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A positive feedback loop between the oncogenic WIP1 phosphatase and the transcription factor GLI1 regulates the Hedgehog signaling

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1. INTRODUCTION

1.1. THE HEDGEHOG-GLI SIGNALING PATHWAY

The Hedgehog (Hh) pathway is a conserved signaling pathway that plays a critical role during embryonic developement, as well as in several human disorders and diseases, including cancer. The first member of the Hedgehog pathway was identified in 1980 by Christiane Nusslein-Volhard and Eric Wieschaus who performed a series of genetic screens on the fruit fly Drosophila melanogaster in the late 1970s and early 1980s (Nusslein-Volhard, C. *et al.*, 1980). The genetic screens were based on chemically induced random mutations that were linked to various phenotypes in the easily observable developmental stages of the fruit fly, especially the larval body segmentation. Based on this technique, the two researchers identified more than 50 genes that were involved in embryonic development, and thus laid the basis for much of our current understanding of development in the fruit fly as well as vertebrates. Their work was recognized in 1995 when they earned the Nobel Prize in Physiology or Medicine together with the developmental geneticist Edward B. Lewis. One of the mutations they induced resulted in a distinct phenotype of short larva where the ventral cuticular denticles formed a dense lawn instead of the distinct segmented bands in the wild type. Due to the close resemblance between this stubby and “hairy” phenotype and a hedgehog, the gene harboring the loss-of-function mutation was named Hedgehog (Hh). Ten years after, it was cloned and its correspondent in vertebrates was discovered. Since then, huge progress has been achieved in disclosing the fundamental roles of Hedgehog in development and diseases.

1.1.1. Hedgehog ligands production, secretion and reception

The Hedgehog (HH) pathway is a highly conserved regulator of development, tissue patterning, cell proliferation and differentiation. In mammals it is activated by the

binding of the three ligands, Sonic HH (SHH), Desert HH (DHH) and Indian HH (IHH) to the transmembrane receptors Patched1 and Patched2 (PTCH1-2). All three proteins undergo several processing steps before being released as functional molecules. After the signal sequence is removed from the Hh precursor protein, the C-terminal domain catalyzes an intramolecular cleavage, with the addition of a molecule of cholesterol (Porter, J.A. *et al.*, 1995, 1996; Lee, J.J. *et al.*, 1994). This allows the association of the cholesterol-modified Hh to the membrane, which is subsequently further modified by the N-terminal addition of a molecule of palmitic acid by the hedgehog acyltransferase Skinny (Buglino, J.A. *et al.*, 2008; Pepinsky, R.B. *et al.*, 1998), which leads to the formation of the mature Hh ligand. Although Hh ligands are associated to the membrane, they can act at distances between 50 and 300μm (Zhu, A.J. *et al.*, 2004), thus creating an autocrine, paracrine and juxtacrine signaling network. Hh ligands are secreted by the producing cells with the involvement of the 12-span transmembrane protein Dispatched (Disp) (Burke, R. *et al.*, 1999; Ma, Y. *et al.*, 2002), metalloproteases (Caspary, T. *et al.*, 2002), the proteoglycans Dally and Dally-like (Dlp) (Beckett, K. *et al.*, 2008; Lum, L. *et al.*, 2003) and the enzymes Sulfateless and Tout velu (Bellaiche, Y. *et al.*, 1998; Toyoda, H. *et al.*, 2000; Koziel, L. *et al.*, 2004). Despite their homology, the three Hh ligands have different expression and functions. Dhh expression is restricted to gonads, including granulosa cells of ovary and sertoli cells of testis, where it is required for the formation of mature sperm (Bitgood, M.J. *et al.*, 1996; Yao, H.H. *et al.*, 2002; Wijgerde, M. *et al.*, 2005). Ihh is specifically expressed in primitive endoderm (Dyer, M.A. *et al.*, 2001), gut (van den Brink, G.R., 2007), and pre-hypertrophic chondrocytes involved in the growth plates of bones (Vortkamp, A. *et al.*, 1996; St-Jacques, B. *et al.*, 1999). Shh is the most broadly expressed mammalian Hh ligand, being expressed during early vertebrate embryogenesis in the node, notochord, and floor plate where it controls the patterning of the left–right and dorso-ventral axes of the embryo (Pagan-Westphal, S.M. *et al.*, 1998; Schilling, T.F. *et al.*, 1999; Meyer, N.P. *et al.*, 2003; Watanabe, Y. *et al.*, 2000). It is also involved in patterning of the distal

elements of the limbs (Riddle, R.D. *et al.*, 1993; Marti, E. *et al.*, 1995; Johnson, R.L. *et al.*, 1994; Chang, D.T. *et al.*, 1994) and in the development of most epithelial tissues during organogenesis.

In absence of ligands, the 12-span transmembrane receptor Patched (Ptch) inhibits the 7-span transmembrane receptor Smoothened (Smo) (Taipale, J. *et al.*, 2002), blocking signal transduction. It is not clear how Ptch regulates Smo, but it seems that Ptch prevents Smo accumulation to primary cilia (Rohatgi, R. *et al.*, 2007; Corbit, K.C. *et al.*, 2005). Because one molecule of Ptch inhibits several molecules of Smo, the inhibition seems to be catalytic (Taipale, J. *et al.*, 2002). The ligand binding to Ptch is regulated by several proteins, such as the Hh-interacting protein (Hip), which negatively regulates Hh signaling by competing with Ptch to bind Hh (Chuang, P.T. *et al.*, 1999) or Cdo and Boc, Gas1, and Glypican-3 which serve as co-receptors of Hh (Allen, B.L. *et al.*, 2007; Zhang, W. *et al.*, 2006; Tenzen, T. *et al.*, 2006; Okada, A. *et al.*, 2006; Seppala, M. *et al.*, 2007; Martinelli, D.C. *et al.*, 2007; Capurro, M.I. *et al.*, 2008; Yao, S. *et al.*, 2006). Upon ligand binding to Ptch, the receptor/ligand complex is translocated out of the primary cilium and internalized in endosomal vesicles, thus triggering the β-arrestin mediated mobilization of Smo into the cilium (Heretsch, P. *et al.*, 2010). Smo is a G-protein-coupled receptor (Ruiz-Gomez, A. *et al.*, 2007) and, upon pathway activation, it inhibits the suppressive action of different kinases on Gli factors. This leads to the activation of the downstream Hh effectors, the glioma-associated (Gli) family of transcription factors. The intracellular events involved in Hh pathway activation are not clear. Several molecules have been identified to be downstream of Smo in *Drosophila* but, despite the high conservation of Hh pathway, the molecular mechanisms of signal transduction from Smo to Gli are distinct between *Drosophila* and mammals.

1.1.2. The last mediators of the Hh pathway; the three Gli transcription factors

The Gli transcription factors (Gli1, Gli2, Gli3 in vertebrates, *Ci* in *Drosophila*) are members of the Kruppel family of transcription factors. They share five conserved C_2-H_2 zinc-finger DNA binding domains and a consensus histidine/cysteine linker sequence between zinc fingers, which bind to the consensus sequence 5'-TGGGTGGTC-3' in the promoter of target genes (Kinzler, K.W. *et al.*, 1988, 1990; Ruppert, J.M. *et al.*, 1991; Sasaki, H. *et al.*, 1997).

GLI1 gene maps in the 12q13.2-q13.3 locus and it spans 12kbp. GLI1 mRNA (NM_005269.2) contains 12 exons and it is translated into a 1065 AA protein (NP_00116108). GLI1 acts as potent transcriptional activator and it is not processed proteolytically like GLI2 and GLI3. GLI1 is a direct target of GLI2 (Ikram, M.S. *et al.*, 2004) and it is also directly regulated by HH signaling, thus representing the best read-out of pathway activation (Lee, J. *et al.*, 1997; Bai, C.B. *et al.*, 2004). GLI1 exists in two other isoforms. GLI1deltaN (NP_001153517.1) lacks the first two exons and it is translated from an internal AUG site. It has an expression level comparable to the GLI1 full length (FL), whereas in tumor cell lines is generally less expressed. Although it responds to HH pathway activation, GLI1DeltaN has generally a weaker capacity to activate transcription and is not localized only in the nucleus. GLI1deltaN is not activated by the dual-specificity tyrosine phosphorylation-regulated kinase 1 (Dyrk1), but it is negatively regulated by Suppressor of Fused (SuFu) (Shimokawa, T. *et al.*, 2008). tGLI1 (NP_001161081.1) lacks an in-frame segment of 123 bases (41 codons) spanning the entire exon 3 and part of exon 4 of the GLI1 gene and it is transcriptionally more potent than GLI1FL. tGLI1 is undetectable in normal cells but is high in glioblastoma multiforme (GBM), where it is associated with increased motility and invasiveness, and in other cancer cells (Lo, H.W. *et al.*, 2009).

GLI2 gene maps in the 2q14 locus and it spans 195kbp. GLI2 mRNA (NM_005270.4 contains 12 exons and it is translated into a 1586 AA protein (NP_005261.2). It has a N-terminal repressor domain and a C-terminal activator domain. GLI2 can act as activator or, in its C-terminal deleted form, as a repressor (Ruiz i Altaba, A., 1999, 1999). *GLI3* gene maps in the 7p13 locus and it spans 276kbp. GLI1 mRNA (NM_000168.5) contains 15 exons and it is translated into a 1580 AA protein (NP_000159.3). It acts mostly a repressor in its C-terminal cleaved form, although it can also have positive effects (Ruiz i Altaba, A., 1999, 1999).

1.1.3. Regulation of the Hedgehog-Gli signaling

Context-dependent differential modulation of the three Glis through cytoplasmic-nuclear shuttling, protein phosphorylation, ubiquitination, acetylation, and protein cleavage or degradation results in different transcriptional responses (Ruiz i Altaba, A. *et al.*, 2007). In absence of Hh ligands Gli2 and Gli3 are sequentially phosphorylated by protein kinase A (PKA), glycogen synthase kinase 3β (GSK3β) and caseine kinase1 (CK1). These events allow their recognition by the F-box protein β-TrCP, an E3 ubiquitin ligase that targets Gli2 and Gli3 to limited proteasome-dependent cleavage. The processing generates the repressor forms (Wang, B. *et al.*, 2000; Pan, Y. *et al.*, 2006; Bhatia, N. *et al.*, 2006), leading to the silencing of Hh targets. Gli1 is retained in the cytoplasm as a microtubule-associated inactive complex with other proteins and degraded by the proteasome. The β-TrCP E3 ubiquitin ligase recognizes two sequences on Gli1 (degron N and C) and induces its proteasome degradation (Huntzicker, E.G. *et al.*, 2006). Also Numb targets Gli1 for proteasome degradation through the Itch E3 ubiquitin ligase (Di Marcotullio, L. *et al.*, 2006). In the presence of Hh ligands, Gli2 and Gli3 are no longer cleaved and act as activators. Gli1 is transcriptionally activated, possibly by preexisting Gli2 and Gli3, and translocates into the nucleus where it transactivates Hh target genes (Fig.1).

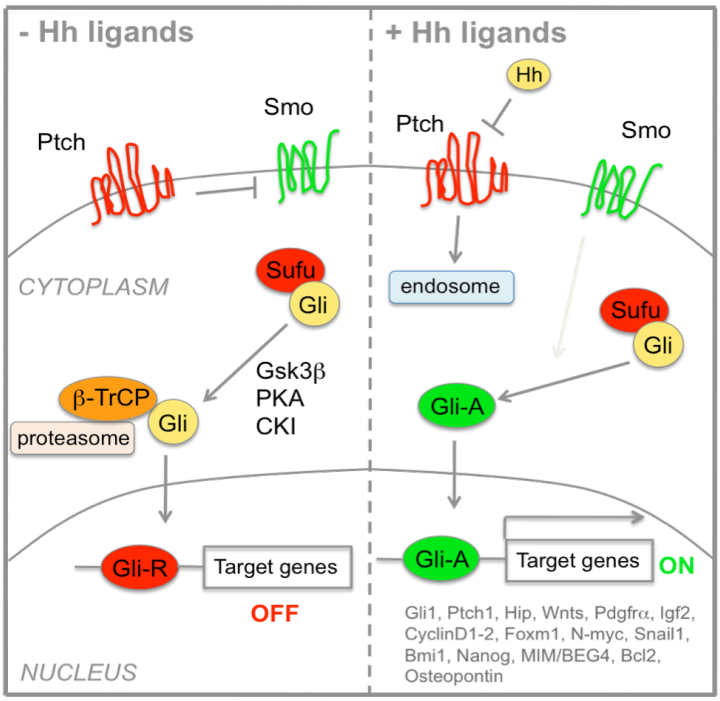


Figure 1 *Hedgehog signaling pathway. Hh ligands bind to Ptch receptor and activate the pathway leading to the induction of the Gli transcription factors target genes.*

The modulation of Hh pathway involves a number of other factors, which affect the localization or modify the Gli proteins. Suppressor of Fused (SuFu) is the main negative regulator of Hh pathway. It associates with and controls the nuclear-cytoplasmic shuttling (Kogerman, P. *et al.*, 1999; Ding, Q. *et al.*, 1999; Dunaeva, M. *et al.*, 2003; Merchant, M. *et al.*, 2004) and the degradation (Yue, S. *et al.*, 2009) of the three Glis. It also recruits the histone deacetylase complex SAP18-mSin3 to occupy the Gli binding sites on the DNA, repressing transcription (Paces-Fessy, M. *et al.*, 2004; Cheng, S.Y. *et al.*, 2002). SuFu recruits GSK3β for Gli3 processing, leading to the formation of the repressor form (Kise, Y. *et al.*, 2009), and interacts with Rab23 to inhibit Gli1 transcriptional activity (Chi, S. *et al.*, 2012). Sufu is phosphorylated at Ser-342 and Ser-346 by GSK3β and PKA, respectively. Phosphorylation at this dual site stabilizes it against SuFu degradation through the ubiquitin-proteasome system induced by Shh signaling (Yue, S. *et al.*, 2009; Chen, Y. *et al.*, 2011).

Similarly to SuFu, REN(KCTD11) negatively regulates the Hh pathway, by counteracting Gli1 nuclear translocation (Di Marcotullio, L. *et al.*, 2004). PKA is another important negative regulator of Hh signaling. It phosphorylates Gli1 protein on Thr374, close to the nuclear localization signal, retaining it in the cytoplasm, inhibiting its transcriptional activity (Sheng, T. *et al.*, 2006). PKA also phosphorylates Gli1 and Gli2 in their C-terminal region, and this modification acts as a priming event for CK1 and GSK3β subsequent phosphorylations. The phosphorylated Glis then interacts with and are ubiquitinated by βTrCP in the SCF ubiquitin-ligase complex, which leads to their degradation by the proteasome (Pan, Y. *et al.*, 2006; Huntzicker, E.G. *et al.*, 2006). PKA also phosphorylates Smo, an this modification induces a further phosphorylation by CKI. Interestingly the de/phosphorylation of Smo seems to be important to transduce graded Hh signaling during development. In fact protein phosphatase 1 (PP1) directly dephosphorylates PKA-phosphorylated Smo site to reduce signaling mediated by intermediate concentrations of Hh, whereas PP2A specifically dephosphorylated PKA-primed, CKI-phosphorylated Smo to restrict signaling by high concentrations of Hh (Su, Y. *et al.*, 2011). The dual specificity Yak1-related kinase 2 (Dyrk2) also inhibits Hh signaling, by directly phosphorylating Gli2 and inducing its proteasome-dependent degradation (Varjosalo, M. *et al.*, 2008). Dyrk1b inhibits Gli2 transcriptional activity and promotes Gli3 processing into the repressor form (Lauth, M. *et al.*, 2010). The regulatory protein 14-3-3 interacts with the three Gli proteins and decreases their transcriptional activity. The interaction depends on their PKA-dependent phosphorylation (Ser640 on Gli1, Ser956 on Gli2, Ser1006 on Gli3) and the phosphorylation sites responsible for the binding to 14-3-3 are distinct from those required for PKA-dependent proteolysis (Asaoka, Y. *et al.*, 2010).

Acetylation of Gli1 and Gli2 has been shown to have an inhibitory effect on their transcriptional activity; in fact, deacetylation of Gli1 and Gli2 mediated by the Hh target HDAC1 promotes transcriptional activation and enhances cellular proliferation and

transformation (Canettieri, G. *et al.*, 2010). Among the positive regulators of Gli factors, the nuclear kinase Dyrk1a enhances Gli1-dependent gene transcription, in part by retaining Gli1 in the nucleus (Mao, J. *et al.*, 2002) and the Ser/Thr kinase ULK3 phosphorylates Gli proteins, increasing their activity (Maloverjan, A. *et al.*, 2010). STK36, the mammalian homolog of *Drosophila* Fused, antagonizes the suppressive function of SuFu (Murone, M. *et al.*, 2000), contributing to Hh pathway activation. The actin-binding protein Missing in Metastasis (MIM), a Hh-responsive gene, is also part of a Gli/SuFu complex and potentiates Gli-dependent transcription (Callahan, C.A. *et al.*, 2004).

HH pathway is also regulated by microRNAs. miR125b, miR324-5p and miR-326 functionally suppress Smo. In cerebellar granule prercursors (GCP) and medulloblastoma miR-324-5p also targets Gli1 (Ferretti, E. *et al.*, 2008) and the miR-17/92 cluster has been shown to synergize with Shh (Northcott, P.A. *et al.*, 2009; Uziel, T. *et al.*, 2009).

Gli targets are only partially known. However, they include genes involved in proliferation and differentiation (e.g. CyclinD1 and D2, N-Myc, Wnts, PdgfRa, Igf2, FoxM1, Hes1) (Kenney, A.M. *et al.*, 2000; Mullor, J.L. *et al.*, 2001; Dahmane, N. *et al.*, 1997; Ingram, W.J. *et al.*, 2008; Teh, M.T. *et al.*, 2002), survival (Bcl2) (Regl, G. *et al.*, 2004), self-renewal (Bmi1, Nanog) (Leung, C. *et al.*, 2004; Clement, V. *et al.*, 2007; Stecca, B. *et al.*, 2009), angiogenesis (Vegf) (Pola, R. *et al.*, 2001), epithelial-mesenchymal transition (Snail1, Sip1, Elk1 and Msx2) (Li, X. *et al.*, 2006; Ohta, H. *et al.*, 2009; Varnat, F. *et al.*, 2009) and invasiveness (Osteopontin) (Das, S. *et al.*, 2009). Interestingly, Gli transcription factors also control the expression of Hh target genes, including Ptch, Hip, Gas1, and Gli1, thus providing negative (by Hh-mediated Ptch and Hip induction) and positive (by Hh-mediated Gli1 induction and Gas1 dowregulation) feedback for Hh signaling.

1.1.4. Modulation of the Hedgehog-Gli signaling by oncogenic inputs

Beside the canonical Hh signaling, GLI1 can be regulated in a ligand-independent manner by several non-Hedgehog pathways, acting downstream of Smo. Oncogenic K-Ras expression increases GLI1 activity through RAF/MEK/MAPK signaling, enhancing its nuclear localization (Stecca, B. *et al.*, 2007; Seto, M. *et al.*, 2009). Consistently, GLI1 ablation also reduces the ability of K-Ras to induce cell transformation, indicating that GLI1 is able to mediate the tumorigenic effects of constitutive K-Ras activation (Ji, Z. *et al.*, 2007). Like K-Ras, also AKT signaling induces nuclear translocation and transcriptional activation of GLI1 in melanoma (Stecca, B. *et al.*, 2007). PI3K/AKT activation inhibits GLI2 phosphorylation mediated by PKA, thus enhancing GLI2-dependent transcription. Consistently, the negative PI3K/AKT regulator PTEN, which is frequently mutated in human cancer, inhibits GLI1 transcriptional activity in glioblastoma (Xu, Q. *et al.*, 2008).

