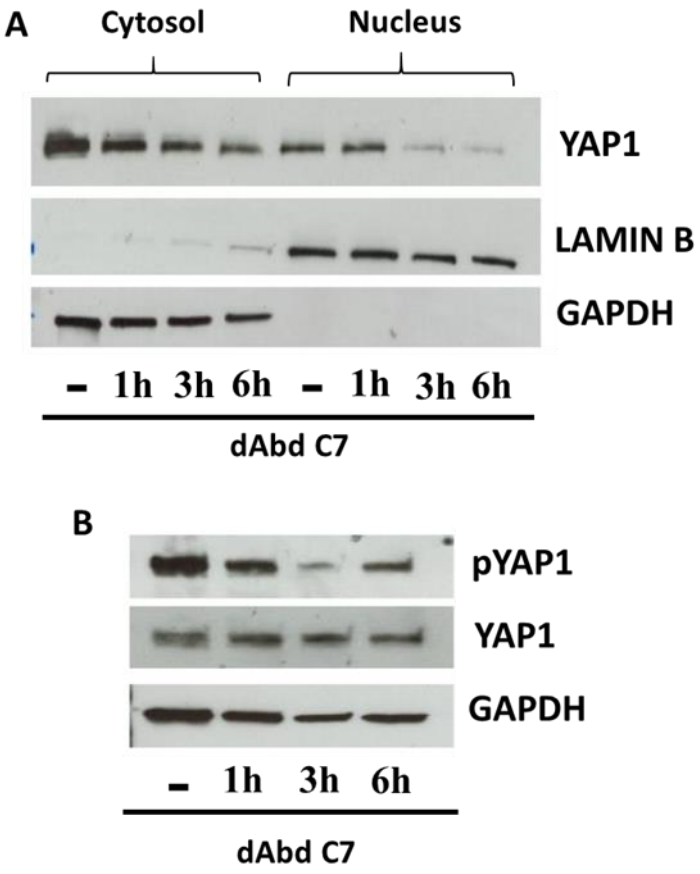


Figure 13. Triggering of CD99 with dAbd C7 modulates YAP1 phosphorylation and location. The 6647 cell line was treated with dAbd C7 (200µg/mL) at the indicated time points (**A**) Representative western blot of YAP1 after nucleus-cytosol separation, equal sample loading was monitored by blotting for GAPDH (cytosol) and Lamin B (nucleus) (**B**) Representative western blot of pYAP1 and YAP1, equal sample loading was monitored by blotting for GAPDH.

3.3.3. Zyxin inhibits Gli1 activity and the modulation is enhanced by CD99 engagement

The Hedgehog (HH) signaling pathway is active during normal development but is deregulated in several tumor contexts (Ruiz i Altaba et al., 2002). The canonical HH signaling pathway relies on the interaction between a ligand of HH family and a cell surface receptor of Patched family that activates the signaling transduction cascade which in turn causes the nuclear translocations of Glis transcriptional factors (Gli1, Gli2, and Gli3) that consequently are able to modulate gene expression (Briscoe & Thérond, 2013; Ruiz i Altaba et al., 2002). Gli1 has drawn our attention because its expression is upregulated by EWS-FLI and its presence is mandatory for EWS transformation (Beauchamp et al., 2009; Joo et al., 2009; Zwerner et al., 2008). In addition, zyxin was found to affect HH signaling in the neural tube of *Xenopus Laevis* by physically interacting with the transcription factor Gli1 (Natalia Y. Martynova et al., 2013).

First, we investigated if zyxin could modulate Gli1 activity in EWS cell lines. By luciferase assay we observed that silencing of zyxin in IOR/CAR causes a significance increase of Gli1 activity (Figure 14A) whilst zyxin over-expression in TC-71 decreases it (Figure 14B). These results are the first evidence of Gli1 inhibition by zyxin in human cells lines, which are coherent with the previous observation made in *Xenopus Laevis* (Natalia Y. Martynova et al., 2013).