Activated mTOR/S6K1 pathway downstream of TNF-α promotes GLI1 transcriptional activity and oncogenic function through S6K1-mediated GLI1 phosphorylation at Ser84, which induces the release of Gli1 from SuFu (Wang, Y. *et al.*, 2012). TGF-β also leads to Hh pathway activation, by inducing in a Smad3 and Smad4-dependent manner GLI2 expression, which in turn increases GLI1 expression (Dennler, S. *et al.*, 2007). A negative reciprocal regulation is observed between GLI1 and the tumor suppressor p53. p53 inhibits the activity, nuclear localization and protein levels of GLI1 Conversely, Hh signaling inhibits p53 by inducing activating phosphorylations on Ser166 and Ser186 on MDM2, thus enhancing p53 degradation (Stecca, B. *et al.*, 2009; Abe, Y. *et al.*, 2008). Notch pathway inhibits Hedgehog signaling. The Notch target Hes1 is a repressive transcription factor that binds GLI1 first intron, thus inhibiting GLI1 expression (Schreck, K.C. *et al.*, 2010). Activation of the ERα pathway promotes cell proliferation by inducing Shh expression and thus

activating the Hh pathway in a ligand-dependent manner (Kameda, C. *et al.*, 2010). In Ewing sarcoma GLI1 is directly induced by the oncogenic transcription factor EWS/FLI derived from the translocation between chromosomes 11 and 22 which characterizes this tumor (Beauchamp, E. *et al.*, 2009). Other positive modulators of Gli function are the tyrosine kinase receptors for the epidermal growth factor (EGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) and insulin growth factor (Kasper, M. *et al.*, 2006; Palma, V. *et al.*, 2004, 2005; Brewster, R. *et al.*, 2000).

1.1.5. The Hedgehog-Gli signaling pathway in development

The developmental processes controlled by Hh signaling in *Drosophila* and vertebrates are highly conserved (Ingham, P.W. *et al.*, 2001). During embryogenesis, specific functions are required at different stages; a single morphogen, Hh, is able to trigger a large number of responses in virtue of the type of the responding cell, the dose of Hh received, and the time the cell is exposed to Hh. The diverse cellular responses are the result of the induction of different sets of target genes, which control cell proliferation, survival, cell fate determination, epithelial-to-mesenchimal transitions (EMT), rearrangement of cell motility and adhesion properties and self-renewal (Rowitch, D.H. *et al.*, 1999; Barakat, M.T. *et al.*, 2010). The pivotal role of Hh signaling during development is evident in mammalian models lacking Hh components. $Shh^{-/-}$ mice show cyclopia, defects in ventral neural tube, distal limb malformation, absence of spinal column and most of the ribs, and failure of lung branching (Chiang, C. *et al.*, 1996). Transgenic mice overexpressing Shh in the skin develop many features of the basal cell nevus syndrome and BCC within the first 4 days of skin development (Oro, A.E. *et al.*, 1997). Shh deletion leads to foregut defects, esophageal atresia/stenosis, tracheoesophageal fistula and lung anomalies. The lung mesenchyme shows enhanced cell death, decreased cell proliferation, and downregulation of Shh target genes, indicating that Shh is required for the growth and differentiation of the esophagus, trachea, and lung (Litingtung, Y. *et al.*, 1998). Shh null mice have

hypoplasia and midline fusion of the first pharyngeal arch due to defective epithelial-mesenchymal signaling (Yamagishi, C. *et al.*, 2006). Ptch1$^{-/-}$ mice die during embryogenesis, have abnormal heart development and open and overgrown neural tubes. Ptch1 and Gli are derepressed in the ectoderm and mesoderm, but not in the endoderm and Shh targets are aberrantly expressed in dorsal and lateral neural tube cells. Ptch1$^{+/-}$ mice are larger than normal, and develop hindlimb defects or cerebellar medulloblastomas (Goodrich, L.V. *et al.*, 1997). They show a phenotype similar to Gorlin syndrome patients, with high incidence of rhabdomyosarcomas and increased incidence of radiation-induced teratogenesis (Hahn, H. *et al.*, 1998). Ptch1$^{+/-}$ mice are predisposed to develop soft tissue tumors, medulloblastoma, and skin tumors (Goodrich, L.V. *et al.*, 1997; Mao, J. *et al.*, 2006). They are susceptible to BCC development following UV irradiation or ionizing radiation, even though they rarely develop full-grown BCCs if kept under normal conditions (Aszterbaum, M. *et al.*, 1999). Smo$^{+/-}$ mice show cyclopia, absence of visible left/right asymmetry, an open gut and a small, linear heart tube (Zhang, X.M. *et al.*, 2001).

Ablation of SuFu in mice leads to embryonic lethality with cephalic and neural tube defects. SuFu$^{-/-}$ mouse embryo fibroblasts (MEFs) show high Gli-mediated Hh pathway activity that can not be modulated by Smo and that can only partially be blocked by PKA activation. SuFu$^{+/-}$ mice develop a skin phenotype with basaloid changes and jaw keratocysts, phenotypes similar to Ptch1 inactivation (Svard, J. *et al.*, 2006). Gli3 mutant mice have central nervous system (CNS) and lung defects and polydactyly associated with increased Shh function (Franz, T., 1994; Grindley, J.C. *et al.*, 1997; Schimmang, T. *et al.*, 1992; Grove, E.A. *et al.*, 1998). Gli2 mutants in the zinc finger domain (Gli2^{zfd}) have numerous skeletal defects (Mo, R. *et al.*, 1997) and abnormal lungs (Motoyama, J. *et al.*, 1998). Gli2$^{-/-}$ mice have diminished response to the notochordal Shh signal; they do not develop the neural tube, the floor plate and the adjacent ventral intermediate region (VIR) cells in the spinal cord, although they still

develop motor neurons (Park, H.L. *et al.*, 2000; Matise, M.P. *et al.*, 1998). Gli2/Gli3 double mutant mice have skeletal and lung defects that are more extreme than either homozygous phenotype (Mo, R. *et al.*, 1997; Motoyama, J. *et al.*, 1998). Mice homozygous for a Gli1 mutation in the zinc finger domain ($Gli1^{zfd}$) are viable and appear normal, although they can not induce SHH targets in the dorsal brain. $Gli1^{zfd/zfd}$;$Gli2^{zfd/+}$, but not $Gli1^{zfd/zfd}$;$Gli3^{zfd/+}$, double mutants die soon after birth and have multiple defects including a variable loss of ventral spinal cord cells and smaller lungs, indicating that during development Gli2 can rescue Gli1 loss (Park, H.L. *et al.*, 2000).

In the adult, the HH pathway is mostly quiescent, but it participates in stem cell maintenance, tissue repair and regeneration (Clement, V. *et al.*, 2007; Teglund, S. *et al.*, 2010). HH pathway is an important regulator of the behavior of neural stem cells in neurogenic niches of the subgranular zone of the hippocampus and the subventricular zone of the lateral ventricle of the forebrain, increasing neurosphere size and number (Ahn, S. *et al.*, 2005; Lai, K. *et al.*, 2003; Machold, R. *et al.*, 2003; Palma, V. *et al.*, 2005) as well as other stem cells (Trowbridge, J.J. *et al.*, 2006). It regulates the growth of the external germinal layer of the cerebellum, which contains granule progenitor cells (GPC). Shh produced by Purkinje neurons induces GPC proliferation (Dahmane, N. *et al.*, 1999; Wechsler-Reya, R.J. *et al.*, 1999; Wallace, V.A. *et al.*, 1999) and the differentiation of the Bergmann glia (Dahmane, N. *et al.*, 1999).

1.1.6. The Hedgehog-Gli signaling pathway in cancer

HH pathway is aberrantly activated in a variety of human cancers. The aberrant activation of HH pathway may be the result of inactivation of the repressors PTCH1 or SUFU, of constitutive activation of the effector SMO, or by GLI1 or GLI2 gene amplification (Hahn, H. *et al.*, 1996; Taylor, M.D. *et al.*, 2002; Johnson, R.L. *et al.*, 1996; Snijders, A.M. *et al.*, 2005; Xie, J. *et al.*, 1998; Kinzler, K.W. *et al.*, 1987). In other cases, HH responses can be triggered by the non-canonical activation of the GLIs by

oncogenes such as AKT or K-RAS, as in the case of melanoma (Stecca, B. *et al.*, 2007). Constitutively active HH signaling affects most aspects of tumorigenesis. It increases cell proliferation, stem cell and cancer stem cell proliferation by inducing cell cycle regulators such as Cyclin-D1, Cyclin-D2, Cyclin-B1, p21, Bmi-1 and telomerase activity; it inhibits cell death and enhances cell survival by controlling the expression of pro-survival genes. Active HH signaling induces EMT and promotes mobility, invasiveness and aggressive metastatic behavior by regulating Snail, metalloproteases (MMPs) and adhesion molecules; it supports angiogenesis by controlling VEGF pathway. It also interacts with other cellular pathways involved in development, resulting in reduced differentiation of tumor cells (Kar, S. *et al.*, 2012).

The HH pathway is deregulated in different types of human cancers. It is required for sustained growth and survival of glioma cancer cells and it regulates the expression of stemness genes and self renewal of CD133+ cancer stem cells (Clement, V. *et al.*, 2007). In medulloblastoma unrestrained HH signaling is associated to loss-of-function mutations of PTCH and SUFU, to gain-of-function mutations of SMO and to 17p deletion, resulting in loss of p53 or activation of HH targets such as Cyclin-D1 and Cyclin-D2. The consequent uncontrolled proliferation of immature cerebellar granule neuron precursors cells (CGNPs) leads to medulloblastoma formation (Ferretti, E. *et al.*, 2005; Michael, L.E. *et al.*, 2008; Rudin, C.M. *et al.*, 2009).

HH signaling plays also a role in the exaggerate de novo lipid synthesis that characterizes this tumor, because SHH induces the E2F1-mediated expression of the lipogenic enzyme fatty acid synthase (FASN) (Bhatia, B. *et al.*, 2011). In neuroblastoma the constitutive HH pathway activation due to elevated expression of HH pathway components and downstream targets leads to increased tumorigenicity and colony formation, continued cell cycle progression and renewal of cancer stem cells (Mao, L. *et al.*, 2009; Shahi, M.H. *et al.*, 2008). In neuroendocrine tumors of the

gastrointestinal tract upregulation of HH targets induces tumor proliferation, reduced apoptosis, EMT and increases metastatic and invasive potential (Fendrich, V. *et al.*, 2007). In breast cancer abnormal HH signaling increases tumor growth and invasiveness and the proliferation of mammary stem cell progenitors, by increasing B lymphoma Mo-MLV insertion region homolog 1 (Bmi-1) levels (Hatsell, S. *et al.*, 2007; Kasper, M. *et al.*, 2009). In colon cancer activation of HH pathway is due to PTCH or SMO mutations as well as to ligand-independent mechanisms, such as K-RAS or B-RAF mutations that increase the RAS/RAF/MEK/ERK signaling. Autocrine HH-GLI1 signaling promotes colon cancer growth, stem cells self-renewal and metastatic behavior (Varnat, F. *et al.*, 2009). In gastrointestinal cancer HH pathway activation leads to poor differentiation, increased proliferation, mobility, invasiveness of cancer cells (Ma, X. *et al.*, 2005; Saqui-Salces, M. *et al.*, 2010). In lung cancer growth is fueled by autocrine HH signaling (Velcheti, V. *et al.*, 2007). Abnormal pathway activation is driven by silencing of SUFU by promoter methylation (Chi, S. *et al.*, 2012), by dysfunction of Rab23, which is localized in the nuclei and by localization of SMO both in the membrane and in the cytoplasm (Huang, T.H. *et al.*, 2011). In ovarian cancer high levels of GLI1 and GLI2 are observed, with consequent increase in cell proliferation, migration, angiogenesis and resistance to cell death (Bhattacharya, R. *et al.*, 2008; Chen, X. *et al.*, 2007; Liao, X. *et al.*, 2009).

The HH signaling pathway plays an essential role in pancreatic cancer. It synergizes with K-RAS signaling in the initiation steps of tumorigenesis and reduces the dependence of tumor cells on the activation of the MAPK and mTOR signaling (Morton, J.P. *et al.*, 2007). Overexpression of the SHH ligand and autocrine stimulation is frequently observed and results from the constitutive activation of NF-kB (Nakashima, H. *et al.*, 2006). Sustained HH activation contributes to the induction of desmoplasia, a characteristic of tumor microenvironment in pancreatic cancer (Xu,

F.G. *et al.*, 2009), and regulates self renewal and resistance to chemotherapy in cancer stem cells (CSCs) (Yang, L. *et al.*, 2010).

Autocrine and paracrine HH stimulation is also observed in prostate cancer, where it drives androgen-independent growth of cancer cells by directly interacting with the androgen receptor, thus compensating or even substituting androgen signaling (Levina, E. *et al.*, 2012; Kumar, S.K. *et al.*, 2008; Chen, G. *et al.*, 2011). Aberrant HH activation is observed in bladder cancer (Fei, D.L. *et al.*, 2010; Mechlin, C.W. *et al.*, 2010), cervical cancer (Chan, D.W. *et al.*, 2011; Xuan, Y.H. *et al.*, 2006), head and neck cancer (Chung, C.H. *et al.*, 2011), liver cancer (Huang, S. *et al.*, 2006; Cheng, W.T. *et al.*, 2009) and uterine endometrial cancer (Feng, Y.Z. *et al.*, 2007; Riobo, N.A. *et al.*, 2006), where constitutive pathway activation increases proliferation, cell motility and invasiveness, and in esophageal cancer (Ma, X. *et al.*, 2006; Yang, L. *et al.*, 2011, 2012) and leukemia (Queiroz, K.C. *et al.*, 2010; Zhao, C. *et al.*, 2009), where it is involved in resistance to chemotherapy or radiation. HH pathway has an important role in skin cancer. Transgenic mice expressing Shh or Gli2 develop multiple BCC-like epidermal proliferations (Aszterbaum, M. *et al.*, 1999; Grachtchouk, M. *et al.*, 2000). Expression of Gli2 under the control of a keratin 5 (K5) promoter in mice results in development of BCC, whereas expression of Gli1 under the same promoter leads to the formation of hair follicle-derived neoplasias, such as trichoepitheliomas, cylindromas, and trichoblastomas and in part of BCC. Inactivating mutations of PTCH1 are found in sporadic basal cell carcinoma (BCC) (Gailani, M.R. *et al.*, 1996; Kim, M.Y. *et al.*, 2002) and in Gorlin syndrome (Hahn, H. *et al.*, 1996; Johnson, R.L. *et al.*, 1996), whose patients are strongly predisposed to the development of BCC in the skin at early age and to medulloblastoma. Beside PTCH1 inactivation, mutations of SMO, SUFU (Xie, J. *et al.*, 1998; Reifenberger, J. *et al.*, 1998) and abnormalities of HH pathway transcription factors GLI1 (Nilsson, M. *et al.*, 2000) and GLI2 (Grachtchouk, M. *et al.*, 2000) have been observed. Alteration of HH pathway associated with PTCH1

mutations has been observed in squamous cell carcinoma (SCC), although the link between SCC and HH pathway is not as strong as in BCC (Michimukai, E. *et al.*, 2001), and in Merkel cell carcinoma, where the signaling is strongly activated (Brunner, M. *et al.*, 2010).

Aberrant HH pathway activation plays an important role in melanoma, although no genetic alterations in HH genes have been found (Chin, L. *et al.*, 2006). HH-GLI signaling is required for the proliferation of human melanocytes and regulates the proliferation and survival of human melanomas; indeed, growth, recurrence and metastasis of melanoma xenografts in mice are prevented by treatment with the SMO antagonist cyclopamine (Stecca, B. *et al.*, 2007). Expression of GLI1 and osteopontin (OPN), a GLI1 direct transcriptional target, is correlated with tumor progression and metastasis of human melanomas (Das, S. *et al.*, 2009). A preclinical study showed that high expression of GLI2 is associated with an invasive and metastatic phenotype *in vitro* and *in vivo*, because melanoma cells with high GLI2 expression metastasize to bone more readily than cells with low GLI2 expression and because GLI2 silencing blocks melanoma bone metastasis (Alexaki, V.I. *et al.*, 2010). High levels of HH pathway components and of embryonic pluripotent stem cell factors SOX2, NANOG, OCT4, and KLF4 are expressed in melanomaspheres, which are enriched in melanoma cancer stem/tumor-initiating cells (CSC/TIC). Inhibition of HH signaling results in a significant decrease in melanoma stem cell self-renewal *in vitro* and tumorigenicity *in vivo* (Santini, R. *et al.*, 2012).

1.1.7. Inhibitors of the Hedgehog-Gli pathway

Because of its heavy implications in human cancer, HH pathway is a good target for cancer therapy, and several HH inhibitors interfere with tumorigenesis and tumor progression. HH inhibitors target the pathway at three levels: *i)* neutralizing the activity of HH ligands; *ii)* inhibiting SMO; *iii)* inhibiting GLIs transcription factors. To

block SHH, a SHH-specific monoclonal antibody (5E1) has been developed and it blocks the growth of some tumors, including small-cell lung carcinoma (Watkins, D.N. *et al.*, 2003).

Most HH inhibitors commonly used target SMO. Cyclopamine, an alkaloid extracted from *Veratum Album*, is the most commonly used SMO antagonist, although it is not suitable as therapeutic agent because of poor solubility, low potency, nonspecific toxicity and chemical instability (Lipinski, R.J. *et al.*, 2008). Other SMO antagonists include SANTs 1-4 (Chen, J.K. *et al.*, 2002), KAAD-cyclopamine (Taipale, J. *et al.*, 2000), compound-2 and compound-5 (Borzillo, G.V. *et al.*, 2005), vitamin D3, that directly binds SMO, and curcumin. CUR-61414 (Curis/Genentech) (Frank-Kamenetsky, M. *et al.*, 2002) failed a Phase I clinical trial, whereas IPI-926 (Olive, K.P. *et al.*, 2009), BMS833923/XL139 (Bristol Myers Squibb/Exelixis), PF-04449913m (Pfizer), TAK-441 (Millennium), LDE225 and LEQ506 (Novartis) are in clinical trials (Low, J.A. *et al.*, 2010). GDC-0449 (Genentech/Roche/Curis) is a potent orally bioavailable SMO inhibitor in Phase II trials effective against BCC and medulloblastoma, although a resistance mechanism based on SMO mutation in medulloblastoma has been described (Yauch, R.L. *et al.*, 2009; Amin, S.H. *et al.*, 2010; Rudin, C.M. *et al.*, 2009).

GANT-58 and GANT-61 are the drugs that block specifically GLI1 by inhibiting its transcriptional activity (Lauth, M. *et al.*, 2007). Also arsenic trioxide (ATO), an already approved therapeutic, directly inhibits GLI proteins. It blocks the accumulation of GLI2 in cilia, ultimately resulting in reduced protein levels (Kim, J. *et al.*, 2010), and by directly binding to and inhibiting GLI1 (Beauchamp, E.M. *et al.*, 2011). Because HH pathway is also activated by other cellular pathways in cancer, and because it sustains the self renewal of CSCs, it is easy to predict that combinatorial therapies with HH

inhibitors and conventional cancer drugs may result in a synergistic inhibition of tumor growth and prevent the tumor relapse.

1.2. THE ONCOGENIC WIP1 PHOSPHATASE

WIP1 (*PP2Cδ, PPM1D*) is a nuclear Ser/Thr phosphatase of the PP2C family of phosphatases identified in a screening of p53-induced genes after DNA damage (Fiscella, M. *et al.*, 1997). *WIP1* gene maps in the 17q23.2 locus and it spans 86kbp. WIP1 mRNA (NM_003620.3) contains 6 exons and it is translated into a 605 AA protein (NP_003611.1). The protein contains an evolutionarily conserved PP2C phosphatase catalytic domain in the central part of the protein (AA 67-368). The N- and C-terminal domains are not present in other eukaryotic PP2C proteins and share no homology with any known proteins; they are likely responsible for the intracellular targeting and for the interactions with other proteins (Choi, J. *et al.*, 2000). Murine *Wip1* gene spans over 36kb of DNA and encodes a 598 aminoacids protein. Human and murine Wip1 proteins show an overall similarity of 86% and have several extensive regions that exhibit complete identity (Choi, J. *et al.*, 2000). The *PPM1D* gene is also conserved in chimpanzee, Rhesus monkey, dog, cow, rat, chicken, and Zebrafish. Recently, a shorter WIP1 splice isoform of 430 AA has been described, which is expressed exclusively in testis and leukocytes, where it might exert a specific function on immune response and/or spermatogenesis. This shorter isoform contains the first 420 aminoacids of the full-length WIP1 plus additional 10 specific aminoacids and it retains phosphatase activity (Chuman, Y. *et al.*, 2009).

1.2.1. Biochemical characterization of WIP1 activity and substrate specificity

WIP1 protein phosphatase activity requires the presence of Mg^{2+}/Mn^{2+} and it is insensitive to treatment with okadaic acid (OA), that inhibits PP1, PP2a and PP2B (Fiscella, M. *et al.*, 1997). Biochemical studies characterized the substrate specificity of

the human WIP1, which preferentially dephosphorylates pSQ/pTQ or pTXpY motifs surrounded by acidic, hydrophobic, or aromatic amino acids, whereas basic aminoacids have a negative influence on substrate dephosphorylation (Yamaguchi, H. *et al.*, 2005, 2007).

1.2.2. Regulation of WIP1 expression

Wip1 is expressed ubiquitously in adult tissues, with the highest levels in testis, suggesting an involvement in basic cellular functions (Choi, J. *et al.*, 2000). It is also expressed throughout mouse pre-implantation development, where it is regulated by p38MAPK pathway (Hickson, J.A. *et al.*, 2007), and during embryonic development (Choi, J. *et al.*, 2000). The constitutive *WIP1* expression is controlled by cAMP-response element-binding protein (CREB) (Rossi, M. *et al.*, 2008) and E2F (Hershko, T. *et al.*, 2006). Indeed, CREB and E2F1 have been reported to directly bind *WIP1* promoter, which contains a CRE (CREB response element) and an E2F response element.

WIP1 expression is induced by many environmental factors. In response to estrogen signaling, estrogen receptor (ER) alpha directly binds to estrogen response elements in WIP1 promoter, inducing the expression of the phosphatase (Han, H.S. *et al.*, 2009). TNFα (tumor necrosis factor-alpha) treatment and LPS (lipopolysaccaride) stimulation increase *WIP1* expression by activating NF-kB, which directly binds a NF-kB site in *WIP1* promoter (Lowe, J.M. *et al.*, 2010). Stressing agents such as ionizing radiation, UV, anisomycin, H_2O_2, methyl methane sulfonate also increase *WIP1* expression (Fiscella, M. *et al.*, 1997; Takekawa, M. *et al.*, 2000; Park, H.K. *et al.*, 2011). Following ionizing radiation and UV exposure, activated p53 binds to a conserved response element in the 5' untranslated region (5'-UTR) of the *WIP1* gene, resulting in the induction of a transcript with a 5'-UTR shorter compared to the one produced by constitutive expression, indicating increased utilization of a downstream

initiation sites (Rossi, M. *et al.*, 2008). UV induction of WIP1 requires p38MAPK activity in addition to wild-type p53, because p38MAPK inhibitors SB203580 and SB202190 prevent WIP1 induction after UV exposure (Takekawa, M. *et al.*, 2000; Song, J.Y. *et al.*, 2010). WIP1 increase after UV irradiation also involves c-Jun, which binds WIP1 promoter at a later time than p53 and whose inhibition prevents WIP1 induction (Song, J.Y. *et al.*, 2010).

WIP1 expression after DNA damage is also controlled by microRNA (miR)-16, which is rapidly induced upon DNA damage and controls the timing of WIP1 mRNA translation, preventing a premature inactivation of ATM/ATR signaling and allowing a functional completion of the early DNA damage response. miR-16 regulation of WIP1 phosphatase is also important in mammary tumorigenesis. Indeed, miR-16 is markedly down-regulated in mammary tumor stem cells and its overexpression suppresses the self-renewal and growth of mouse mammary tumor stem cells and sensitizes MCF-7 human breast cancer cells to the chemotherapeutic drug doxorubicin (Zhang, X. *et al.*, 2009). Regulation of WIP1 expression is also controlled by miR-29, which is up-regulated after DNA damage induced by drugs such as doxorubicin or by aging (Ugalde, A.P. *et al.*, 2011).

1.2.3. WIP1 as a homeostatic regulator of stress responses

WIP1 expression is induced by environmental stresses. However several studies indicated that WIP1 acts as a homeostatic regulator of the DNA damage response, facilitating the return of the cell to a normal pre-stress state following repair of damaged DNA (Lu, X. *et al.*, 2005, 2008). This homeostatic function may be partially responsible for the oncogenic effects of WIP1 when it is amplified and overexpressed in human tumors and can easily be explained by the nature of WIP1 targets, most of which are involved in DNA-damage response. The main WIP1 target is the tumor suppressor p53, that it is directly dephosphorylated on Ser15 and inactivated by WIP1

(Lu, X. *et al.*, 2005). The mutual regulation between WIP1 and p53 is tightly regulated. UVC irradiation, for example, produces a dose-dependent response of WIP1, that correlates with the cellular response. Low doses of UVC, which stimulate intra-S phase cell cycle arrest, transiently induce WIP1 levels in a p53-dependent manner and WIP1 dephosphorylates p53 after damage repair. In contrast, higher doses of UVC fail to induce WIP1 expression, and the cell undergo apoptosis (Xia, Y. *et al.*, 2011). Also under non-stress conditions, WIP1 plays a critical role in p53-mediated cell/tissue homeostasis. WIP1 induces G2/M arrest in cells with wild-type but not mutant p53, thus enabling normal cells to be ready for mitosis and avoid mitotic catastrophe (Park, H.K. *et al.*, 2011) .

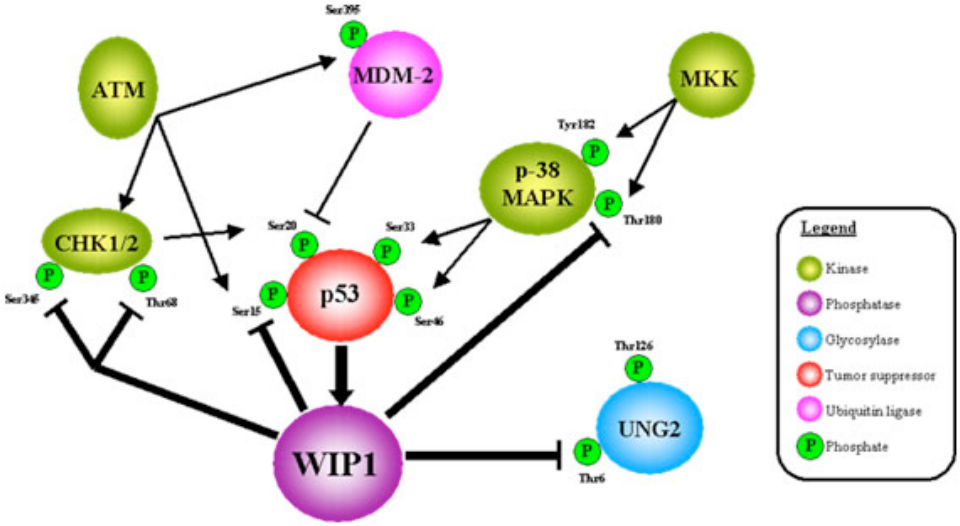


Figure 2 *WIP1 directly and indirectly controls p53 phosphorylation (from Schito, M.L. et al., 2006).*

WIP1 inhibits p53 activity not only by directly dephosphorylating it on Ser15, but also by controlling the activity of a variety of proteins that in turn activate or stabilize p53, such as CHK1, CHK2, ATM, p38MAPK, HDM2, MDMX (Fig.2). WIP1 dephosphorylates CHK1 on Ser345 and CHK2 on Thr68, leading to decreased CHK1 and CHK2 kinase activity and reduced intra-S and G2/M checkpoint arrest in response to DNA damage induced by ultraviolet and ionizing radiation (Lu, X. *et al.*, 2005; Oliva-Trastoy, M. *et al.*, 2007; Fujimoto, H. *et al.*, 2006). WIP1 dephosphorylates Ser1981 of

ATM (which in turn dephosphorylates CHK1, CHK2, HDM2, MDMX and p53) and inhibits its kinase activity, thus resulting in impaired DNA damage response (Shreeram, S. *et al.*, 2006; Lu, X. *et al.*, 2005) (Fig.3). WIP1 is critical for regulating the ATM-mediated tumor surveillance network because $Wip1^{-/-}$ mice show delayed onset of Eμ-myc-induced B cell lymphomas (Shreeram, S. *et al.*, 2006). Consistently, $Wip1^{-/-}$ and $Atm^{-/-}$ mice do not show radiation sensitivity, fertility defects and high incidence of T-cell lymphoma caused by ATM deficiency and show enhanced p53 and DNA damage responses and reduced chromosomal instability compared to $Atm^{-/-}$ mice (Darlington, Y. *et al.*, 2012). WIP1 dephosphorylates p38MAPK on Thr180 and inactivates it. This results in reduced p38MAPK-mediated p53 phosphorylation at Ser33 and Ser46 after UV radiation and creates a negative feedback regulation of p38MAPK-p53 signaling that contributes to suppress the UV-induced apoptosis (Takekawa, M. *et al.*, 2000) (Fig.3). Wip1 directly dephosphorylates Mdm2 on Ser395 (Lu, X. *et al.*, 2007) and MdmX on Ser403 (Zhang, X. *et al.*, 2009), two sites phosphorylated by the ATM kinase. This leads to stabilization of Mdm2 and MdmX proteins, thus resulting in increased p53 ubiquitination and degradation.

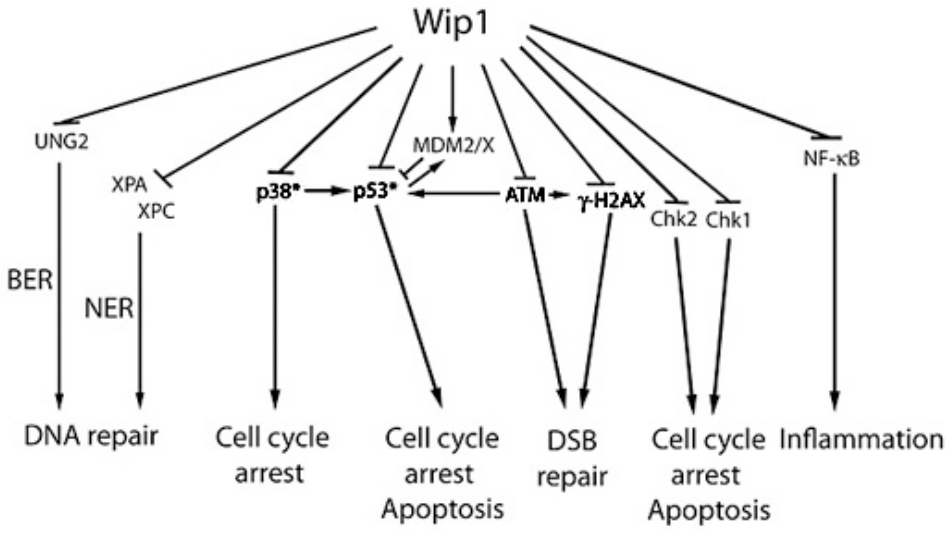


Figure 3 *The targets and functional consequences of Wip1 signaling (from Lowe, J. et al., 2012).*

WIP1 also interferes with DNA damage repair systems. It suppresses base excision repair (BER) by dephosphorylating Thr6 of UNG2 and reducing its activity (Lu,

X. *et al.*, 2004) and nucleotide excision repair (NER) by dephosphorylating XPA on Ser196 and XPC on Ser892 (Nguyen, T.A. *et al.*, 2010). WIP1 dephosphorylates γ-H2AX on Ser139, which is phosphorylated by ATM soon after induction of double strand breaks (DSB) (Fig.3). This prevents the recruitment to the DNA damage sites of repair factors such as MDC1 and 53BP1 and the activation of cell cycle checkpoints that protects the cell from replication of damaged DNA (Macurek, L. *et al.*, 2010; Moon, S.H. *et al.*, 2010, 2010). WIP1 is also a negative regulator of NF-kB, which dephosphorylates on Ser536, essential for the transactivation function of the p65 subunit of NF-kB. Consistently, $Wip1^{-/-}$ mice show enhanced inflammation after lipopolysachharide (LPS) stimulation compared to the control (Chew, J. *et al.*, 2009) (Fig.3). In p53-negative tumors WIP1 increases the sensitivity to anticancer drugs by increasing the BAX/BCL-XL ratio. The negative regulation of NF-kB leads to reduced BCL-XL expression, whereas the dephosphorylation of Ser432 of RUNX2 results in enhanced BAX expression (Goloudina, A.R. *et al.*, 2012). In normal tissue, however, WIP1 protects from apoptosis induced by anticancer drugs through attenuation of the p53 response (Goloudina, A.R. *et al.*, 2012).

1.2.4. Phenotype of *Wip1*$^{-/-}$ mouse models

$Wip1^{-/-}$ mice are viable but show some postnatal abnormalities, such as reduced body weight, variable male runting, reduced serum insulin-like growth factor 1 (IGF-1), reduced male fertility and longevity and male reproductive organ atrophy, suggesting an important role for Wip1 in spermatogenesis (Choi, J. *et al.*, 2002; Nannenga, B. *et al.*, 2006). $Wip1^{-/-}$ mice had increased susceptibility to pathogens, diminished T- and B-cell function, and fewer splenic T cells. Their thymi were smaller, contained significantly fewer cells, and failed to undergo age-dependent involution compared with wild-type animals. The abnormal thymic phenotype of Wip1-deficient mice was reversed in the absence of p53, suggesting that Wip1 down-regulates p53 activation in the thymus and is required for normal alpha-beta T cell development (Schito, M.L. *et al.*, 2006). Wip1 is

preferentially expressed in neutrophils among immune cells and its deficiency results in impaired development and maturation of myeloid progenitors to neutrophils. This involved the p38MAPK-STAT1 pathway and it is p53-independent (Liu, G. *et al.*, 2012).

Wip1$^{-/-}$ mouse embryo fibroblasts (MEFs) show decreased proliferation rates, premature senescence and appear to be compromised in entering mitosis (Choi, J. *et al.*, 2002). Indeed, WIP1 allows the cell to re-enter cell cycle during an ongoing DNA damage response in G2, by antagonizing the repression of Cyclin B and Plk1 by p53 (Lindqvist, A. *et al.*, 2009). Wip1 regulates the generation of new neural cells in adult olfactory bulb by controlling the p53-dependent M-phase entry in neural stem/progenitors (NPCs). During neurogenesis, Wip1 deficiency results in decreased new cell formation in adult olfactory bulb and decreased NPC amplification, stem cell frequency and self-renewal (Zhu, Y.H. *et al.*, 2009). In the central nervous system (CNS), Wip1 is also involved in the modulation of dendritic morphology and in memory processes through p38MAPK pathway. Wip1 deficiency results in impaired object recognition tasks and contextual memory, effects that are reversed in Wip1$^{-/-}$ p38MAPK$^{-/-}$ mice (Fernandez, F. *et al.*, 2012). Wip1 also regulates the homeostasis of intestinal stem cells, where it is highly espressed and controls p53-dependent apoptosis (Demidov, O.N. *et al.*, 2007).

WIP1 appears to play a role in organismal aging. Wip1 levels drop with aging in NPC, explaining the aging-induced increase in p53 phosphorylation observed and the fact that Wip1$^{-/-}$ NPCs behave as though their aging program has been accelerated both *in vitro* and *in vivo* (Zhu, Y.H. *et al.*, 2009). In pancreatic islets the age-related decrease of Wip1 expression is associated to increased p38MAPK activity and p16Ink4a expression. This leads to the induction of the permanent cell cycle arrest (senescence), decline in β-cell proliferation and reduced resistance to drug-induced diabetes in aged mice (Wong, E.S. *et al.*, 2009). The Wip1–p38MAPK–p16Ink4a

pathway is also implicated in proliferative senescence of human mesenchymal stem cells (hMSC) during *in vitro* passaging. Indeed, Wip1 overexpression abolished the increase of p16Ink4a and rescued the proliferative capacity of mesenchymal stem cells (Lee, J.S. *et al.*, 2009).

1.2.5. WIP1: an oncogenic phosphatase

In recent years WIP1 has emerged as an important player in tumorigenesis. WIP1 was first found is amplified/overexpressed in breast cancer (Li, J. *et al.*, 2002; Bulavin, D.V. *et al.*, 2002; Yang, D.H. *et al.*, 2010) and its high expression was associated to low p38MAPK activity and p16Ink4a (Yu, E. *et al.*, 2007). WIP1 amplification was associated with ERBB2 expression, usually observed in tumours with poor prognosis (Rauta, J. *et al.*, 2006). High WIP1 expression was observed in medulloblastoma where it antagonized p53-mediated apoptosis after etoposide treatment (Castellino, R.C. *et al.*, 2008). Wip1 increased Mdm2 levels thus enhancing medulloblastoma proliferation (Buss, M.C. *et al.*, 2012). High levels of WIP1 are found also in neuroblastoma, where its silencing suppresses cancer growth leading to apoptotic cell death (Saito-Ohara, F. *et al.*, 2003). WIP1 was also found amplified/overexpressed in several other types of human cancer such as ovarian clear cell carcinoma (Hirasawa, A. *et al.*, 2003), lung adenocarcinoma (Satoh, N. *et al.*, 2011), pancreatic adenocarcinoma (Loukopoulos, P. *et al.*, 2007), glioma (Liang, C. *et al.*, 2012), gastric carcinoma (Fuku, T. *et al.*, 2007), suggesting that it may be used as a prognostic factor for several types of cancer. Interestingly, tumors with WIP1 amplification rarely harbored mutated p53 (Rauta, J. *et al.*, 2006; Yu, E. *et al.*, 2007; Bulavin, D.V. *et al.*, 2002), indicating that WIP1 overexpression is sufficient to reduce the selective pressure for p53 mutation during tumor progression.

Although WIP1 is frequently amplified in human cancer, its overexpression by itself is not sufficient to transform normal cells (Bulavin, D.V. *et al.*, 2002; Li, J. *et al.*,

2002). In fact, WIP1 cooperates with known oncogenes to promote tumorigenesis. Wip1 accelerates the transformation of rodent primary fibroblasts induced by oncogenes such as E1A, MYC, NEU1 or RAS (Bulavin, D.V. *et al.*, 2002; Li, J. *et al.*, 2002), enhancing their anchorage-independent growth and foci formation abilities in soft agar and increasing their tumorigenic potential *in vivo*. Wip1 overexpression in the mammary gland of transgenic breast cancer-prone mice expressing the ErbB2 oncogene results in increased tumorigenesis, which is mediated by WIP1 attenuation of the MKK6/p38MAPK pathway (Demidov, O.N. *et al.*, 2007). Consistently, Wip1 deficiency suppresses polyp formation in a mouse model of APC(Min)-driven polyposis by affecting p53 control of intestinal stem cells apoptosis (Demidov, O.N. *et al.*, 2007) and require Chk2, Cdkn2a and Gadd45a (Demidov, O.N. *et al.*, 2012).