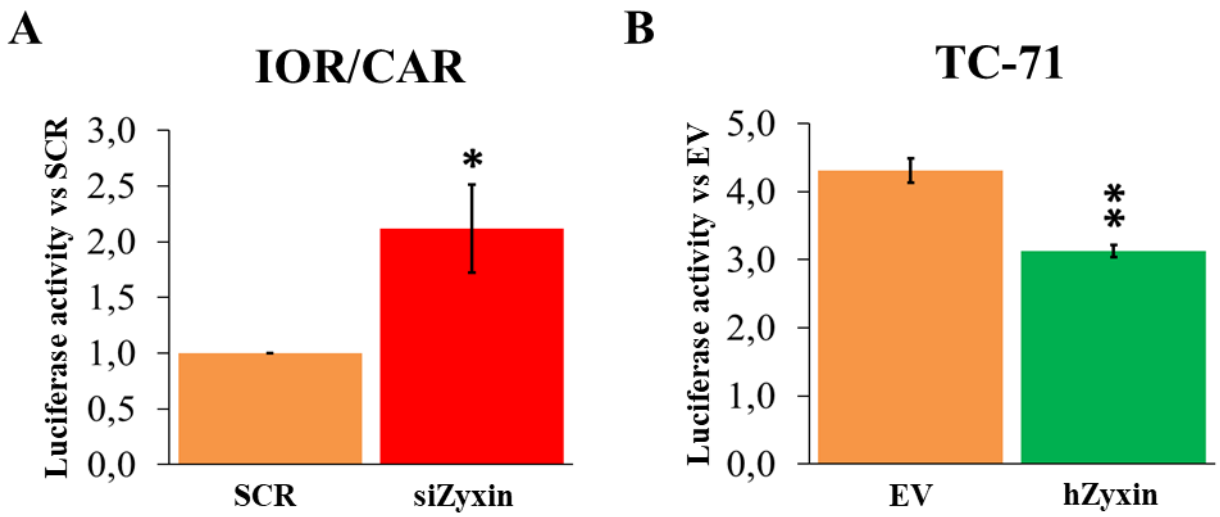


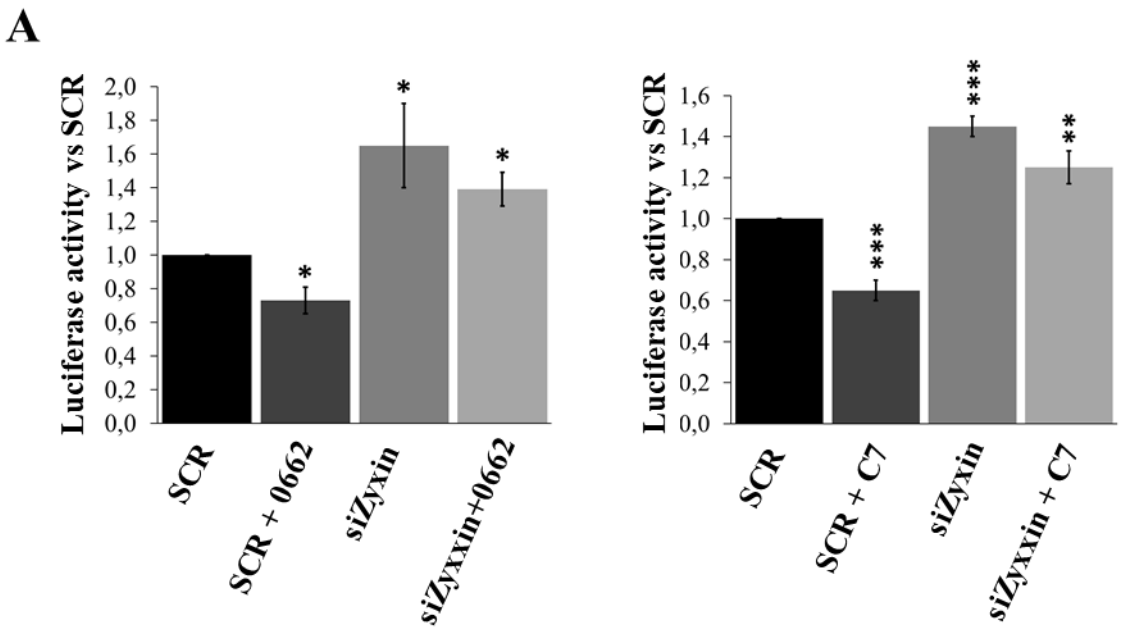
Figure 14. Zyxin modulates Gli1 activity. Gli1 driven luciferase activity was tested in response to (**A**) Zyxin transient silencing in IOR/CAR cell line (**B**) Forced stable expression of zyxin in TC-71 cell line; the bars represent mean ± SE from at least three independent experiments; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Student's t test.

Consequently, we wanted to verify if there was any relation between CD99 and Gli1 and, if present, if it was zyxin-mediated. The use of the anti-CD99 antibody dAbd C7 in the cell line 6647 causes in 3 hours a strong decrease of Gli1 activity, assessed by luciferase assay (Figure 15A).

Accordingly, triggering of CD99 with mAb 0662 gave similar results (Figure 15B). But, in presence of zyxin silencing (Figure 15C) the effects of both the anti-CD99 antibodies on Gli1 activity are completely abrogated (Fig 15A, B).

To assess the impact of triggering CD99 on Gli1 transcriptional capability we evaluated mRNA levels of four well-established targets of Gli1 in EWS: NKX2-2, Patched 1 (PTCH1), GAS1 and Cyclin D1 (CCND1) (Joo et al., 2009). Treatment with dAbd C7 induces a time-dependent expression decrease