$Wip1^{-/-}$ mice are resistant to spontaneous tumorigenesis over their entire lifespan, in part because of increased stress response following DNA damage (Nannenga, B. *et al.*, 2006). $Wip1^{-/-}$ MEFs are more resistant to oncogene-induced transformation. Wip1 loss confers significant resistance to the appearance of mammary tumors induced by ErbB2 and HRas1 in Wip1 null mice, due to increased p38MAPK activation and consequent higher expression of p16Ink4a and p19ARF (Bulavin, D.V. *et al.*, 2004). $Wip1^{-/-}$ and $Wip1^{+/-}$ mice show delayed onset of Eμ-Myc-induced B cell lymphoma, due to the modulation of ATM and p53 function by Wip1 (Shreeram, S. *et al.*, 2006).

Wip1 is implicated also in other pathological conditions, such as obesity and atherosclerosis. Wip1 modulates the non-canonical Atm-mTOR signaling pathway and regulates the autophagy-dependent cholesterol efflux from macrophage foam cells, which leads to the development of atherosclerotic claque (Brichkina, A. *et al.*, 2012; Le Guezennec, X. *et al.*, 2012).

1.2.6. Inhibitors of WIP1 activity

The oncogenic behavior of WIP1 makes it a promising drug target for cancer therapy. The biochemical characterization of WIP1 substrate specificity allowed the development of specific WIP1 inhibitors, which block its phosphatase activity (Yagi, H. *et al.*, 2012; Yamaguchi, H. *et al.*, 2006; Hayashi, R. *et al.*, 2011). Arsenic trioxide (ATO), a chemotherapeutic agent used for treatment of acute promyelocitic leukemia, has been found to inhibit WIP1 *in vitro* and *in vivo*, resulting in p38MAPK/p53 pathway activation (Yoda, A. *et al.*, 2008). At present, however, only one specific WIP1 inhibitor is commercially available, CCT007093, although its use is limited to *in vitro* systems (Zhang, X. *et al.*, 2010). Few molecules have been found to inhibit WIP1 and to be able to reduce growth of breast cancer cells both *in vitro* and *in vivo*. However, because they modestly inhibit also PP2A and PP2Cα phosphatases, they only serve as possible lead compounds for the development of specific WIP1 inhibitors (Belova, G.I. *et al.*, 2005).

1.3. THE E2F FAMILY OF TRANSCRIPTION FACTORS

The E2F family of transcription factors includes 8 genes that encode for 9 proteins in mammals, classified as activators or repressors (Trimarchi, J.M. *et al.*, 2001) (Fig.4). All E2Fs proteins contain a DNA binding domain and a dimerization domain. The primary function of “activating E2Fs” (E2F1, E2F2, and E2F3a) is to induce S-phase entry in quiescent cells (Johnson, D.G. *et al.*, 1993; DeGregori, J. *et al.*, 1997; Qin, X.Q. *et al.*, 1994) and overcome arrest mediated by the p16Inka cyclin-dependent kinase inhibitor (CDK1) (Lukas, J. *et al.*, 1996).

The activating E2Fs contain a cyclin A binding domain, a nuclear localization signal (NLS) and a transactivation and pocket protein binding domain. The “repressive E2Fs” (E2F3b, E2F4, E2F5, E2F6, E2F7 and E2F8) are involved in cell cycle exit and terminal differentiation processes such as adipogenesis (Fajas, L. *et al.*, 2002; Landsberg, R.L. *et al.*, 2003) and erythrocyte maturation (Humbert, P.O. *et al.*, 2000).

Figure 4 *Structural features of the E2F (top panel) and pRb (bottom panel) family members. The conserved domains of the proteins are shown (from Blais, A. et al., 2004).*

The repressive E2Fs lack the cyclin-A binding domain E2F6, E2F7, and E2F8 also lack the transactivation and pocket protein binding domain. In place of a NLS, E2F4 and E2F5 contain a nuclear export signal (NES) and their nuclear localization depends on the heterodimerization with the DRTF polypeptide (DP) proteins. The pocket-protein binding domain in E2Fs1-5 allows the interaction with the “pocket protein” family proteins pRB, p107 and p130, which regulate E2Fs activity. They inhibit E2Fs transcriptional activity by masking key residues required for transcriptional activation and by recruiting repressor complexes on the DNA. E2F6 inhibits transcription by interacting with members of the Polycomb complex (PcG) (Trimarchi, J.M. *et al.*, 2001). E2F7 and E2F8 function similarly to E2F6 and they are the unique E2Fs that bind DNA without the DP subunit, thanks to a second DNA binding domain (Di Stefano, L. *et al.*, 2003). E2Fs1-5 form heterodimers with DP-1 and DP-2 (Girling, R. *et al.*, 1993; Wu, C.L. *et al.*, 1995; Zhang, Y. *et al.*, 1995), which are homolog to E2Fs and share the same dimerization and DNA binding domains. Whereas the DNA binding specificity depends on the E2F subunit of the E2F-DP complexes (Wu, C.L. *et al.*, 1995), the interaction with the DP proteins increases their DNA binding activity (Bandara, L.R. *et al.*, 1993; Krek, W. *et al.*, 1993) and may influence the nuclear

accumulation of E2Fs in the nucleus (Magae, J. *et al.*, 1996). E2Fs transcription factors bind the consensus sequence [T/C]TT [C/G][G/C]C G[C/G] (Slansky, J.E. *et al.*, 1993) in a great number of E2F responsive promoters (Xu, X. *et al.*, 2007; Bieda, M. *et al.*, 2006). The specificity and the correct timing of expression of an E2F target gene depends on the cell-cycle phase (Sardet, C. *et al.*, 1995; Muller, H. *et al.*, 1997) and on the interaction of E2Fs with other transcription factors (e.g. Sp1 (Karlseder, J. *et al.*, 1996; Lin, S.Y. *et al.*, 1996)) or growth regulatory factors, which may allow E2Fs to bind to promoters lacking an E2F consensus sequence, thus greatly increasing the E2F family target genes (reviewed in Black, A.R. *et al.*, 1999).

1.3.1. E2Fs and cell cycle regulation

Among E2Fs targets there is great number of genes implicated in cell replication, whose expression varies throughout the cell cycle in response to the different activities of the E2Fs proteins (Fig.5). During G_0 and early G_1, E2F4 and E2F5 are bound preferentially to p130 and repress E2F-responsive genes, while the activating E2Fs are bound and inactivated by pRB. In late G_1 phase, pRB and p130 are phosphorylated first by cyclin-D/CDK (Kato, J. *et al.*, 1993) and later by cyclin-E/CDK (Lundberg, A.S. *et al.*, 1998), thus releasing the activating E2Fs, which increase the transcription of S phase genes, including cyclin-E and cyclin-A.

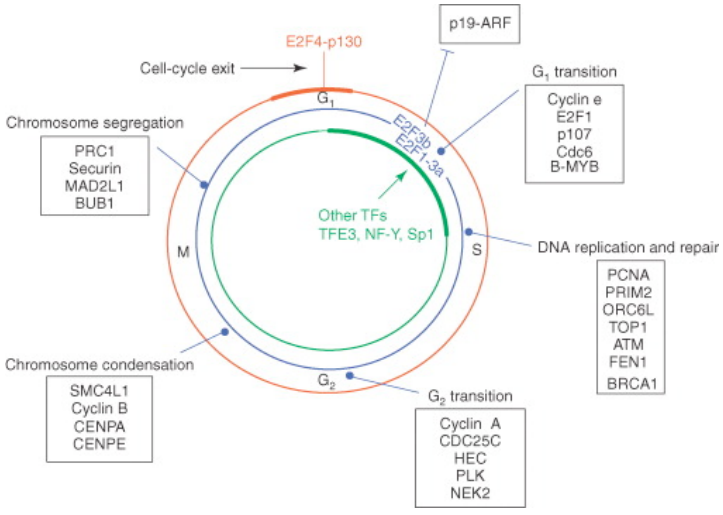


Figure 5 *E2F targets regulate many aspects of cell growth and proliferation (from Blais, A. et al., 2004).*

At the same time, p130 is targeted for ubiquitin-mediated degradation (Tedesco, D. *et al.*, 2001), and E2F4 and E2F5 shuttle from the nucleus to the cytoplasm (Lindeman, G.J. *et al.*, 1997).

E2Fs proteins have partially overlapping, but distinct functions in the control of cell cycle. For instance, both E2F1 and E2F3 are required for cell cycle entry, but only E2F3 is required for sustained cell proliferation (Kong, L.J. *et al.*, 2007) E2Fs are also involved in development, differentiation and tissue homeostasis (Stanelle, J. *et al.*, 2006; McClellan, K.A. *et al.*, 2007). E2F1 and 3, for example, are involved in neuronal migration (Jiang, S.X. *et al.*, 2007; Chen, C. *et al.*, 2007; McClellan, K.A. *et al.*, 2007) and E2F4 in differentiation of adipocytes (Fajas, L. *et al.*, 2002; Landsberg, R.L. *et al.*, 2003) and of respiratory epithelium (Danielian, P.S. *et al.*, 2007). E2Fs are also implicated in human cancer, where hyperactive E2Fs leading to uncontrolled cell proliferation have been observed in almost any human malignancy. Deregulation of E2Fs activity occurs through several mechanisms that interfere with pRB pathway, such as functional pRB loss, cyclin-D amplification, p16Inka loss, expression of the human papillomavirus (HPV) protein E7 (Sherr, C.J. *et al.*, 2002), of the Epstein-Barr virus nuclear antigen 3C (Knight, J.S. *et al.*, 2005) or of the human megalovirus pp71 protein (Kalejta, R.F. *et al.*, 2003).

1.3.2. The E2F1 transcription factor

E2F1 (RBAP1, RBBP3, RBP3) is the founding member and the best studied component of the E2F family of transcription factors. *E2F1* gene maps in the 20q11.2 locus and it spans 14kbp. E2F1 mRNA (NM_005225.2) contains 7 exons and it is translated into a 437AA protein (NP_005216.1). Like other E2Fs, E2F1 is activated by mitogenic signals, such as serum stimulation or growth factors, and promotes cell proliferation. However, paradoxically E2F1 is also able to induce apoptosis in response to DNA damaging agents (ionizing radiation, UV radiation, chemotherapeutic drugs)

(Blattner, C. *et al.*, 1999; Huang, Y. *et al.*, 1997; O'Connor, D.J. *et al.*, 2000) and to enhance autophagy after nutrient or growth factor deprivation (Polager, S. *et al.*, 2008) (Fig.6).

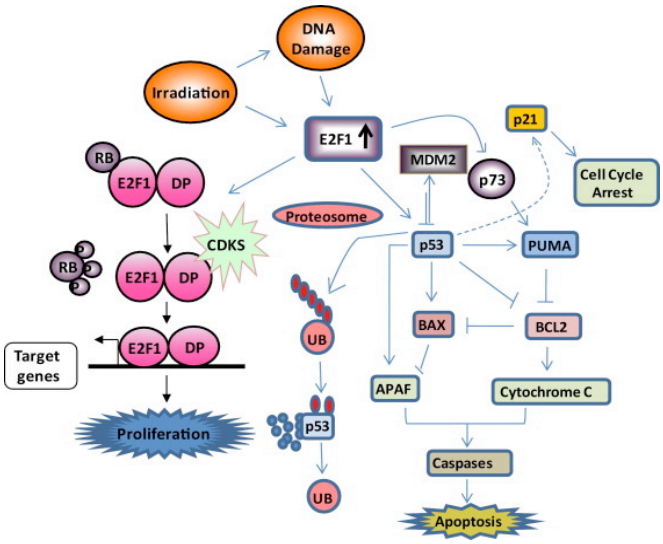


Figure 6 *E2F1 controls both proliferation and apoptosis (from Udayakumar, T. et al., 2010).*

1.3.3. Regulation of E2F1 expression

E2F1 promoter contains responsive elements for a variety of transcription factors, including E2Fs itself. Indeed, E2F1 transactivates its own promoter, by binding to E2F responsive elements just upstream the transcription start site (Johnson, D.G. *et al.*, 1994). On the other hand, after DNA damage E2F7 and E2F8 repress E2F1 expression, thus providing a mechanism to finely tune E2F1 levels after genotoxic stress (Zalmas, L.P. *et al.*, 2008). The oncogenic Myc protein directly binds to E2F1 promoter and contribute to its induction at pre-S phase (Fernandez, P.C. *et al.*, 2003). The binding of NF-Y to E2F1 promoter induces its expression, resulting in enhanced p53 activation and induction of apoptosis (Gurtner, A. *et al.*, 2010). PAX8 binds to and transactivates E2F1 promoter; however, because PAX8 is required for pRB stabilization, it creates a negative feedback loop, which tightly controls E2F1 function (Li, C.G. *et al.*, 2011).

E2F1 expression is also regulated by the Wnt/Tcf-pathway, as E2F1 promoter activity is inhibited by sodium butyrate and by overexpression of beta-catenin/TCF. The regulation is directly mediated by TCF, which binds to a responsive element in E2F1 promoter (Abramova, M.V. *et al.*, 2010). E2F1 expression in breast cancer has also been found to be regulated by c-Fos, a component of the transcription factor AP-1, and ERα, which bind regulatory elements in E2F1 promoter (Dahlman-Wright, K. *et al.*, 2012).

E2F1 levels are also regulated by several microRNAs. In the cluster miR-17-92, which is up-regulated by activator E2Fs (in particular E2F3), miR-17-5p and miR-20a target E2F1 (Sylvestre, Y. *et al.*, 2007; Woods, K. *et al.*, 2007). Similarly, E2F1 induces the miR-106b-25 and miR-449 clusters which includes miR-106b, miR-93 and miR-449, targeting E2F1 itself (Lize, M. *et al.*, 2010; Petrocca, F. *et al.*, 2008; Yang, X. *et al.*, 2009). E2F1 is also targeted by miR-205, which is significantly suppressed in melanoma compared with nevi and is correlated inversely with melanoma progression (Dar, A.A. *et al.*, 2011).

1.3.4. Regulation of E2F1 protein

E2F1 undergoes several post-translational modifications during cell cycle progression or in response to external stimuli. Cyclin-A/CDK2 complex phosphorylates E2F1 at Ser375, thus increasing its affinity for pRB (Peeper, D.S. *et al.*, 1995). Other inhibitory phosphorylations occur at Ser403 and Thr433 by TFHII and trigger E2F1 degradation (Vandel, L. *et al.*, 1999). After DNA damage E2F1 is modified at different sites. ATM and ATR phosphorylate E2F1 on Ser31, allowing the binding of 14-3-3, which blocks E2F1 ubiquitination and degradation by the proteasome (Blattner, C. *et al.*, 1999; O'Connor, D.J. *et al.*, 2000; Lin, S.Y. *et al.*, 1996; Wang, B. *et al.*, 2004). E2F1 stabilization is also increased by phosphorylation of Ser364 by CHK2 (Stevens, C. *et al.*, 2003), whereas phosphorylation on Ser403 after doxorubicin treatment leads

to increased E2F1 transcriptional activity (Real, S. *et al.*, 2010). Acetylation of Lys117, Lys120 and Lys125 near the DNA binding domain by either P/CAF or p300 acetyltransferases enhances E2F1 stabilization and DNA-binding activity, particularly on the p73 promoter, important for the apoptotic response after DNA damage (Galbiati, L. *et al.*, 2005; Ianari, A. *et al.*, 2004; Pediconi, N. *et al.*, 2003, 2009). Consistently, E2F1 deacetylation by SIRT1 reduces p73 expression (Pediconi, N. *et al.*, 2003). E2F1 is also methylated on Lys185 by the SET9 methyltransferase, which inhibits its transcriptional activity, and it is activated by demethylation by LSD1 after doxorubicin treatment (Kontaki, H. *et al.*, 2010).

E2F1 function is significantly controlled by protein-protein interactions. Like other E2Fs, E2F1 selectively binds to the A and B domain (pocket site) of the hypophosphorylated pRB form through its C-terminal pocket-protein binding domain (Chellappan, S.P. *et al.*, 1991; Helin, K. *et al.*, 1993; Shan, B. *et al.*, 1992); this interaction masks E2F1 transactivation domain and the resulting pRB/E2F1 complex recruits a number of co-repressors such as histone deacetylases (HDACs). Upon mitogenic stimulation, cyclin-dependent kinases (CDKs), particularly cyclin-D and cyclin–E-associated kinases, phosphorylate pRB on multiple sites. E2F1 is released and can interact with chromatin modifying enzymes such as histone acetyltransferases (HAT) (e.g. CBP) (Brehm, A. *et al.*, 1998), thus activating the transcription of genes involved in DNA replication and cell cycle progression. E2F1 also binds specifically to a second site within the C-terminal domain of pRB and this interaction inhibits E2F1-induced apoptosis (Dick, F.A. *et al.*, 2003). After DNA damage, however, the region of pRB involved in this interaction is acetylated, E2F1 is released and induces the expression of pro-apoptotic genes (Markham, D. *et al.*, 2006). DNA damage also leads to the formation of E2F1-containing protein complexes that mediate different activities. CHK1 and CHK2-mediated phosphorylation of pRB on Ser612 results in the formation of a transcriptionally active complex pRB-E2F1-P/CAF that drives the expression of

proapoptotic genes (Ianari, A. *et al.*, 2009). Induction of p73, BRCA1, CHK1 or Caspase-7 after DNA damage is increased by the interaction of the E2F1-DP dimer with microcephalin (MCPH1), which enhances E2F1 transcriptional activity (Yang, S.Z. *et al.*, 2008). E2F1-induced apoptosis is enhanced by Jab1, which interacts with the marked box domain of E2F1 (Hallstrom, T.C. *et al.*, 2003), and by the ribosomal RNA processing 1 homolog B (RRP1B), a specific E2F1 target that acts as coactivator to prime cells for E2F1-dependent killing (Paik, J.C. *et al.*, 2010). Transcriptional repression after DNA damage occurs on promoters of cell cycle genes, through recruitment of HDACs by pRB-E2F1 (Ianari, A. *et al.*, 2009). pRB-independent repression of E2F1 transcriptional activity is also mediated by TopBP1, which binds E2F1 on the same region bound by MCPH1 and recruits on E2F1 target promoters Brg1 and hBrm, components of the SWI/SNF chromatin remodeling complex (Liu, K. *et al.*, 2004). The interaction between E2F1 and TopBP1 also plays a role in DNA repair, because the E2F1-TopBP1-GCN5 complex is recruited to DNA double strand breaks and UV-induced damage sites (Guo, R. *et al.*, 2011).

1.3.5. E2F1 and the apoptotic response

Unique among E2Fs proteins, E2F1 is able to induce apoptosis after genotoxic stress by several mechanisms (Fig.7). The first described mechanism of E2F1-mediated induction of apoptosis is through the activation of p53. Although E2F1 does not bind to p53 promoter, E2F1 induces p14ARF, which binds to HDM2 and prevents p53 degradation (Bates, S. *et al.*, 1998; Hiebert, S.W. *et al.*, 1995). E2F1, like all other activator E2Fs, directly binds to and stabilize p53 via their Cyclin-A binding domain; in response to DNA damage, in fact, the decrease of Cyclin-A levels allows the E2F1-p53 complex formation (Hsieh, J.K. *et al.*, 2002). E2F1 leads to p53 activation also by induction of ATM, CHK1 and CHK2 (Berkovich, E. *et al.*, 2003; Yang, S.Z. *et al.*, 2008) and of AMP kinase α2 (AMPα2), which all phosphorylate p53 (Hallstrom, T.C. *et al.*, 2003, 2008; Jones, R.G. *et al.*, 2005). E2F1 enhances p53 function by up-regulating

p53 apoptotic co-factors, such as ASPP1, ASPP2, JMY and TP53INP1 (Hershko, T. *et al.*, 2005), but can also induce apoptosis in a p53-independent manner. E2F1 directly induces a large number of pro-apoptotic genes, such as the p53-homolog p73 (Irwin, M. *et al.*, 2000; Stiewe, T. *et al.*, 2000) or APAF1 (Furukawa, Y. *et al.*, 2002). E2F1 increases caspase activation by transcriptionally inducing caspase-3, -7, -8, -9 (Cao, Q. *et al.*, 2004; Nahle, Z. *et al.*, 2002) and by blocking caspase inhibition by direct transactivation of Smac/DIABLO (Xie, W. *et al.*, 2006). The induction of NOXA, PUMA, BID, BIK, BIM, BNIP3, HRK, BOK (Cao, Q. *et al.*, 2004; Hershko, T. *et al.*, 2004; Real, P.J. *et al.*, 2006; Stanelle, J. *et al.*, 2002; Rodriguez, J.M. *et al.*, 2006) makes E2F1 an important mediator of the mitochondrial outer membrane permeabilization, that triggers the mitochondrial apoptotic pathway. E2F1 induction of FOXO3 leads to the increase of BIM, NOXA and PUMA (Dijkers, P.F. *et al.*, 2000; Obexer, P. *et al.*, 2007; You, H. *et al.*, 2006) and to p53 activation (You, H. *et al.*, 2006). Mitochondrial death pathway is also enhanced by E2F1 induction of E1AF, which interacts with BAX promoter (Wei, Y. *et al.*, 2008), the serine protease inhibitor maspin (SERPINB5) (Ben Shachar, B. *et al.*, 2010) and the Kruppel-like transcription factor KLF10 (Engelmann, D. *et al.*, 2010), that mediates the E2F1-induced sensitization of tumor cells to genotoxic agents.

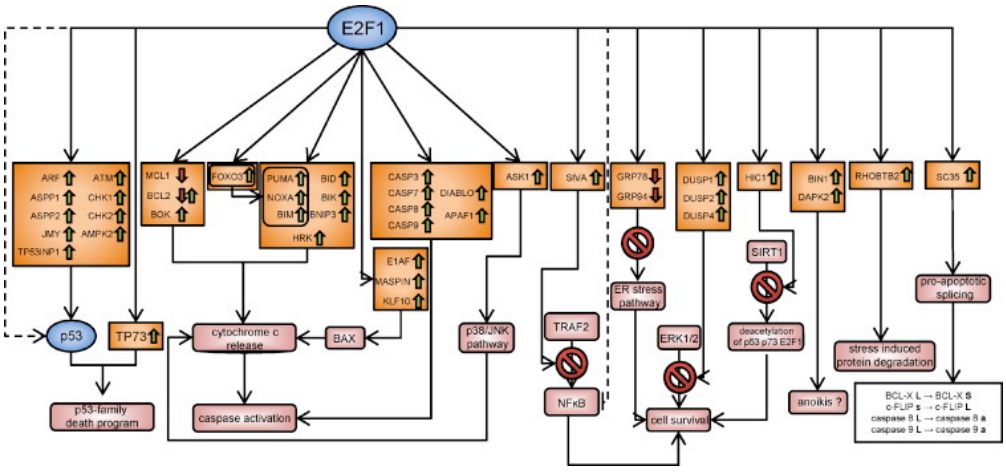


Figure 7 *E2F1 induces apoptosis by p53-dependent and p53-independent mechanisms (from Engelmann, D. et al., 2010).*

E2F1 controls the apoptotic response also by inhibiting survival signaling. It down-regulates the anti-apoptotic Bcl-2 family member Mcl-1 and interferes with Bcl-2

itself (Croxton, R. *et al.*, 2002). E2F1 can inhibit NF-kB signaling by interacting with and reducing the DNA binding activity of the p65 subunit (Tanaka, H. *et al.*, 2002) and by decreasing TRAF2 protein levels, thus inhibiting the anti-apoptotic NF-kB function; this effect is mediated by the induction of SIVA, which promotes TRAF2 degradation (Fortin, A. *et al.*, 2004). In response to oxidative stress, E2F1 induces the expression of DUSP1, DUSP2 and DUSP4, which, in turn, dephosphorylate ERK, thus blocking cell survival signals (Wang, J. *et al.*, 2007). E2F1 participates in the apoptotic response induced by unfolded proteins by repressing the endoplasmic reticulum chaperons GRP78 and GRP94 (Racek, T. *et al.*, 2008; Li, J. *et al.*, 2006). Finally, some of the E2F1-regulated miRNAs have been implicated in apoptosis induction by antagonizing the expression of pro-survival genes and/or cooperating with p53 in the integration of apoptotic signals. For instance, miR-449a/b diminished SIRT1 deacetylase expression, thus leading to p53 increased apoptotic response (Lize, M. *et al.*, 2010).

1.3.6. E2Fs and other cellular processes

Beside cell cycle regulation and apoptosis induction, E2F1 is involved in several other cellular processes. It modulates the activity of signal transduction pathways by transcriptionally regulating their components or controls metabolic signaling. E2F1 modulates the MAPK-p38 by inducing the apoptosis-signal-regulating kinase (ASK1) and WIP1 phosphatase, which respectively activate and inactivate p38MAPK (Hershko, T. *et al.*, 2006). It also positively modulates the PI3K-AKT signaling by inducing the adaptor protein GAB2 (Chaussepied, M. *et al.*, 2004). E2F1 sensitizes cells to basic fibroblast growth factor (bFGF) by upregulating the FGF receptor 1 (Tashiro, E. *et al.*, 2003) and to platelet-derived growth factor (PDGF) by activation of the MEK-ERK pathway (Korotayev, K. *et al.*, 2008). Recent data demonstrate that E2F1 plays a role in the induction of autophagy. E2F1 up-regulates the expression of four crucial autophagy genes LC3, ATG1, ATG5, DRAM (Polager, S. *et al.*, 2008) and AMPα2, a nutrient energy sensor (Hallstrom, T.C. *et al.*, 2008). E2F1 is involved in

cellular metabolism, as it directly regulates pyruvate dehydrogenase kinase 4 (PDK4), a nutrient sensor and modulator of glucose homeostasis which is chronically elevated in obesity and diabetes and induced under the metabolic stress of starvation or fasting (Hsieh, M.C. *et al.*, 2008). E2F1 also drives lipogenesis, by inducing the fatty acid synthase (FASN) and this regulation is particularly relevant in medulloblastoma, where SHH signaling drives E2F1-dependent lipogenesis typical of this tumor (Bhatia, B. *et al.*, 2011).

1.3.7. Phenotype of *E2f1*$^{-/-}$ and E2f1 transgenic mouse models

Because of its proliferative properties and its ability to induce p53-dependent and independent apoptosis, E2F1 displays both tumor promoting and tumor suppressing capabilities in mice, indicating that this dual function is highly context dependent and influenced by the cellular status. E2f1$^{-/-}$ mouse embryo fibroblasts (MEFs) show impaired cell cycle entry (Wang, Z.M. *et al.*, 1998), because of role of E2f1 in controlling the G_0-S checkpoint. Consistently, cells transfected with E2f1 can form colonies in soft agar, induce tumor formation in nude mice and transform rat embryo fibroblasts in cooperation with activated Ras (Johnson, D.G. *et al.*, 1994). *In vivo*, E2f1 overcomes transforming growth factor-beta (TGF-β)-mediated suppression (Schwarz, J.K. *et al.*, 1995) and increases the susceptibility to skin tumours (Pierce, A.M. *et al.*, 1998, 1998) in p53-deficient mice. Transgenic E2f1 expression under the control of a keratin 5 (K5) promoter induces spontaneous tumors of epithelial basal cell origin (Pierce, A.M. *et al.*, 1999), whereas conditional expression of E2f1 in the liver leads to formation of hepatocellular adenomas and large cell dysplasias (Conner, E.A. *et al.*, 2000). E2f1$^{-/-}$ mice are viable and fertile, but show testicular atrophy and exocrine gland dysplasia (Yamasaki, L. *et al.*, 1996). They display hypercellularity of the thymus because of a defective negative selection, due to impaired induction of p14Arf and p53 (Field, S.J. *et al.*, 1996; Zhu, J.W. *et al.*, 1999). Despite the role of E2f1 in promoting cell proliferation *in vitro*, mice lacking E2f1 develop a broad spectrum of tumors,

including histiocytic sarcomas, hemangiosarcomas, lymphomas, hepatocarcinomas, and lung tumors, demonstrating that E2f1 also functions as a tumor suppressor *in vivo* (Yamasaki, L. *et al.*, 1996). Consistently, E2f1 inactivation significantly accelerates tumor development in Myc transgenic mice (Rounbehler, R.J. *et al.*, 2002). Targeted E2f1 expression in the squamous epithelium leads to p53-dependent apoptosis and E2f1-dependent apoptosis suppresses carcinogenesis in a Ras-driven skin carcinogenesis model (Pierce, A.M. *et al.*, 1998). Conditional E2f1 activation in mouse testes results in the activation of E2f1 target genes and p53-independent apoptosis, leading to testicular atrophy, and causes also premalignant changes similar to those observed in carcinoma *in situ* in humans (Agger, K. *et al.*, 2005).

Nevertheless, loss of E2f1 is not always associated with enhanced tumor development. In fact, in $pRb^{+/-}$ mice E2f1 loss impairs the development of pituitary and thyroid tumors, and tumor incidence increases within certain tissues and decreases in others (Yamasaki, L. *et al.*, 1998). To explain this discrepancy, it is important to take into account that any genetic experiment designed to define the specific function of an individual E2F is complicated by the extensive compensation by other E2Fs occurring in E2F-mutant cells.

1.3.8. E2F1 in cancer

In humans, E2F1 expression is frequently associated with high grade tumors, poor patient survival prognosis and tumor progression. Overexpression of E2F1 in lung and liver metastases of human colon cancer is associated with gene amplification and correlates with levels of thymidylate synthase and resistance to therapy (Banerjee, D. *et al.*, 2000; Iwamoto, M. *et al.*, 2004). E2F1 is overexpressed in small cell lung carcinoma (SCLC) and in large cell neuroendocrine carcinoma (LCNEC) whereas it is undetectable in adenocarcinoma and squamous carcinoma; E2F1 overexpression in neuroendocrine lung tumors is also associated with a high proliferative index and a

BCL2:BAX ratio >1 (Eymin, B. *et al.*, 2001). In high-grade neuroendocrine (HGNE) lung carcinomas the high expression of E2F1 and of its target SKP2 are associated with advanced stages and nodal metastasis (Salon, C. *et al.*, 2007). E2F1 is highly expressed in non-small cell lung carcinoma (NSCLC) where it co-localizes with phospho-pRb (Gorgoulis, V.G. *et al.*, 2002) or is associated with aberrant pRb status (Imai, M.A. *et al.*, 2004). In this type of cancer high E2F1 expression is frequently associated with deregulation of the p53-MDM2 pathway and patients with increased E2F1 positivity had a poorer outcome. In pancreatic ductal carcinoma high E2F1 expression is observed in less differentiated carcinomas, correlates with MIB1 expression and is associated to a shorter disease-associated survival time after resection (Yamazaki, K. *et al.*, 2003). In bladder cancer patients high expression of E2F1 and of its targets is associated to superficial to invasive tumors progression (Lee, J.S. *et al.*, 2010; Zacharatos, P. *et al.*, 2004). E2F1 is up-regulated in papillary and anaplastic thyroid cancers and correlates with Cyclin-D1 and Ki67 expression (Onda, M. *et al.*, 2004; Saiz, A.D. *et al.*, 2002). In breast carcinomas, E2F1 expression correlated with proliferation and growth index (Zacharatos, P. *et al.*, 2004; Zhang, S.Y. *et al.*, 2000). Its expression was shown to predict a worse outcome in lymph node positive breast cancer patients treated with adjuvant chemotherapy, suggesting that E2F1 expression may be used as a biological marker of treatment response (Han, S. *et al.*, 2003). In melanoma there is a high expression of E2F1, due to increased gene copy number (Nelson, M.A. *et al.*, 2006) and to suppression of miR-205, which targets E2F1 (Dar, A.A. *et al.*, 2011). E2F1 levels are associated to high expression of its targets Cyclin-D1, Cyclin-A2, and Cyclin-E, and to the presence of hyperphosphorylated forms of pRb, p107, and p130 (Halaban, R. *et al.*, 2000). E2F1 is expressed at higher levels in lymph node metastases than in primary tumors of melanoma patients (Nelson, M.A. *et al.*, 2006) and it is required for development of melanoma metastasis, but not for proliferation. E2F1 in fact directly induces the

expression of epidermal growth factor receptor (EGFR) which is required for melanoma invasiveness (Alla, V. *et al.*, 2010).

Because the actions of E2F1 are mainly dependent on the functionality the pRb and p53 tumor suppressor pathways, their status might explain why in some types of tumors E2F1 behaves as an tumor suppressor. In colon cancer E2F1 overexpression is a relatively common event (Suzuki, T. *et al.*, 1999), but it seems to act as a tumor suppressor by inducing the apoptotic pathway (Zacharatos, P. *et al.*, 2004). Indeed, E2F1 expression is inversely correlated with tumor growth, being expressed in lesions with high apoptotic incidence and low proliferation (Bramis, J. *et al.*, 2004). In prostatic carcinomas E2F1 is absent in the cancerous areas, whereas it is expressed in the normal and hyperplastic glands (Zacharatos, P. *et al.*, 2004). This observation can be explained by the fact that high androgen levels driving prostate tumor growth may increase pRb activity, thus hindering E2F1 action (Hofman, K. *et al.*, 2001). In human glioblastoma there is a correlation between E2F1 and hTERT expression and lower E2F1 levels are significantly associated with longer overall survival (Alonso, M.M. *et al.*, 2005), in accordance with the observation that E2F1 overexpression in glioma triggers apoptosis and suppresses tumor growth (Fueyo, J. *et al.*, 1998; Mitlianga, P.G. *et al.*, 2002). In diffuse large B-cell lymphomas E2F1 acts as tumor suppressor, because low E2F1 expression is associated with treatment failure, and thus may be used as a prognostic marker (Moller, M.B. *et al.*, 2000).

1.3.9. E2F1 and cancer therapy

There are evidences showing that E2F1 might represent an endogenous chemosensitizer in cancer patients and that, thanks to its ability to induce apoptosis, its overexpression might be exploited as anticancer therapy. In gastric and colon cancer patients treated with adjuvant chemotherapy E2F1 expression was associated with improved survival (Belvedere, O. *et al.*, 2004; Lee, J. *et al.*, 2008). Moreover a study on

breast cancer patients revealed that the E2F1-positive group had less tumor recurrences, lymph node metastases during follow-up, and distant metastases than the E2F1-negative group (Kwon, M.J. *et al.*, 2010). The effect of E2F1 overexpression alone and in combination with chemotherapeutic drugs or radiotherapy on tumor cells has been evaluated in most types of human cancer, including glioma, melanoma, esophageal cancer, breast- and ovarian carcinoma, head and neck squamous cell cancer, gastric cancer, pancreatic carcinoma, fibrosarcoma, osteosarcoma, leukemia and non-small-cell lung cancer (Banerjee, D. *et al.*, 1998; Engelmann, D. *et al.*, 2010; Fueyo, J. *et al.*, 1998; Ben Shachar, B. *et al.*, 2010; Yang, L. *et al.*, 2011; Dong, Y.B. *et al.*, 1999; Elliott, M.J. *et al.*, 2002; Gomez-Manzano, C. *et al.*, 2001; Hunt, K.K. *et al.*, 1997; Kuhn, H. *et al.*, 2002; Liu, T.J. *et al.*, 1999; Nguyen, K.H. *et al.*, 2005; Nip, J. *et al.*, 1997; Parr, M.J. *et al.*, 1997; Rodicker, F. *et al.*, 2001). These studies revealed that induction of apoptosis by the combination treatments leads to increased responsiveness of tumor cells to chemotherapy, with modest effects on normal tissues. However, during tumorigenesis the cellular pathway leading to apoptosis are often inactivated, with the result that E2F1 overexpression *in vivo* could be rather oncogenic than anti-neoplastic.

2. AIM OF THE STUDY

GLI1 transcription factor is negatively controlled by p53 (Stecca, B. *et al.*, 2009). Therefore we hypothesized that WIP1 might modulate HH pathway by affecting p53 phosphorylation status. The consequent reduced activity of p53 would then lessen its inhibition of GLI1, thus resulting in the activation of HH pathway. Previous reports indicate that ligand-mediated HH signaling activation increases E2F1-mediated effects in medulloblastoma (Bhatia, B. *et al.*, 2011) and that E2F1 controls WIP1 expression at transcriptional level (Hershko, T. *et al.*, 2006). Therefore we speculated that HH pathway activation in cancer might be sustained by a positive regulatory loop involving WIP1-GLI1 and E2F1.

The aim of this study is to demonstrate that in human cancer cells the activation of HH pathway is sustained by a positive regulatory loop involving WIP1, GLI1 and E2F1 and that it controls cancer cell growth, self-renewal and tumorigenicity. We propose the following aims:

1. Test whether the oncogenic WIP1 phosphatase regulates the HH signaling pathway. In particular, we will focus on three transcription factors GLI1, GLI2 and GLI3, the last effectors of the HH pathway. We will investigate the molecular mechanisms of this regulation and the involvement of p53, a known WIP1 target.
2. Test whether GLI1 regulates WIP1 through the transcription factor E2F1.

3. RESULTS

3.1. WIP1 REGULATES ACTIVITY AND FUNCTIONS OF GLI1

3.1.1 WIP1 positively modulates the activity of GLI1, but not of GLI2 and GLI3

We first tested the effect of WIP1 on the transcriptional activity of GLI1, GLI2 and GLI3 by luciferase reporter assay. We used as reporter p8x3GLI-BS (GLI-BS) vector (Sasaki, H. *et al.*, 1997), which contains 8 direct repeats of the GLI consensus sequence cloned upstream the luciferase gene. We activated the reporter by co-transfecting GLI-BS with low amount of GLI1, GLI2 or GLI3 expression constructs in HEK-293T cells, patient-derived melanoma cells SSM2c and in the breast cancer cell line MCF7, which all express high levels of WIP1. Knock-down of WIP1 was performed using replication incompetent lentiviruses expressing two independent short hairpin RNA (shRNA) specifically targeting WIP1 (*shWIP1*): *shWIP1 39*, targeting the 3'UTR region of WIP1 mRNA and *shWIP1 40*, targeting exon 6. Both of them reduced WIP1 protein level in MCF7 cells (Fig.8a), which harbor *WIP1* gene amplification (Bulavin, D.V. *et al.*, 2002; Liang, C. *et al.*, 2012).