of the up-regulated Gli1 targets NKX2-2, PTCH1 and CCND1, while increases the expression of the down-regulated target GAS1 (Figure 15D). Silencing of zyxin had opposite effects on the gene expression and, in addition, completely abrogated the inhibition of Gli1 activity promoted by CD99 engagement with dAbd C7 (Figure 15D), confirming the luciferase data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Luciferase activity vs SCR
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siZyxin
siZyxxin+0662
Luciferase activity vs SCR
1,6
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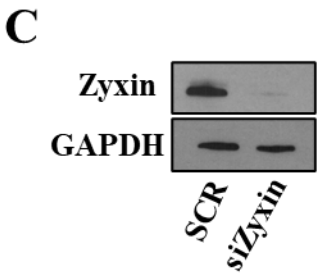
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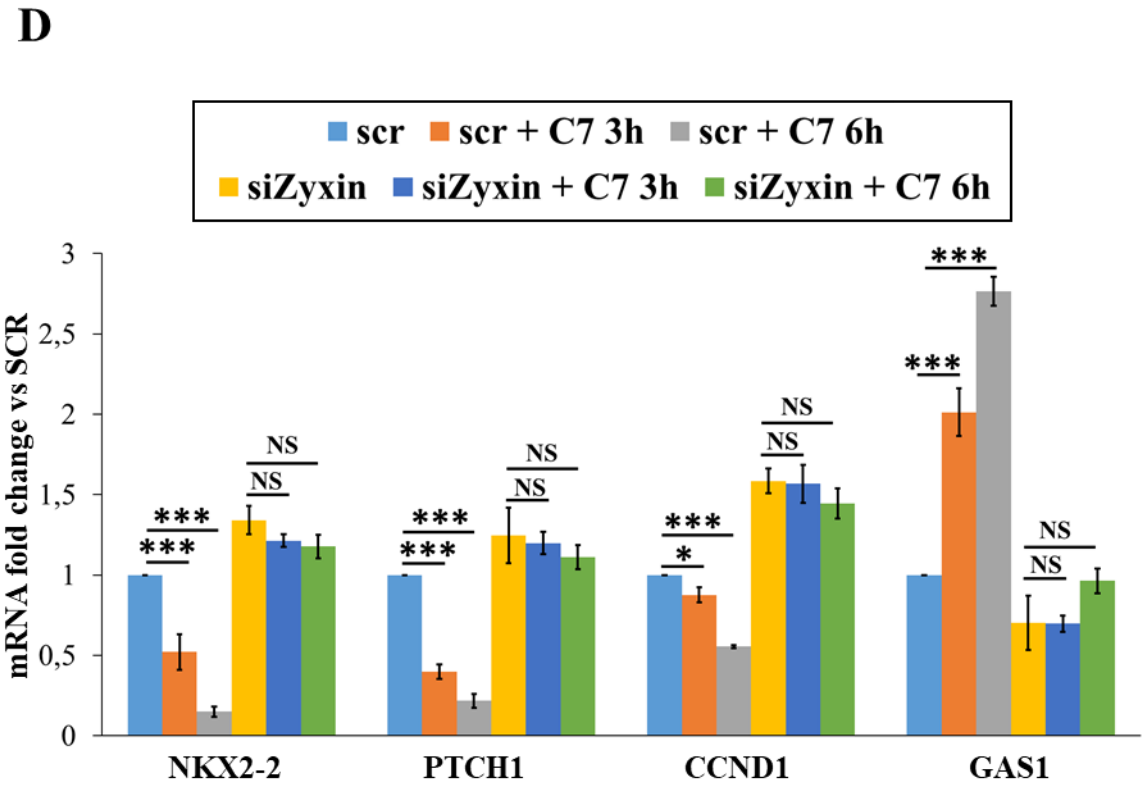
D
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mRNA fold change vs SCR
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2
1,5
1
0,5
0
NKX2-2
PTCH1
CCND1
GAS1
NS

Figure 15. Triggering of CD99 with specific antibodies decreases Gli1 activity through zyxin. Gli1 driven luciferase activity was tested in response to zyxin transient silencing in 6647 cell line followed by a 3 hours treatment with (**A**) dAbd C7 (200μg/mL) (**B**) mAb 0662 (3μg/mL); the bars represent mean ± SE from at least three independent experiments; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Student's t test vs SCR (**C**) Representative western blot of zyxin after zyxin transient silencing in 6647 cell line, equal sample loading was monitored by blotting for GAPDH; (**D**) gene expression of the indicated targets in 6647 transiently silenced for zyxin and treated with dAbd C7 (200μg/mL) for the reported time points. Evaluation by RT-qPCR using GAPDH as housekeeping gene; the bars represent mean ± SE from at least three independent experiments; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Student's t test

Finally, we performed an analysis of gene expression microarray on the precedent described model of A673p6TR/shCD99 with inducible silencing of CD99 through treatment with tetracycline (Figure 9). Specifically, we carried out Gene Set Enrichment analysis (GSEA) (Subramanian et al., 2005) using PID_HEDGEHOG_GLI_PATHWAY (Schaefer et al., 2009) as gene set in untreated A673p6TR/shCD99 cells (CTR), in cells treated with tetracycline (TET) and cells in which tetracycline was removed after 24 and 48 h (TET wo 24h and 48h). Interestingly, the results showed that expression profile of A673p6TR/shCD99 TET was negatively correlated with the genes involved in Hedgehog signaling mediated by Gli proteins (Figure 16A). In particular, amongst the core genes, we found that NKX2-2, CCND1 as well others Gli1 up regulated targets (MYCN, MEOX1, FOXM1 and ABCG2) are down-regulated with CD99 silencing and recovered their expression during the wash out (Figure 16B). These results indicate that CD99 loss of function causes inhibition of Gli1 signaling, recapitulating and amplifying the effects obtained by CD99 engagement with specific antibodies. In addition, we also observed that the temporary inhibition of CD99 downregulates various cyclins, inducing an anti-proliferative phenotype, lost after recovery of CD99 expression (Figure 16B).

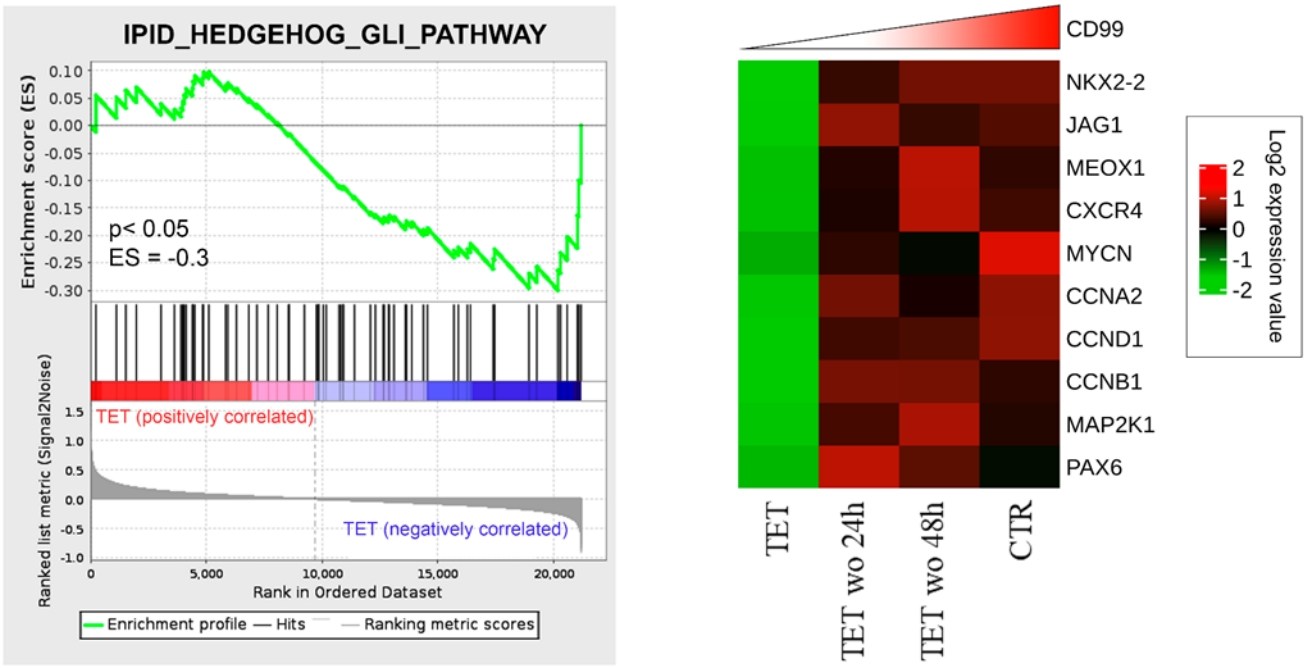

Figure 16. CD99 modulates Gli1 activity. A673p6TR/shCD99 cells were treated with tetracycline (50 µg/mL) for 48 h (TET) to induce CD99 silencing; subsequently tetracycline was removed to promote regain of CD99 expression and the cells were harvested after 24 and 48 h (TET wo 24h and 48 h). (**A**) GSEA revealed significant enrichment of genes downregulated in A673p6TR/shCD99 TET respects to A673p6TR7shCD99 TET wo 24 h and 48 h using PID_HEDGEHOG_GLI_PATHWAY as gene sets. The enrichment score curve was obtained from GSEA software. In the enrichment plot, the x-axis shows the rank order of genes. Vertical black line indicates the position of the enriched genes (Hit) comprising the gene set. The graph on the bottom shows the ranked list metric (signal-to-noise ratio) for each gene as a function of the rank in the ordered dataset. (**B**) Heatmap of log2 expression value of core enrichment genes from GSEA

4. Discussion

In this work we have proved the tumour suppressor role of zyxin in EWS. This was confirmed by functional studies conducted *in vitro* that reported zyxin as a negative regulator of cell migration, adhesion and growth, which are crucial elements in tumour progression. Still, the mechanism used by zyxin to affect these processes remained unknow. In an effort to fill this gap in our knowledge, we focused our attention on the relation between CD99 and zyxin. We proved that CD99 has a key role in keeping zyxin at low levels and, consequently, CD99 loss of function by engagement with specific antibodies (mAb 0662 and dAbd C7) or by using shRNA, induces a large increase of zyxin protein and promotes its nuclear translocation. At this point a wide range of possibilities has opened up. Indeed, once in the nucleus zyxin assumes a remarkable series of interactions with numerous partners (Kotb et al., 2018; Y. X. Wang et al., 2019), which could help to understand the mechanisms that allow zyxin to counteract the malignant phenotype of EWS. This is especially true in the context of this tumour, which is characterized by a stable genome and, apart the main typical genomic aberration EWS-ETS, other mutations are fairly uncommon (Grünewald et al., 2018; Helman & Meltzer, 2003; Turc-Carel et al., 1983). Therefore, the tumour progression of EWS is not driven by multiple mutations but from the deep reprogramming of its gene expression, executed mainly by EWS-FLI1. Indeed, the chimera influencse the cell gene expression in several ways, from chromatin remodelling to post-transcriptional regulation (Sand et al., 2015; Toomey et al., 2010). Thus, any potential tumour suppressor, in order to reduce the malignant phenotype of EWS, is likely to impairs EWS-FLI1 functions; this inhibition could happen both by direct inactivation of EWS-FLI1 or by targeting its downstream effectors. Applying this hypothesis to our study, we tried to understand how zyxin could interfere with EWS-FLI1. The first idea was the possibility of a direct interaction between zyxin and EWS-FLI1, but has been excluded by experimental data, that clearly showed no influence of zyxin on EWS-FLI1 transcriptional activity. However, is remarkable the fact that the engagement of CD99 with mAb 0662 impairs EWS-FLI1 binding to the promoters of its target genes, because to date no direct cross talk between the signalling of the two proteins was so robustly demonstrated. In addition, our data also offer a valid explanation for the requirement of CD99 high expression in the cell to create a permissive context for EWS-FLI1-induced transformation (Manara et al., 2018; Pasello et al., 2018): The low expression or lack of CD99, in a still undiscovered way, inhibits EWS-FLI1 ability to modulate its target genes and consequently is restrained from applying its gene expression reprogramming, necessary for the transformation of the cell.