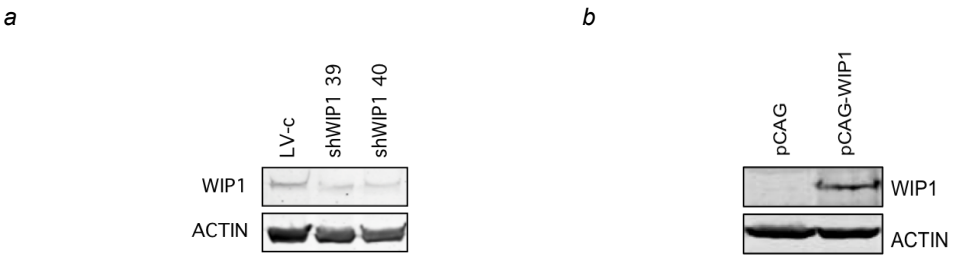


Figure 8 *Modulation of WIP1 levels. (a) Western blot analysis of control (LV-c) and shWIP1 transduced MCF7 cells showing the reduction of WIP1 expression with two independent shRNA. (b) Western blot analysis of control (pCAG) and WIP1 transfected HCT116 cells showing the expression of exogenous WIP1. ACTIN served as loading control.*

WIP1 overexpression was performed by transiently transfecting the colon cancer cell line HCT116, which expresses low level of WIP1, with pCAG-WIP1 vector, where WIP1 expression is driven by chicken β-actin promoter (Fig.8b). Knock-down of WIP1

significantly reduced GLI1 transcriptional activity in HEK-293T (p=0.0001), SSM2c (p=0.0001), MCF7 (p<0.0001) and HCT116 (P<0.001) cells (Fig.9a-d), but did not significantly affect GLI2 nor GLI3 (Fig.9a-c). Consistently, overexpression of WIP1 in HCT116 cells increased GLI1 transcriptional activity of more than 80% (p<0.0002) and failed to increase the activity of GLI2 and GLI3 (Fig.9d).

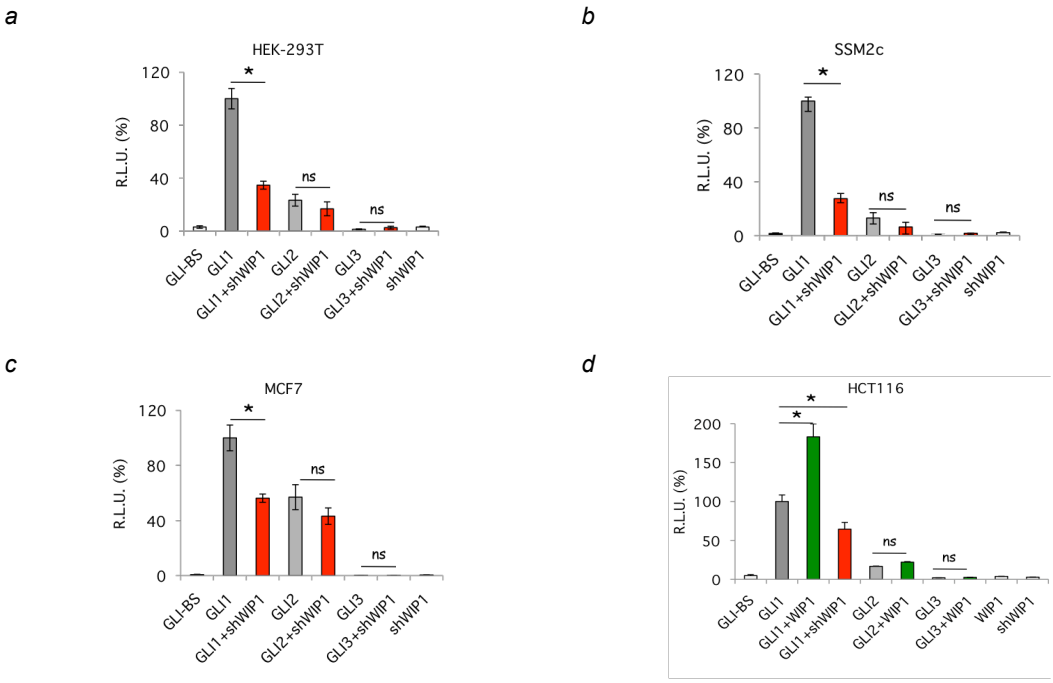


Figure 9 *Regulation of GLI transcriptional activity by WIP1. Quantification of GLI-dependent luciferase reporter assays. Relative luciferase units (R.L.U.) were GLI-dependent reporter firefly/renilla control ratios, with the level induced by GLI1 equated to 100%. (a-d). (a-d) WIP1 silencing (shWIP1) reduced transcriptional activity of GLI1 in HEK-293T, SSM2c, MCF7 and HCT116 cells, but not that of GLI2 and GLI3. (d) WIP1 increased transcriptional activity of GLI1, but not that of GLI2 and GLI3 in HCT116 cells. The data represent mean±SEM of at least 3 independent experiments. *p<0.05*

The modulation of GLI1 transcriptional activity by WIP1 was dose dependent, because transfection of increasing amount of *shWIP1* progressively reduced GLI1 transcriptional activity (Fig.10a). WIP1 overexpression did not change GLI1 transcriptional activity in the presence of a mutated GLI-dependent reporter (GLI-BS mut) (Fig.10b), thus confirming the specificity of our assay.

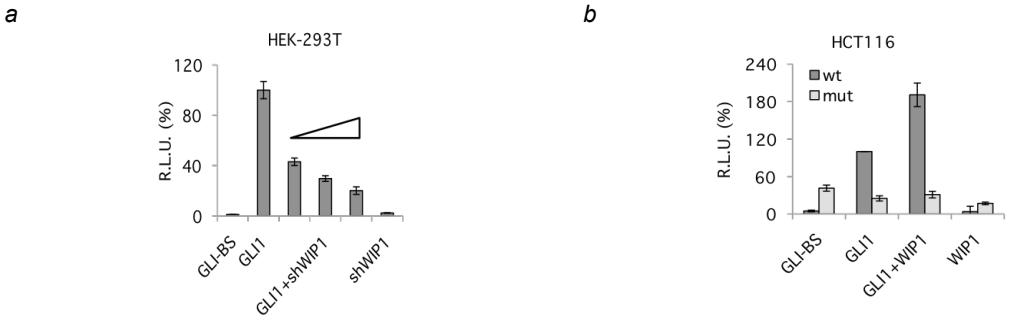


Figure 10 *(a) Quantification of GLI-dependent luciferase reporter assays showing a concentration dependent inhibition of GLI1 transcriptional activity by shWIP1 in HEK-293T cells. GLI1/shWIP1 DNA ratios were 1/0.5, 1/1,1/2 (triangle). (b) Quantification of GLI luciferase reporter assays with a wild type (wt, dark grey) and a mutant GLI-binding site reporter (mut, light grey) in HCT116 cells. GLI1 activated wt GLI-BS, but not mut GLI-BS, and WIP1 enhanced GLI1 transcriptional activity with wt but not with mut GLI-BS. R.L.U. were GLI-dependent reporter firefly/renilla control ratios with the level induced by GLI1 equated to 100%. Shown is the mean±SEM of 3 independent experiments.*

To investigate the requirement of WIP1 phosphatase activity for GLI1 modulation, we performed GLI-BS luciferase reporter assay with catalytically inactive WIP1 (D314A) (Fujimoto, H. *et al.*, 2006) or with the specific WIP1 inhibitor CCT007093 (CCT) (Rayter, S. *et al.*, 2008). WIP1 D314A mutant did not change exogenous GLI1 transcriptional activity in HCT116 cells (Fig.11a). Consistently, treatment of MCF7 cells with CCT reduced by more than 60% GLI1 transcriptional activity ($p<0.0001$) (Fig.11b), mimicking the effect of *shWIP1*. Altogether, these results indicate that WIP1 positively regulates transcriptional activity of GLI1 and that modulation of HH-GLI1 by WIP1 depends on its phosphatase activity.

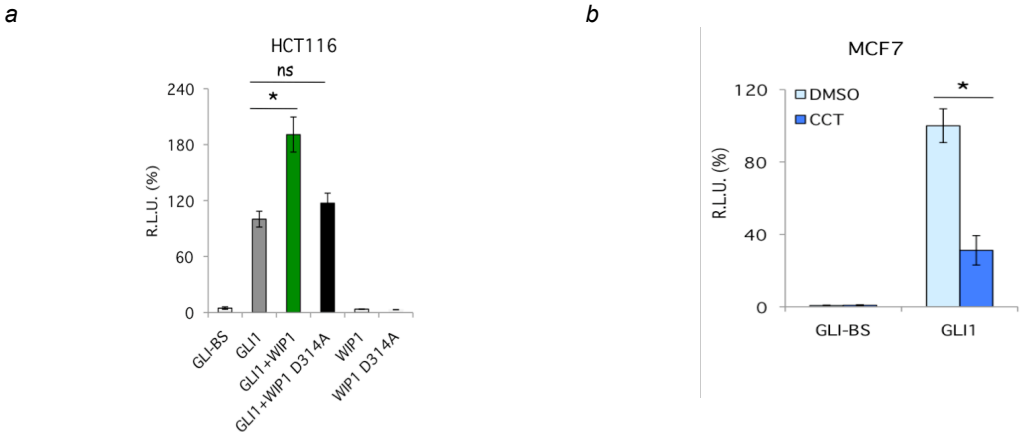


Figure 11 *Quantification of GLI-dependent luciferase reporter assay in HCT116 (a) and MCF7 (b) cells. R.L.U. were GLI-dependent reporter firefly/renilla control ratios, with the level induced by GLI1 equated to 100%. (a) Wild type WIP1 increased the transcriptional activity of GLI1 in HCT116 cells whereas catalytically inactive WIP1 (D314A) did not. (b) Treatment of MCF7 cells with WIP1 inhibitor CCT007093 (CCT, 10μM) reduced GLI1 transcriptional activity. The data represent mean±SEM of at least 3 independent experiments. *p<0.05*

3.1.2 WIP1 modulates the endogenous HH signaling

To investigate the effect of WIP1 on the endogenous HH pathway, we tested the protein level and transcriptional activity of endogenous GLI1 protein after WIP1 silencing/inhibition or overexpression. Western blot analysis showed that endogenous GLI1 was greatly decreased upon WIP1 knock-down in MCF7 cells (Fig.12a). Consistently, WIP1 overexpression significantly increased endogenous GLI1 protein levels in M26c melanoma cells (Fig.12b).

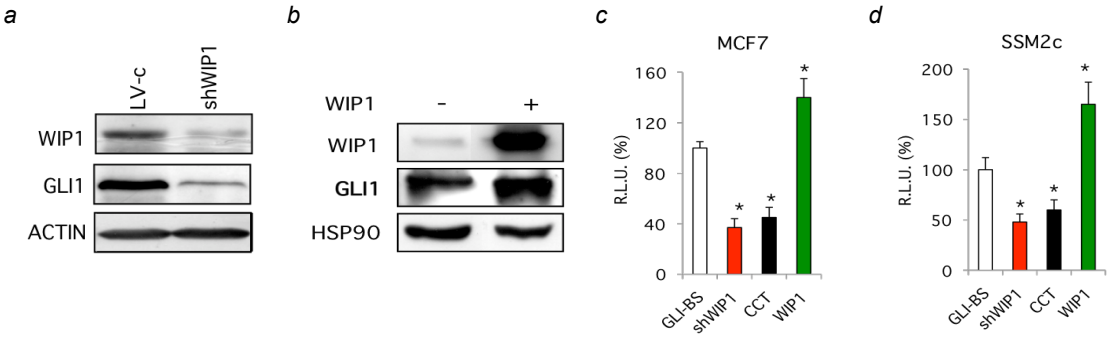


Figure 12 *WIP1 modulates the activity of the endogenous HH pathway in cancer cells. (a, b) Western blot analysis showing endogenous GLI1 and WIP1 proteins after WIP1 silencing in MCF7 cells (a) or overexpression in M26c cells (b). ACTIN and HSP90 served as loading control. (c, d) Quantification of GLI-dependent luciferase reporter assay in MCF7 (c) and SSM2c (d) cells after WIP1 inhibition with shWIP1 or CCT (10μM, 16hrs) and transfection with WIP1 expressing vector. R.L.U. were GLI-dependent reporter firefly/renilla control ratios, with the level of GLI-BS equated to 1. *p<0.05*

To measure the transcriptional activity of endogenous HH-GLI pathway, we performed a GLI-dependent luciferase reporter assay in MCF7 and SSM2c cells and we inhibited or overexpressed WIP1. The assay showed that WIP1 overexpression increased endogenous reporter activity of 40-50%, whereas chemical (CCT) or genetic (*shWIP1*) inhibition of WIP1 decreased by 50-60% endogenous reporter activity in both MCF7 and SSM2c cells (Fig.12c, d).

WIP1 silencing significantly repressed endogenous mRNA levels of *GLI1, PTCH1*, *FOXM1* and *SNAI1* (two HH targets), in both MCF7 and SSM2c cells (Fig.13a), confirming that it was able to control the endogenous HH pathway. Consistently, WIP1 overexpression increased GLI1 mRNA (Fig.13b), the best read out to measure HH pathway activation.

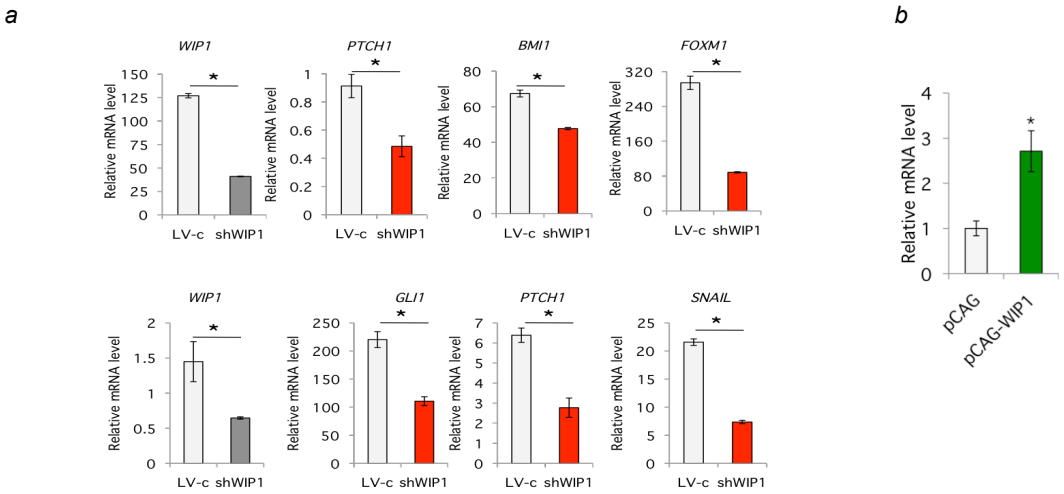


Figure 13 *WIP1 controls the activity of the endogenous HH pathway in cancer cells. (a) Expression of WIP1 and HH pathway components in MCF7 (top panel) and SSM2c (bottom panel) cells transduced with LV-c or shWIP1, measured by qPCR. (b) Endogenous GLI1 expression in SSM2c cells transfected with pCAG or pCAG-WIP1. LV-c and pCAG were set to 1. The y-axis represents expression ratio of gene/(GAPDH+βACTIN average). The data represent mean±SEM of 3 independent experiments. *p<0.05*

To investigate the function of Wip1 on the Hh-Gli pathway under physiological conditions, we used the NIH3T3 cells. These murine cells are very responsive to the activation of HH pathway and are one of the best models currently used to study the endogenous HH signaling. NIH3T3 cells were transfected with the GLI-BS luciferase reporter and treated with the Smo agonist SAG to activate the pathway.

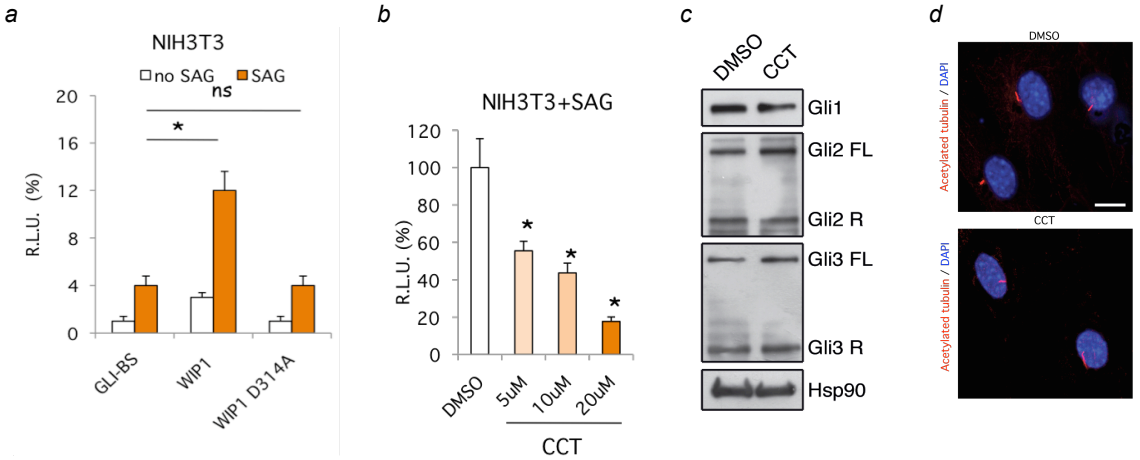


Figure 14 *Wip1 controls endogenous Hh pathway in NIH3T3 cells. (a) Quantification of endogenous GLI-dependent luciferase reporter assay after overexpression of wild type or D314A mutant WIP1, showing that WIP1 phosphatase activity is required to enhance HH pathway.(b) Quantification of endogenous GLI-dependent luciferase reporter assay after inhibition of endogenous Wip1 with CCT at the doses indicated for 16hrs. Cells were treated with SAG (100nM) for 48hrs. R.L.U. were GLI-dependent reporter firefly/renilla ratio, with the level induced by control (GLI-BS) equated to 1 in (a) and to 100% in (b). *p<0.05 (c) Effect of the chemical inhibition of Wip1 on endogenous Gli proteins. Western blot analysis of endogenous Gli1, Gli2 and Gli3 proteins in NIH3T3 cells treated with SAG (100nM) and CCT (10μM) for 48hrs. Hsp90 served as loading control. (d) Effects of CCT on primary cilia in SAG treated (100nM) NIH3T3 cells stained with acetylated tubulin. Scale bar=15μm.*

Overexpression of wild type, but not of D314A mutant WIP1 increased the transcriptional activity of endogenous HH pathway, which had been activated with the Smo agonist SAG (Chen, J.K. *et al.*, 2002) (Fig.14a). Wip1 inhibitor CCT suppressed endogenous Hh signaling in a dose-dependent manner (Fig.14b). At protein level, it slightly reduced the levels of endogenous Gli1, but it did not change Gli2 and Gli3 protein levels nor their processing (Fig.14c).

Primary cilia play an important role in physiological Hh pathway signal transduction (e.g. Corbit, K.C. *et al.*, 2005; Rohatgi, R. *et al.*, 2007). To investigate whether Wip1 might affect cilia formation, we inhibited Wip1 in NIH3T3 cells where cilia formation was stimulated by serum starvation. Staining for acetylated tubulin showed that cilia were not disturbed in morphology and frequency in NIH3T3 cells exposed to CCT (Fig.14d).

3.1.3. Modulation of GLI1 transcriptional activity by WIP1 does not require p53

A previous report showed that p53 negatively regulates GLI1 (Stecca, B. *et al.*, 2009). This finding, along with the ability of WIP1 to dephosphorylate p53 at Ser15 (Lu, X. *et al.*, 2005), and thus to decrease its activity, suggested that p53 might mediate the modulation of GLI1 by WIP1. In fact WIP1 could dephosphorylate and inactivate p53, thus relieving its inhibition on GLI1. To investigate this possibility we performed a GLI-dependent luciferase reporter assay silencing either p53, WIP1 or both. p53 silencing in HEK-293T and M26c patient-derived melanoma cells increased GLI1 transcriptional activity ($p=0.0001$ in both cells), as expected (Stecca, B. *et al.*, 2009), but it did not reverse the effect of WIP1 silencing on GLI1 (Fig.15a, b). Consistently, WIP1 enhanced and shWIP1 decreased GLI1 transcriptional activity in both HCT116 p53wt

and p53ko isogenic cell lines (Fig.15c) (p<0.0002), suggesting that the modulation of GLI1 by WIP1 did not require p53.