We also examined the Hippo signalling pathway as a possible target of zyxin modulation. The core of the Hippo pathway consists in the kinases MST1/2 that phosphorylates and activates LATS1/2 which, in turn, phosphorylates YAP1/TAZ promoting their cytosol accumulation and degradation. When YAP1/TAZ are not phosphorylated, they shift into the nucleus and bind transcription factors, predominantly TEADs proteins, activating the expression of several genes involved in cell proliferation, survival, or migration (S. Ma et al., 2019; B. Zhao et al., 2011). In tumours, included EWS, YAP1/TAZ have a clear oncogenic role (Kovar et al., 2020; Rodríguez-Núñez et al., 2020). In breast cancer zyxin promotes YAP1 nuclear translocation favouring the epithelial-mesenchymal transition (B. Ma et al., 2016) and in colon cancer enhances the mitotic potential by upregulating YAP1 activity via CDK8 (Zhou et al., 2018). Our results demonstrated that the engagement of CD99 with dAbd C7 causes recruiting of YAP1 in the cytosol, followed by probably degradation, in a zyxin-independent manner, contrasting with our starting hypothesis.

Our quest for a partner that could explain the tumour suppressor role of zyxin ended with the finding of Gli1, a prominent member of the Hedgehog (HH) signalling pathway, which is normally active during the development but its function is lost in the adults (Briscoe & Thérond, 2013; Ruiz i Altaba et al., 2002). The activation of the pathway starts when a member of the HH ligands (Sonic Hedgehog [SHH], Desert Hedgehog [DHH] or Indian Hedgehog [IHH]) binds its cell surface receptor, a protein of the Patched family, Patched 1 (PTCH1) or Patched 2 (PTCH2) (Beachy et al., 2010). This interaction activates Patched, that frees Smooth (SMO) from the PTCH-SMO complex (Taipale et al., 2002), allowing it to release Glis (Gli1, Gli2 or Gli3) from its complex with Suppressor of Fused (SUFU). In turn Glis, transcriptional factors belonging to the Kruper family of zinc finger proteins, translocate into the nucleus and promote the transcription of genes involved in homeostasis and differentiation (Briscoe & Thérond, 2013; Machold et al., 2003; Ruiz i Altaba et al., 2002). As usually happens for several developmental pathway, HH signalling pathway is reactivated in a large number of cancers, where it sustains tumour progression by promoting cell survival, growth and migration (Briscoe & Thérond, 2013; Ruiz i Altaba et al., 2002). Consequently, Gli1 is appointed as a crucial effector of EWS malignant phenotype. However, unlike in other cancers, in EWS the reactivation of the pathway is non-canonical, because mainly relies on EWS-FLI1 driven-transcription of Gli1, without any effects on its upstream target (Beauchamp et al., 2009). Accordingly, the application of conventional drugs against HH signalling pathway that target effectors upstream Gli1 are almost completely ineffective in EWS; on the opposite, the inhibition of its activity by treatment with drugs directed against it, such as Arsenic Trioxide (ATO), GANT58 and GANT61, or by using shRNAs, robustly impairs cell survival and growth in anchorage-independent. (Beauchamp et al., 2009; Joo et

al., 2009; Matsumoto et al., 2014; Zwerner et al., 2008). Association between zyxin and Gli1 has been observed in the neural tube of *Xenopus Laevis* embryos, where nuclear zyxin binds directly to Gli1 preventing its transcriptional activity, demonstrated both by luciferase assay and ChIP. This interaction allows a fine regulation of HH signalling pathway and subsequently the correct development of the neural tubes (Natalia Y. Martynova et al., 2013). Here we proved that zyxin is able to affect Gli1 function also in human cells, specifically in EWS cell lines. Zyxin up-regulation inhibits Gli1 activity, while zyxin repression promotes it. Moreover, the treatment with dAbd C7 or mAb 0662, that induces zyxin increase and nuclear translocation, strongly impairs Gli1 activity, which reaches a minimum after 3 hours when zyxin is almost exclusive into the nucleus. Confirming zyxin role as mediator of this processes, the use of shRNAs against zyxin block the inhibition of Gli1 caused by CD99 engagement. Accordingly to these data, the triggering of CD99 modulates via zyxin the expression of Gli1 target genes and microarray analysis demonstrates that the use of shRNA against CD99 has similar results. Looking more closely to the several genes involved, NKX2-2, GAS1 and the cyclins have attracted our attention, because their modulation explains the anti-tumour phenotype promoted by zyxin in EWS.

NKX2-2 is a transcriptional factor and a major promoter of EWS tumour progression (Smith et al., 2006); EWS-FLI1 directly up-regulated NKX2-2 and almost half of the targets genes of the latter are also EWS-FLI1 targets, indicating that the chimera uses NKX2-2 to amplifying its effects on the transcriptional processes (Owen et al., 2008; Shi et al., 2020; Smith et al., 2006). Indeed, in EWS NKX2-2 is crucial for the maintenance of a undifferentiated phenotype, repressing mesenchymal features such as cell adhesion and cytoskeletal organization, whilst loss of NKX2-2 leads to the formation of more actin stress and focal adhesions (Fadul et al., 2015). Interestingly, zyxin, which we have just proved down-regulates indirectly NKX2-2, is a repressed transcriptional target of NKX2-2 itself (Fadul et al., 2015), suggesting the presence of a negative loop where NKX2-2 enhances its own activity by keeping low zyxin levels, which in turn cannot inhibit Gli1, leading to further increase of NKX2-2 function.

Several cancers modulate the activity of cyclins to bypass the normal regulation of the cell cycle, promoting cell growth and survival. This is also the case of EWS, especially for Cyclin D1, a direct target of EWS-FLI1. Thereby, zyxin capability to modulate through Gli1 the expression of several cyclins and of the growth arrest protein 1 (GAS1) explains its robust negative effect on EWS cell growth (Cheung et al., 2007; Zhang et al., 2004).

Collectively with this work we discovered a new role for zyxin in EWS, recapitulated in Figure 17: In stable conditions (Figure 17A) EWS-FLI1 and CD99 maintain zyxin in the cytosol at low levels, allowing Gli1 to promote tumour progression, especially maintaining a more mesenchymal phenotype through up-regulation of NKX2-2 and supporting survival thanks to down-regulation of GAS1 and up-regulation of cyclins. But loss of function of CD99 (Figure 17B), by shRNAs or by engagement with mAbs, is sufficient to increase zyxin levels and to promote its nuclear translocation, where zyxin inhibits Gli1 activity and establishes an anti-tumorigenic phenotype, decreasing cell growth, migration and promoting adhesion.

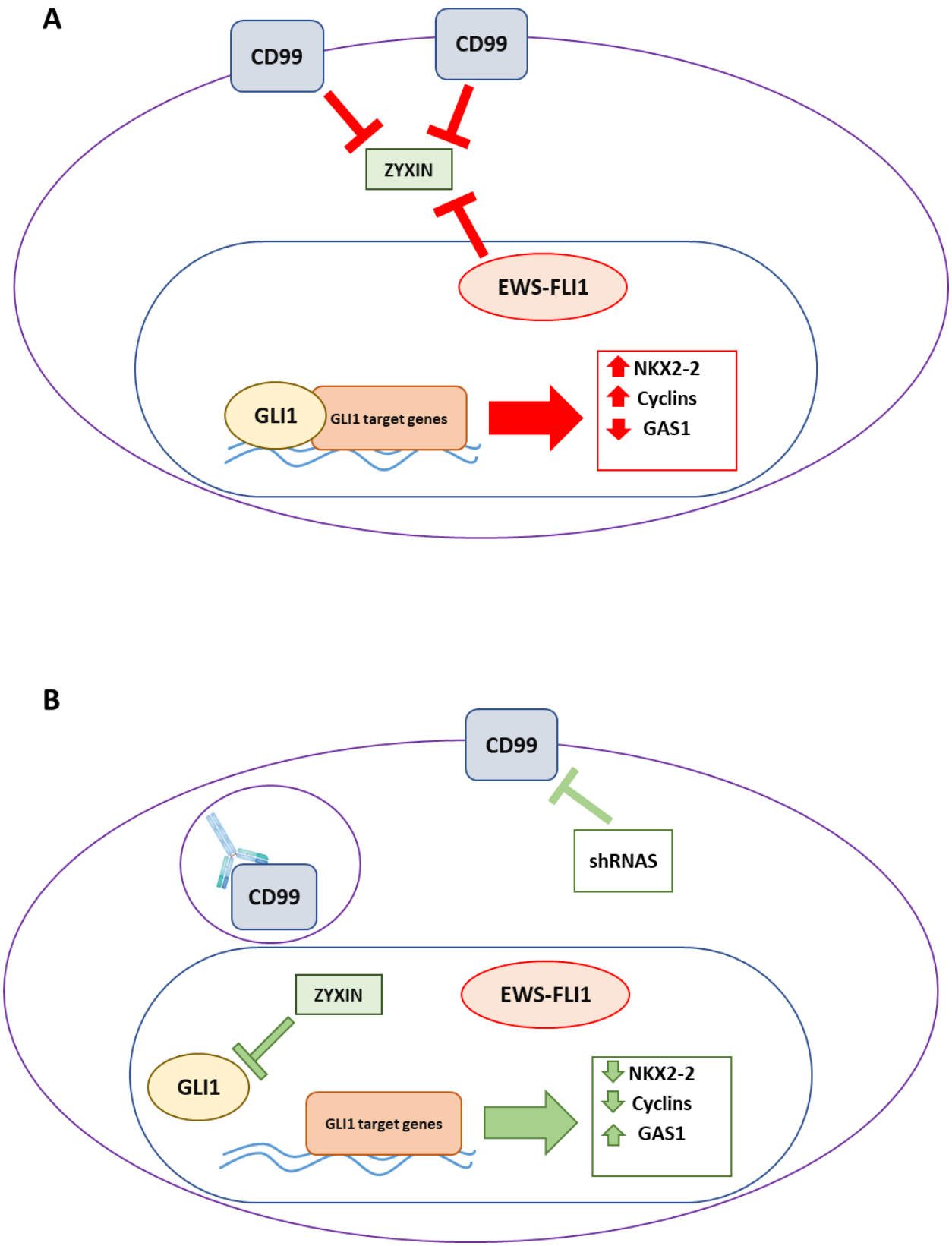

Figure 18. Schematic representation of zyxin role in EWS. (**A**) In stable conditions (**B**) after CD99 loss of function by engagement with mAbs or use of shRNAs

5. Material and methods

Cell lines and primary cultures

The lines 6647 and TC-71 were kindly provided by T.J. Triche (Children's Hospital, Los Angeles, CA, USA); SK-N-MC, SK-ES-1, and RD-ES were provided by American Type Culture Collection, ATCC (Rockville, MD, USA); IOR/CAR and LAP-35 were previously established in our laboratory (Bagnara et al., 1990; Guerzoni et al., 2015); the A673 sarcoma cell line was provided by Dr. H. Kovar (St. Anna Kinderkrebsforschung, Vienna Austria), the cell lines PDX-EW#2-C and PDX-EW#4-C were obtained by the respective EWS PDX after the first passage in animal as previously described (Nanni et al., 2019); the human mesenchymal stem cells hBM-MSC 223, hBM-MSC 244 and DP-15 were kindly provided by E. Lucarelli (Istituto Ortopedico Rizzoli, Bologna, Italy) and cultured as previously described (Amaral et al., 2014; Sciandra et al., 2014); the Phoenix cell line, producer of amphotropic retroviruses was a kind concession of Dr. B. Calabretta (Thomas Jefferson University, PA). The EWS cells were cultured in Iscove's modified Dulbecco's medium (IMDM; EuroClone, Milan, Italy) enriched with 10% fetal bovine serum (FBS) (EuroClone) supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin and incubated at 37 °C in a humidified atmosphere at 5% CO2.