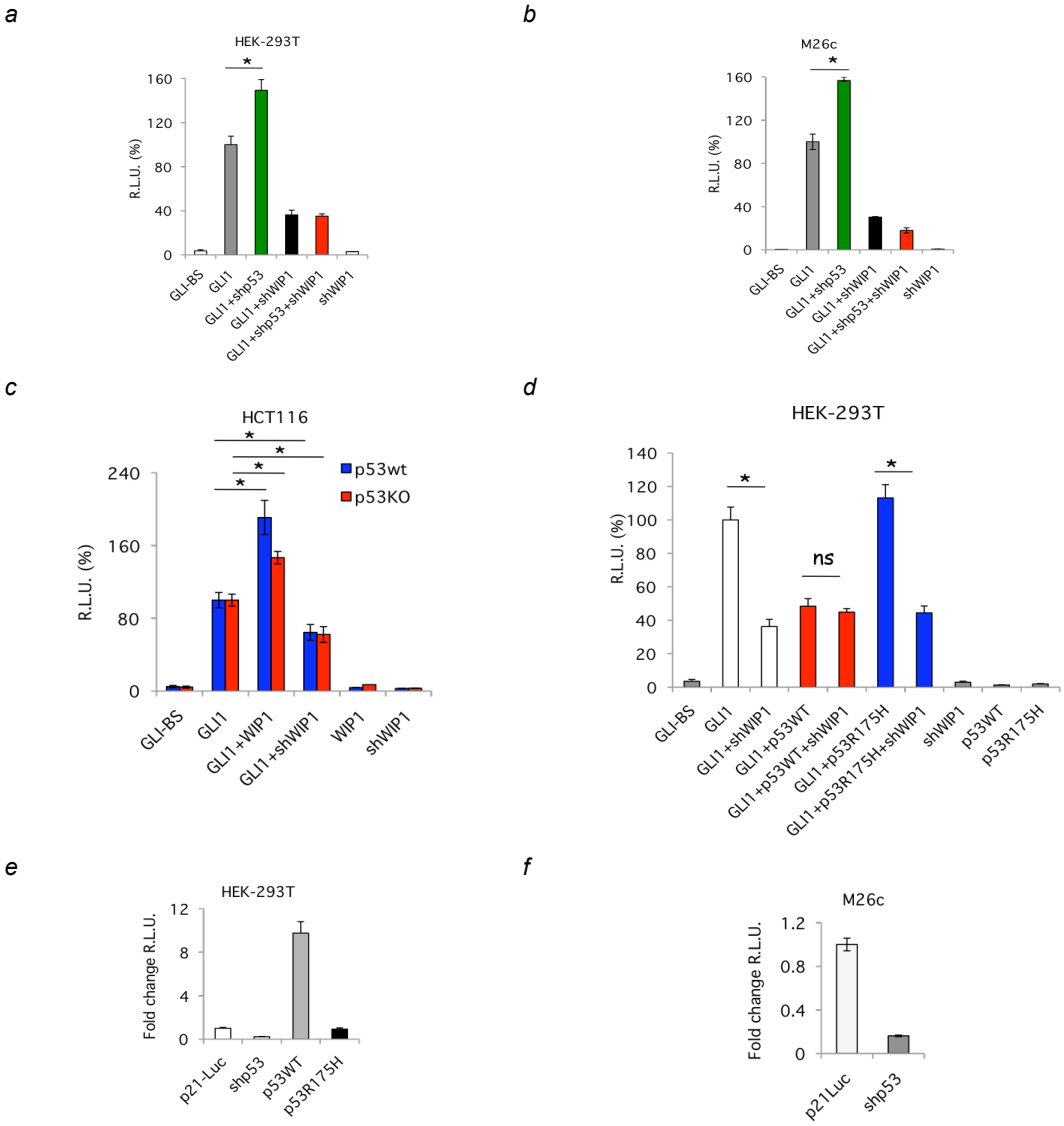


Figure 15 *Enhancement of GLI1 transcriptional activity by WIP1 does not require p53. (a-d) Quantification of GLI-dependent luciferase reporter assays. (a, b) p53 silencing in HEK-293T and M26c cells increased GLI1 transcriptional activity, but it did not reverse the effect of WIP1 silencing. (c) WIP1 overexpression increased and WIP1 silencing reduced GLI1 transcriptional activity in both p53wt (blue) and p53KO (red) isogenic HCT116 cells. (d) Overexpression of p53 wt (red), but not mutant p53R175H (blue), reduced GLI1 transcriptional activity in HEK-293T cells. WIP1 silencing reduced GLI1 transcriptional activity independently of co-expression of p53 wt or mutant p53R175H. p53/GLI1 DNA ratios were 0.5/2 in all cases. R.L.U. were GLI-dependent reporter firefly/renilla control ratios, with the level induced by GLI1 equated to 100% (e, f) Quantification of p53-dependent luciferase reporter assay (p21-Luc) to test the efficacy of shp53 and the transcriptional activity of p53 wt and the mutant p53R175H. The data represent mean±SEM of 3 independent experiments. *p<0.05*

To further confirm our results, we overexpressed wild type (wt) or mutant p53 in combination with shWIP1. p53 wt efficiently reduced GLI1 activity (by 52%, p<0.0001), whereas R175H (conformational mutant) did not have any effect, indicating that functional p53 is required to inhibit GLI1 (Fig.15d). WIP1 silencing, however, still

inhibited GLI1 activity in presence of R175H p53 mutant (by 60%) ($p<0.001$ in both wild type and mutant p53) (Fig.15d).To confirm the efficiency of shp53 and the activities of the p53 variants we used a p53-inducible luciferase reporter driven by $p21^{WAF1/CIP1}$ promoter (p21-Luc), widely used to measure p53 transcriptional activity (Fig.15e, f). This revealed an endogenous p53 transcriptional activity in M26c (p53 wt) and HEK-293T (p53 wt), despite the presence of SV40 large T-antigen in the latter. Altogether, these data suggest that modulation of GLI1 transcriptional activity by WIP1 does not require p53.

3.1.4. WIP1 increases stability and nuclear localization of GLI1 and interacts with it

GLI2 and GLI3, but not GLI1, are regulated by proteolytic cleavage to convert them from full length transcriptional activators to cleaved repressor forms in absence of HH ligands (Pan, Y. *et al.*, 2007). We then asked whether WIP1 might affect the processing and levels of GLI proteins.

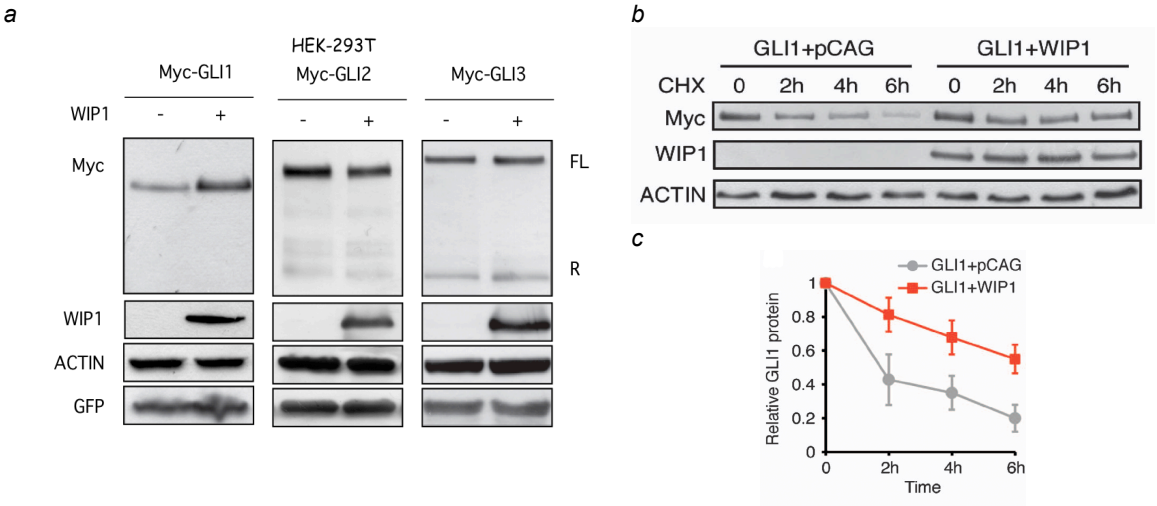


Figure 16 *WIP1 positively modulates GLI1 protein and interacts with it. (a) Western blot analysis showing GLI1, GLI2 and GLI3 proteins after WIP1 overexpression in HEK-293T cells. GFP served as control for transfection and ACTIN as loading control. (b) Western blot analysis showing the increase in GLI1 protein stability in presence of WIP1 after cycloheximide treatment (CHX) (b) and densitometric quantification of the data in b (c). The y-axis represents GLI1 protein levels normalized on ACTIN. The x-axis represents hours of CHX treatment. Shown is the mean±SEM of 3 independent experiments. The half-lives of GLI1 alone or with WIP1 co-expression are approximately 2 and 6 hrs, respectively.*

Co-transfection of WIP1 and Myc-tagged GLI1, GLI2 and GLI3 showed that WIP1 induced by two fold GLI1 protein level, did not change GLI2 and slightly increased levels of GLI3-FL and GLI3-R proteins without changing the ratio between full-length and repressor forms (Fig.16a).

Stabilization of GLI1 protein is a key event for HH signaling in cancer (Huntzicker, E.G. *et al.*, 2006), therefore we tested GLI1 protein stability in presence of WIP1. Treatment with cycloheximide to block *de novo* protein synthesis indicated that co-expression of WIP1 significant increased GLI1 stability (Fig.16b, c).

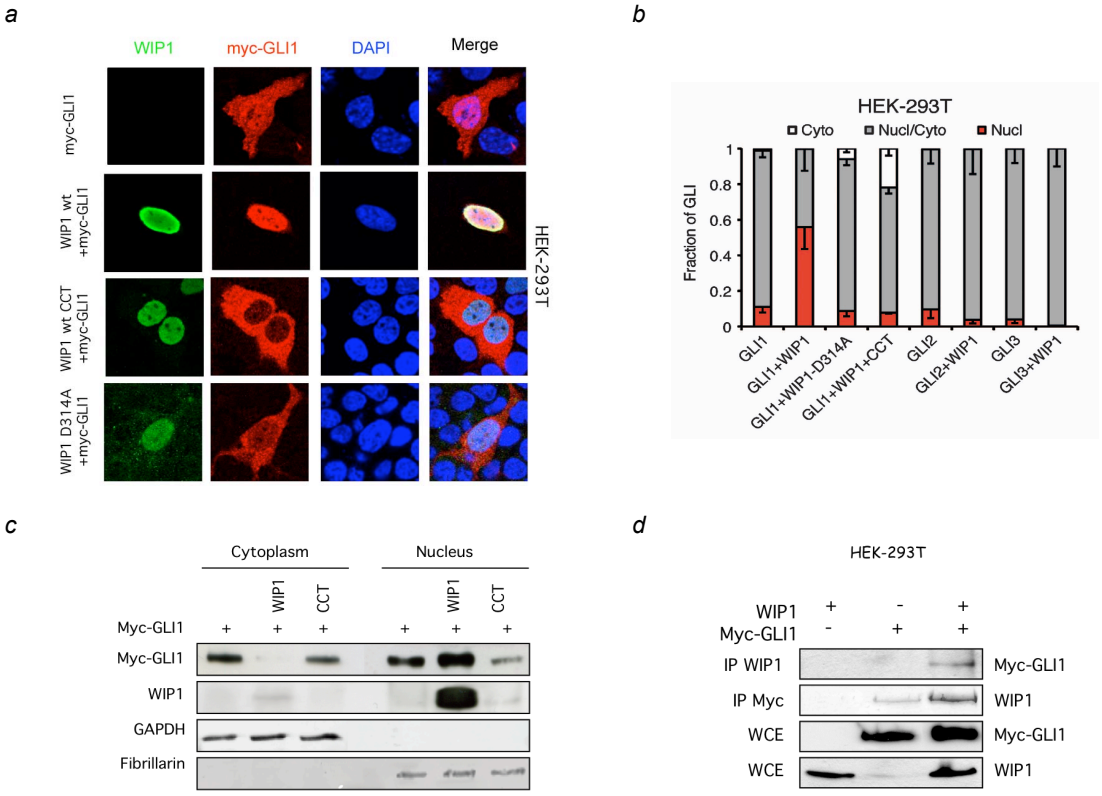


Figure 17 *WIP1 enhances GLI1 nuclear localization. (d-e) Representative images (d) and quantification (e) of GLI1, GLI2 and GLI3 subcellular localization after co-transfection with WIP1 in HEK-293T cells. Immunolocalization was with anti-Myc antibody for Myc-tagged GLI1, GLI2 and GLI3 (red) and anti-WIP1 antibody for WIP1 (green). Shown is the mean±SEM of at least 3 independent experiments. Nuclei were counterstained with DAPI. Over 500 cells were counted in each case. (f) HEK-293T cells were transfected with low doses of GLI1 in combination with WIP1 or CCT (10μM, 16hrs). Cell fractionation was performed and lysates were subjected to WB with the antibodies anti-Myc (for GLI1), anti-WIP1, anti-GAPDH (control for cytoplasmic proteins) and anti-fibrillarin (control for nuclear proteins). (g) Reciprocal co-immunoprecipitation (IP) experiments showing that exogenous WIP1 and GLI1 are in a complex. GLI1 and WIP1 expression in whole-cell extract (WCE) was determined by WB. Scale bar=15μm.*

WIP1 is a nuclear phosphatase (Fiscella, M. *et al.*, 1997), whereas GLI1 shuttles between the nucleus and the cytoplasm (Kogerman, P. *et al.*, 1999). Thus, we tested whether WIP1 might affect intracellular trafficking of GLI1, GLI2 and GLI3

proteins. Epitope-tagged GLI1 was nuclear and cytoplasmic in HEK-293T cells, whereas co-expression of WIP1 greatly enhanced nuclear localization of GLI1 (Fig.17a, b). GLI2 and GLI3 localization was not affected by WIP1 (Fig.17b). Catalytically inactive WIP1 D314A mutant or treatment with WIP1 inhibitor CCT slightly increased the cytosolic fraction of GLI1 (Fig.17a, b), suggesting that WIP1 phosphatase activity is required for GLI1 nuclear localization.

We corroborated these findings by performing cell fractionation in HEK-293T cells transfected with low doses of GLI1 in combination with WIP1 or CCT. Overexpression of GLI1 in combination with WIP1 resulted in the disappearance of GLI1 in the cytoplasm and increase of GLI1 level in the nucleus (Fig.17c). Consistently, CCT treatment reduced GLI1 protein levels in both nuclear and cytoplasmic fractions.

Co-localization of WIP1 and GLI1 in the nucleus suggested that WIP1 might interact with GLI1. Thus, we tested whether WIP1 and GLI1 were physically associated. When Myc-tagged GLI1 and WIP1 were overexpressed in HEK-293T cells, GLI1 was immunoprecipitated by an anti-WIP1 antibody (Fig.17d). Reciprocal experiments showed that WIP1 was immunoprecipitated by GLI1 (Fig.17d), indicating that WIP1 and GLI1 are in a complex.

3.1.5. WIP1 is required for HH-induced cancer cell growth and cancer stem cell self-renewal

Our data suggest that WIP1 positively regulates the HH pathway by enhancing GLI1 function. To understand the potential role of WIP1 in regulating HH-induced cell growth, we mimicked HH pathway activation by silencing *PTCH1* (Stecca, B. *et al.*, 2009) and knocked-down *WIP1* in MCF7 and SSM2c cells. The silencing was achieved by transducing the cells with a replication incompetent lentivirus expressing a shRNA specifically targeting *PTCH1* (*shPTCH1*, targeting exon 18 of *PTCH1* mRNA) or *WIP1*

(*shWIP1*). MCF7 cells were transduced with LV-c (control lentivector), *shPTCH1*, *shWIP1* or both (*shPTCH1/shWIP1*) and allowed to form colonies. *shPTCH1* led to a 60% increase in the number of colonies compared to LV-c (p=0.049). *shWIP1* significantly reduced the number of colonies compared to LV-c (p<0.0001), and, surprisingly, it drastically diminished the effect induced by HH pathway activation (*shWIP1* versus *shPTCH1/shWIP1*, p=0.53) (Fig.18a, b).

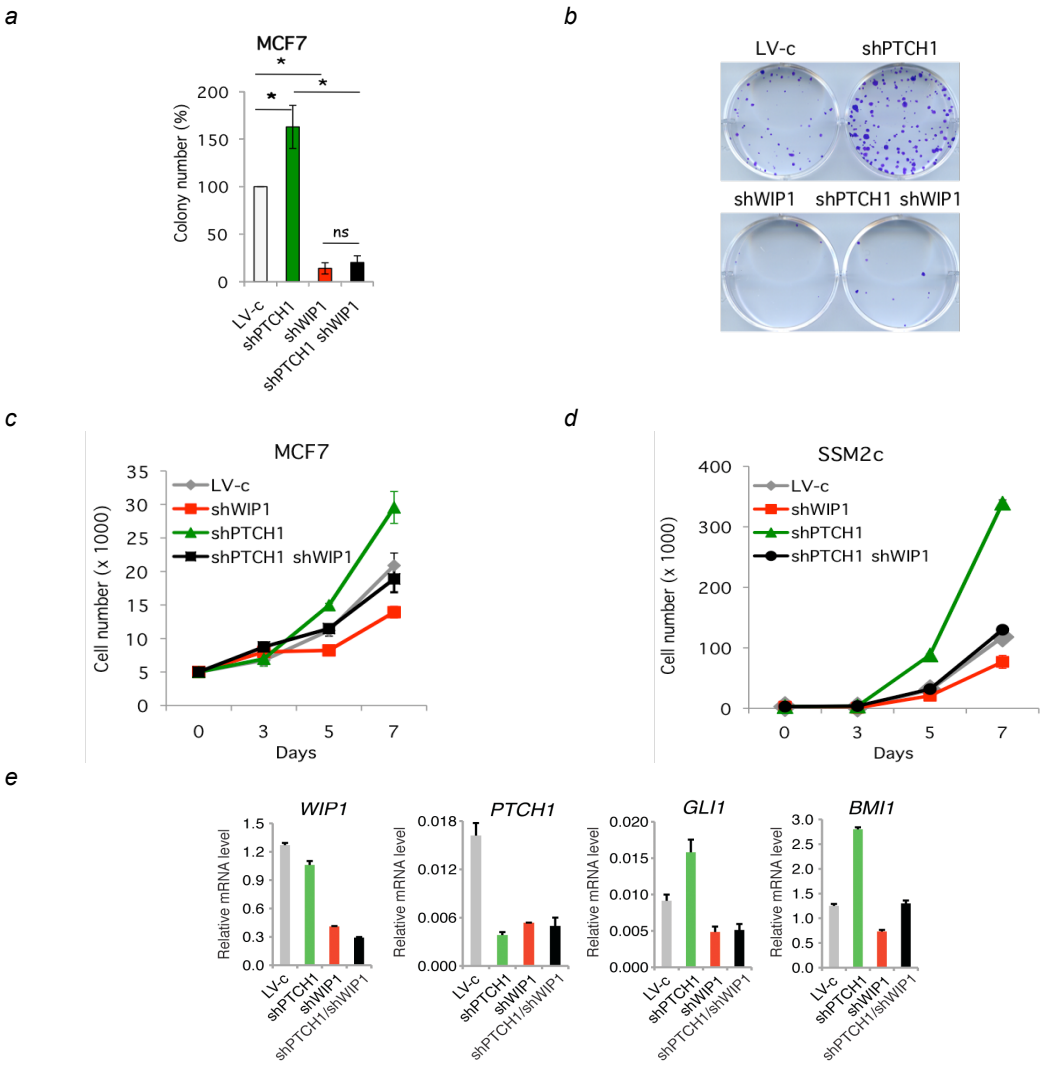
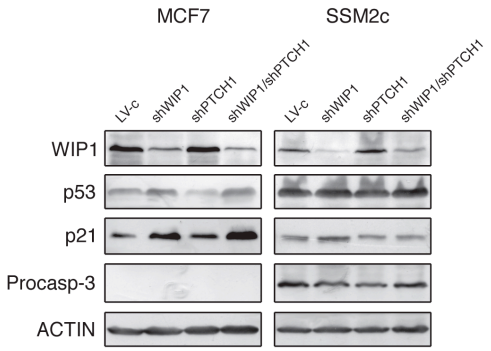