All cell lines were tested for mycoplasma contamination (MycoAlert Mycoplasma Detection Kit, Lonza) and authenticated by short tandem repeat (STR) polymerase chain reaction (PCR) analysis using a PowerPlex ESX Fast System kit (Promega, Madison, WI, USA).

Transient and stable transfections

Transfections were performed using TransIT-X2 (Mirus, Madison, WI, USA) or Lipofectamine 2000 (Life Technologies) according to the manufacturer’s protocol.

For transient zyxin silencing, IOR/CAR cells were seeded and after 24 hours transfected with small interfering RNA (siRNA) sequences targeting zyxin (ON-TARGETplus SMARTpool, Human zyxin, Dharmacon) or irrelevant targets (ON-TARGETplus Non-targeting siRNA). After 48 hours from transfection the cells were used for the *in vitro* experiments.

For stable silencing of CD99, a plasmid pSilencer 2.1-U6 Neo vector (encoding for the shRNAs against CD99) was created, and IOR/CAR and TC-71 cells were transfected. Stable transfectants expressing shRNA-CD99 (TC-CD99-shRNA#1 and #2 or CAR-CD99-shRNA#1 and #2) or negative controls (TC-CTR-shRNA or CAR-CTR-shRNA) were obtained after selection in neomycin (500 μg/ml).

The TET ON model of inducible silencing of CD99 A673p6TR/shCD99 was obtained through sequential transfection of the A673 cell line with the plasmid p6TR (encoding the tetracycline-sensible repressor) and the plasmid pTER (encoding for the shRNAs against CD99). The cells were cultured in absence of tetracycline (TET) and selected with blasticidin (2 μg/mL) and zeocin (50μg/mL). A673p6TR/shCD99 cells were incubated with TET (50μg/mL) for 48 hours to induce CD99 silencing; subsequently TET was removed (wash-out) to guarantee the rescue of CD99 expression and the cells were harvested after 24 and 48 hours.

Co-transfection of the packaging cell line Phoenix

The plasmids pMSCV-hZyxin, kind concession of Dr. M. Beckerle (University of Utah, UT, US), pMSCV-empty vector and plasmid-helper Sara3, both provided by Dr. B. Calabretta (Thomas Jefferson University, PA, US), were used for the co-transfection of the amphotropic cell line Phoenix. The transfections were performed using Lipofectamine 2000 (Life Technologies) according to the manufacturer's protocol. After 48 hours from transfection, the supernatants containing the pseudoviral particles were harvested, centrifuged (1500 rpm x10') and filtered (0.22μM, low protein binding PES) to eliminate cells and suspension debris.

Infection of the 6647 and TC-71 cell lines

For zyxin forced stable expression, 6647 and TC-71 cells were infected with the vector supernatants of pMSCV-hZyxin or control pMSCV-empty vector described above. The cells were seeded in 6-wells plates and after 48 hours the culture medium was substituted with the pseudoviral supernatant added of polybrene (Sigma-Aldrich). The plates were centrifuged (1800 rpm x 45') and incubated for 2 h at 32 °C; subsequently, the pseudoviral supernatant was substituted with fresh one and the plates were centrifuged again (1800 rpm x 45') and incubated for 4 h at 32 °C. Finally, the medium was substituted with IMDM plus 10% FBS and puromycin (2.5 μg/ml), the latter added for the selection.

Treatments with anti-CD99 antibodies

The anti-CD99 0662 monoclonal antibody (mAb) was produced in the Unité INSERM 343, Hospital de l'Archet, France. The anti-CD99 diabody C7 (dAbd C7) was produced by Diatheva srl, Italy. Treatments with 0662mAb (3μg/ml) and C7 dAbd (200μg/ml) were performed in adhesion or at the concentration of 10 × 106/ml cells.

In vitro parameters of malignancy

Migration ability in the EWS cell lines was assessed using transwell chambers (Costar, Cambridge, MA) with an 8 μm pore size and polycarbonate filters. A cell suspension of 100,000 cells was seeded in the upper compartment, whereas IMDM plus 10% FBS was placed in the lower compartment of the chamber as a chemoattractant. Incubation was performed overnight at 37 °C in a humidified atmosphere. The migrated cells were fixed with methanol, stained with Giemsa dye and counted at 10 × magnification.

Anchorage-independent growth was determined in 0.33% SeaPlaque Agarose (Lonza) with a 0.5% agarose underlay. Colonies were counted after 7 days.

Luciferase reporter gene assay

A luciferase assay was used to evaluate the transcriptional activity of Gli1. Cells were seeded in standard medium in 24-well plates (30,000 cells/well) previously coated with fibronectin (3 μg/cm2; Sigma-Aldrich). Transfection was performed 24 h before assessment of luciferase activity with 250 ng of Gli1 signal reporter using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's instructions. The cells were lysed, and luciferase activity was measured according to the manufacturer's protocol using a Dual-Glo Luciferase Assay System (Promega) with a GloMax Luminometer (Promega). The firefly luciferase luminescence signal was normalized to that produced by Renilla luciferase, which was included as an internal control.

Immunoblotting analysis

Western blot experiments were performed according to standard protocols. Samples were lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mm Tris-HCl, pH 7.4, 150 mm NaCl, 0.1%

sodium dodecyl sulfate (SDS), 1% Triton X-100, 5 mm ethylenediaminetetraacetic acid (EDTA), 1% deoxycholate) supplemented with protease inhibitors. Equivalent amounts of lysates were run on SDS gels under denaturing conditions and blotted onto nitrocellulose membranes. The membranes were incubated overnight with anti-Zyxin (ab109316; Abcam), anti-CD99 (sc-53148; Santa Cruz Biotechnology) anti-phosphoYAP (Ser127) (Cell Signaling, #4911), anti-YAP (Cell Signaling, #14074), anti-GAPDH (sc-25778; Santa Cruz Biotechnology), anti-Lamin B (sc-6216; #2250; Santa Cruz Biotechnology) primary antibodies. Anti-rabbit (NA9340V, GE Healthcare), anti-mouse (NA9341V, GE Healthcare) or anti-goat (sc-2020, Santa Cruz Biotechnology) antibodies conjugated to horseradish peroxidase were used as secondary antibodies. The proteins were visualized with an ECL Western Blotting Detection System (GE Healthcare).

Immunofluorescence

Cells were fixed in 4% paraformaldehyde and were permeabilized with 0.15% Triton X-100 (Sigma) in phosphate-buffered saline and were incubated with the antibody anti-Zyxin (sc-271134, dilution 1:100). Anti-rabbit FITC (Thermo Scientific, #31569, dilution 1:100) was used as secondary antibodies. Images were acquired using a Nikon ECLIPSE 90i microscope and were then analyzed with NIS-Elements software (Nikon).

RNA extraction and qPCR

Total RNA from cell lines was extracted with a TRIzol extraction kit (Life Technologies, Grand Island). Nucleic acid quality and quantity were assessed with a NanoDrop spectrophotometer (NanoDrop Technologies, ThermoFisher Scientific). Total RNA for each sample was reverse transcribed into cDNA using a High-Capacity cDNA Reverse Transcription Kit (Life Technologies) according to the manufacturer's protocols. qPCR was performed on a ViiA7 system (Life Technologies) using Syber PCR Master Mix or TaqMan PCR Master Mix (Life Technologies). Predesigned TaqMan probes (Life Technologies) were used for Zyxin (Zyxin, Hs00899658_m1), Cyclin D1 (Hs00765553) and GAPDH (Hs99999905_m1). The following primers were used for: PATCHED1 forward 5′-AATGGGTCCACGACAAAGCCGACTA-3′ and reverse 5′-TCCCGCAAGCCGTTGAGGTAGAAAG-3; GAS1 forward 5′-GAAGGGATGGTTGGGGATAC-3′ and reverse 5′-TGCAGACGAGTTGGGAGTTTC-3; GAPDH forward 5′-GAAGGT GAAGGTCGGAGTC-3′ and reverse 5′-GAAGATGGTGATGGGATTTC-3'. Relative

quantification was performed with the ΔΔCT method, and the expression levels of the target genes were normalized to those of the housekeeping gene GAPDH.

Chromatin Immunoprecipitation

ChIP assays were performed as previously described (Rocchi et al., 2010) using the antibody anti-FLI1 (ab15289, Abcam) for immunoprecipitation.

The following primers were used for the detection of the binding site of EWS-FLI1 on the promoters of: LOX forward 5'-CTGTGTGTAGGTAATATGAGAAATGG-3' and reverse 5'-GGCATTTT GAAAAAGAGACAGG-3'; NR0B1 5'-GTTTGTGCCTTCATGGGAAATGGTTATTC-3' and reverse 5'- CTAGTGTCTTGTGTGTCCCTAGGG -3'; EZH2 forward 5'-GACACGTG CTTAGAACTACGAACAG-3' and reverse 5'-TTTGGCTGGCCGAGCTT-3'.

The primers were used in Real Time PCR with the SYBR Green method and the quantification analysis was calculated with the following formula: % of recruitment = 2ΔCt × input chromatin percentage where ΔCt = Ct (INPUT) - Ct (IP).

Microarray analysis

RNA from A673 cell line, A673p6TR7shCD99 was hybridized on Agilent whole human genome microarray, which represents 60k unique human transcripts. Briefly, RNA quality was assessed by Bioanalyzer to have a RNA integrity number (RIN) higher than 7. Labeled cRNA was synthesized starting from 100 ng of total RNA using the Low Input Quick-Amp Labeling Kit, one color in the presence of cyanine 3-CTP. Hybridization was performed at 65 °C for 17 hours in a rotating oven. Images at 5 μm resolution were generated by Agilent scanner, Feature Extraction 10.7.3.1 software was used to obtain the microarray raw-data (Agilent Technologies). Data were normalized using quintile normalization and $\log_2$ transformed by GeneSpring GX v.14.8 software (Agilent Technologies). We carried out Gene Set Enrichment analysis (GSEA) (Subramanian et al., 2005) using PID_HEDGEHOG_GLI_PATHWAY (Schaefer et al., 2009) from The Molecular Signature Database (MsigDB) and additional genes related to Gli pathway (Skoda et al., 2018) as gene set. Using $\log_2$ expression value matrix, we evaluated which class between A673p6TR/shD99 TET and A673p6TR/shCD99 TET wo 24/48 h was enriched in the above considered gene set. The Heatmap of genes included in the core enrichment was obtained adopting ComplexHeatmap R package (Gu et al., 2016).

Statistical analyses

All statistical analyses were performed using Prism version 6.0 (GraphPad Software, La Jolla, CA). Differences among means were evaluated by one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test, whereas two-tailed Student's t-test was used for comparisons between two groups. Correlations analysis were performed using Spearman correlation test. The data were considered statistically significant at $p < 0.05$.

6. References

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