Figure 18 *WIP1 is required for cancer cell growth induced by HH pathway activation. (a) Histogram of the quantification and (b) representative images of the colony assay in MCF7 cells transduced with LV-c, shPTCH1, shWIP1 or shPTCH1/shWIP1 lentiviruses. The y-axis represents the percentage of colony number with the number of colonies of LV-c-transduced cells equated to 100%. *p<0.05 (c,d) Growth assay in MCF7 (c) and SSM2c cells (d) transduced with LV-c, shPTCH1, shWIP1 or shPTCH1/shWIP1 lentiviruses, showing that WIP1 silencing reduced the increase in cancer cell proliferation induced by shPTCH1 at day 7. (e) qPCR analysis of WIP1 and HH pathway components in MCF7 cells transduced with lentiviruses as indicated. The y-axis represents expression ratio of gene/(GAPDH+βACTIN average).*

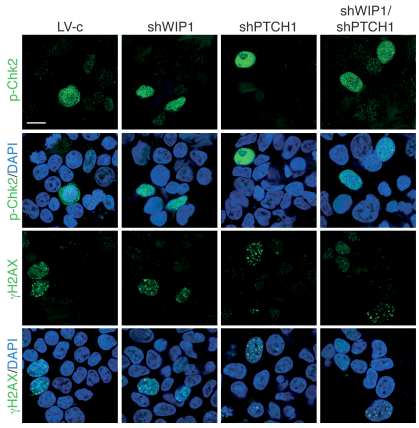
As a complementary approach, we assessed proliferation by viable cell count. In both MCF7 and SSM2c cells *shPTCH1* increased cell number compared to LV-c

(p<0.05 in both cell types), whereas *shWIP1* reduced it (p<0.05 in both cell types). Consistently with the results obtained in colony assays, *shWIP1* suppressed the effect of *shPTCH1* in both cell types (LV-c versus *shPTCH1/shWIP1*, p=0.41 in MCF7, p=0.38 in SSM2c) (Fig.18c, d). To confirm that these differences were dependent on the modulation of HH pathway, we measured the expression of HH components by qPCR and we found that WIP1 knock-down reduced the expression of GLI1 and the target gene BMI1 (Stecca, B. *et al.*, 2007) in MCF7 cells (Fig.18e).

a



b



c

MCF7

Time	SubG0	G1	S	G2-M
LV-c	0.35	69.06	14.70	15.89
shWIP1	1.17	71.84	11.01	15.99
shPTCH1	0.29	67.47	20.37	11.87
shWIP1/shPTCH1	2.29	74.68	8.6	14.43

SSM2c

Time	SubG0	G1	S	G2-M
LV-c	3.28	40.98	21.64	34.10
shWIP1	3.56	60.75	19.90	15.79
shPTCH1	5.72	47.93	21.59	24.76
shWIP1/shPTCH1	2.04	57.60	19.77	20.59

d

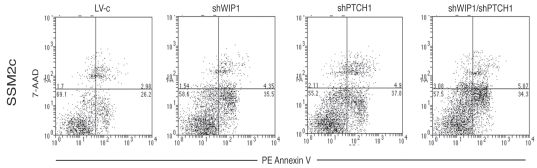


Figure 19 *Effect of LV-c, shWIP1, shPTCH1 and shWIP1/shPTCH1 on DNA damage response in MCF7 and SSM2c cells. (a) Western blot analysis of MCF7 and SSM2c cells transduced with control (LV-c), shWIP1, shPTCH1, shWIP1/shPTCH1 lentiviruses, showing expression of WIP1, p53, p21 and procaspase-3 proteins. ACTIN served as loading control. (b) Confocal microscopy of SSM2 cells transduced as indicated. Cells were fixed, permeabilized and immunostained with anti-pChk2 and anti-γH2AX. Nuclei were counterstained with DAPI. Scale bar=15μm. (c) Effect of LV-c, shWIP1, shPTCH1 and shWIP1/shPTCH1 on cell cycle distribution and apoptosis in MCF7 and SSM2c cells. (a) Cell cycle distribution by propidium iodide staining. (d) Annexin V/7-AAD staining in SSM2c cells. Data were from the representative experiments. The experiments were conducted 3 times with similar results.*

We ruled out the possibility that WIP1 silencing by itself would trigger a DNA damage response and apoptosis. In fact, *shWIP1* did not increase phosphorylated CHK2 or γH2AX (Fig.19a, b) and did not induce changes in the amount of apoptotic cells in both SSM2c and MCF7 cell types, consistently with a previous study

(Parssinen, J. *et al.*, 2008) (Fig.19c, d). However, *shWIP1* produced a slight reduction of cells in S phase (MCF7) and an increase of cells in G1 phase (SSM2c) (Fig.19c). These data indicate that WIP1 silencing reverses the effect of increased proliferation obtained by HH pathway activation, supporting the hypothesis that endogenous WIP1 is critical to maintain cancer cell growth induced by activation of the HH signaling.

The HH signaling regulates cancer stem cell (CSC) self-renewal in several instances (e.g. Eberl, M. *et al.*, 2012; Clement, V. *et al.*, 2007; Peacock, C.D. *et al.*, 2007; Liu, S. *et al.*, 2006; Varnat, F. *et al.*, 2009; Santini, R. *et al.*, 2012). Recent data indicate that WIP1 inhibition suppresses self-renewal and growth of mouse mammary CSC (Zhang, X. *et al.*, 2010). To test for a possible role of WIP1 in controlling HH-mediated CSC self-renewal, we used melanoma and breast CSC cultures (melanomaspheres and mammospheres) from, respectively, SSM2c and MCF7 cells, transduced with *shWIP1* and/or *shPTCH1*. These cells, seeded in non-adherent culture conditions form spheres enriched in stem and progenitor cells able to self-renew. As a measure of self-renewal, we quantified the ability of dissociated, single cells (plated at limiting dilution) to generate secondary spheres. Silencing of *PTCH1* increased by 9-fold the number of mammospheres ($p<0.0001$) (Fig.20a, b) and by 2-fold the number of melanomaspheres ($p=0.001$) (Fig.20c, d) compared to LV-c. Silencing of *WIP1* slightly decreased the number of melanomaspheres ($p=0.03$) but did not change the number of MCF7-derived mammospheres ($p=0.21$); however, it reversed the increase in self-renewal induced by *shPTCH1* in both cell types (LV-c vs *shPTCH1/shWIP1*, $p=0.56$ in SSM2c cells; LV-c vs *shPTCH1/shWIP1*, $p=0.11$ in MCF7 cells) (Fig.20a, c). Similar results were obtained by inhibiting WIP1 with the CCT inhibitor in SSM2c-derived melanomaspheres, where CCT treatment prevented the increase in self renewal induced by *PTCH1* silencing (LV-c versus *shPTCH1*, $p=0.0014$; LV-c CCT versus *shPTCH1* CCT, $p=2141$) (Fig.20d). These data suggest that endogenous WIP1 is

required for the maintenance of CSC self-renewal induced by activation of the HH signaling.

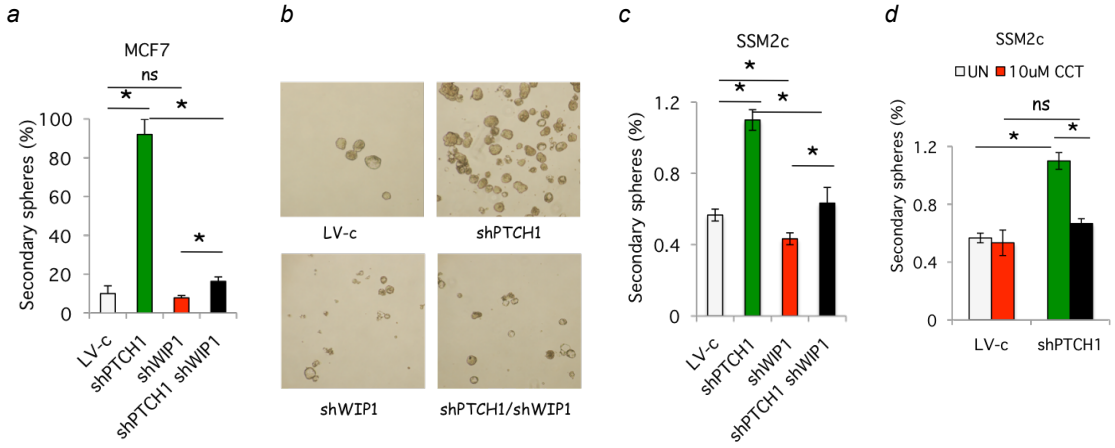


Figure 20 *WIP1 is required for stem cell self renewal induced by HH pathway activation. Quantification of the self-renewal assay in MCF7 (a) and SSM2c (c) cells transduced with LV-c, shPTCH1, shWIP1 or shPTCH1/shWIP1 and in SSM2c cells transduced with LV-c or shPTCH1 and treated with CCT007093 (d). The y-axis represents the percentage of secondary spheres formed over the number of cells seeded at 1cell/μl. The data represent mean±SEM values of 3 independent experiments. (b) Representative images of self-renewal assay performed in MCF7 cells plating dissociated cells at 1 cell/μl. Scale bar=150μm. *p=0.05*

3.1.6. *WIP1* correlates with the expression of HH pathway components in human melanomas

Recent data indicate that WIP1 enhances Sonic hedgehog-dependent medulloblastoma formation (Doucette, T.A. *et al.*, 2012) and that human melanomas require an active HH pathway (Stecca, B. *et al.*, 2007).

Table 1. *Clinical features of melanoma patients and corresponding short-term melanoma cultures*

Sample	***Gender***	***Age***	***Type***[a]	***Site***	***Breslow***[b]	***Stage***
M16	M	88	PM	Cheek	10	IIB
SSM2	M	55	CM	Trunk	8.5	IIIB
SSM2c						
M3	F	84	LNM	Groin	6.4	IIIC
M5	M	65	CM	Arm	1.1	IIIB
M6	M	79	LNM	Arm	n.a.	IIIC
M11	M	76	CM	Trunk	9.3	IIIC
M14	F	79	CM	Leg	8.1	IIIC
M15	M	85	CM	Scalp	11	IIC
M15c						
M21	M	87	CM	Trunk	5	IIIC
M25	F	80	CM	Leg	2.6	IIIC
M26	F	79	LNM	Groin	0.47	IV
M26c						
M27	M	71	LNM	Armpit	2.35	IIIC

[a] *PM: primary melanoma, CM: Cutaneous Metastasis; LNM: Lymph Node Metastasis.* [b] *Thickness (mm) of the primary melanoma from which metastasis originated. n.a.: data not available. SSM2c, M15c and M26c were cloned from the original metastases (SSM2, M15 and M26 respectively).*

To investigate the significance of HH modulation by WIP1 in melanoma we evaluated the expression of *WIP1* mRNA and of components of the HH pathway in a panel of 15 patient-derived short-term melanoma cultures (1 from a primary and 14 from metastatic melanomas) (Table 1) (Santini, R. *et al.*, 2012). qPCR analysis revealed a positive correlation between the expression of *WIP1* and *GLI1* (R^2=0.807), *PTCH1* (R^2=0.867) and *SMO* (R^2=0.787) (Fig.21). No correlation was found between the expression of *WIP1* and *GLI2* (R^2=0.004), nor between *WIP1* and microphtalmia-associated transcription factor (*MITF-M)* (R^2=0.088), a melanoma marker.

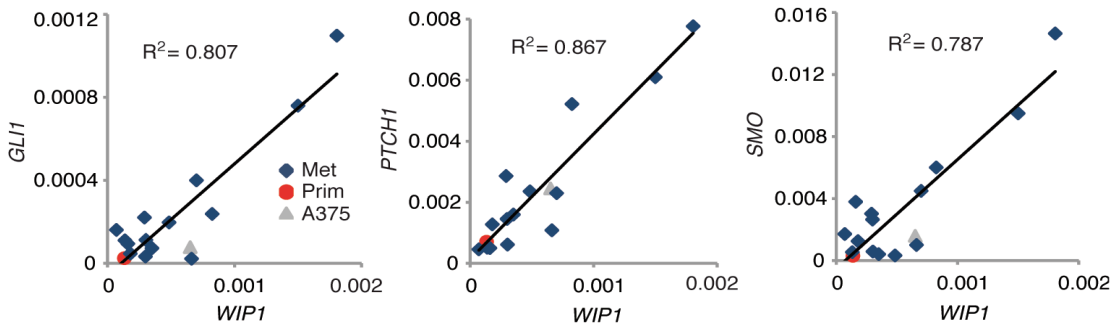


Figure 21 *Linear correlation analysis of WIP1 with GLI1, PTCH1, SMO (a), GLI2 and MITF-M (b) transcripts expression, measured by qPCR, in the A375 melanoma cell line and in 15 patient-derived short-term melanoma cultures, 1 of which from a primary melanoma (Prim) and 14 from metastases (Met). Each sample is represented by a dot. Axes in each graph represent expression ratio of gene/(GAPDH+βACTIN average). The extent of the correlation is indicated by R^2 coefficient.*

3.1.7. WIP1 is required for melanoma xenograft growth induced by activation of the HH pathway

To test for a role of WIP1 in regulating HH-induced melanoma xenograft growth *in vivo*, 10^3 SSM2c cells stably transduced with LV-c, *shPTCH1* and/or *shWIP1* were engrafted subcutaneously into athymic nude mice. FACS-sorted GFP+ SSM2c cells expressing *shPTCH1* yielded more than 2 fold larger xenografts than control GFP+ cells (LV-c) (p=0.041) (Fig.22a). Combined with the results of growth curve assays (Fig.18d), these data suggested that activation of the HH pathway increases melanoma cancer cell growth *in vitro* and in a xenograft model. WIP1 silencing decreased melanoma xenograft growth compared to LV-c (p=0.046) and drastically reduced tumor

growth induced by *shPTCH1* to levels comparable to LV-c (*shWIP1* versus *shPTCH1/shWIP1*, p=0.52; LV-c versus *shPTCH1/shWIP1*, p=0.33 at day 32) (Fig.22a, b), consistent with *in vitro* data (Fig.18d). These results suggest that endogenous WIP1 is critical in regulating melanoma xenograft growth induced by activation of the HH pathway.

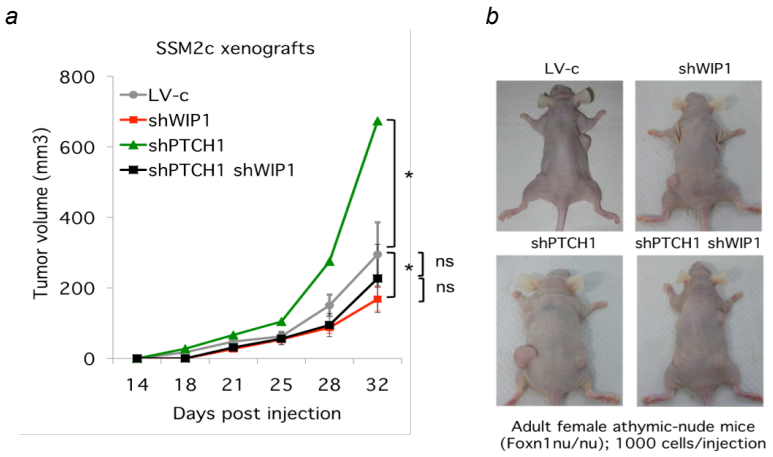


Figure 22 *Interference with WIP1 prevents HH-induced melanoma xenograft growth Effect of WIP1 silencing on HH pathway activation in SSM2c melanoma xenografts. SSM2c cells were transduced with LV-c, shPTCH1, shWIP1 or shPTCH1/shWIP1 lentivectors and injected s.c. in athymic-nude mice. (a) Quantification of the tumor volume over time (n=12/group), showing that WIP1 silencing reverted the increase in tumor growth induced by shPTCH1. (b) Representative images of SSM2c xenografts growth, as indicated.*

Because WIP1 enhances GLI1 activity, we considered the possibility that combined inhibition of WIP1 and HH signaling might produce a more potent pathway inhibition, resulting in reduced cancer cell growth. To test this hypothesis, we treated patient-derived melanoma cells SSM2c, M21 and M26c, and MCF7 cells with low doses of cyclopamine (Cyc, 2.5μM), a SMO inhibitor (Taipale, J. *et al.*, 2000), or of CCT (10μM), a WIP1 inhibitor (Rayter, S. *et al.*, 2008), or both. Single treatments were of limited efficacy (Fig.23); however, Cyc and CCT combined treatments produced synergistic inhibition of cell growth in both melanoma and breast cancer cells (Fig.23, Excess over Bliss Additivism > 8), consistent with a convergent action of the two agents.

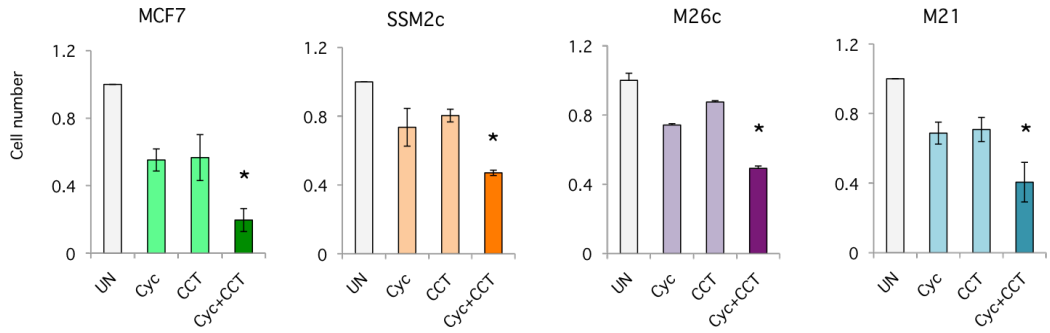


Figure 23 *Synergistic reduction of cell number in the breast cancer cell line and in patient-derived human melanoma cells SSM2c, M21 and M26c after combined treatment with WIP1 inhibitor CCT (10μM) and SMO antagonist cyclopamine (Cyc, 2.5μM) for 72hrs. Cyc and CCT alone modestly reduced cell growth, whereas Cyc+CCT combined treatment showed synergism (Excess over Bliss Additivism >8).*

3.2. GLI1 REGULATES WIP1 THROUGH THE TRANSCRIPTION FACTOR E2F1

3.2.1. E2F1 controls WIP1 expression in melanoma

A previous report showed that E2F1 induces WIP1 expression in U2OS, H1299 and WI38 cells by directly binding to WIP1 promoter (Hershko, T. *et al.*, 2006). To confirm that E2F1 was able to modulate WIP1 expression also in patient-derived melanoma cells, we transduced SSM2c cells with two replication incompetent lentiviruses expressing shRNAs specifically targeting E2F1 (*shE2F1 50 and 53*, both targeting exon 7 of *E2F1* mRNA).

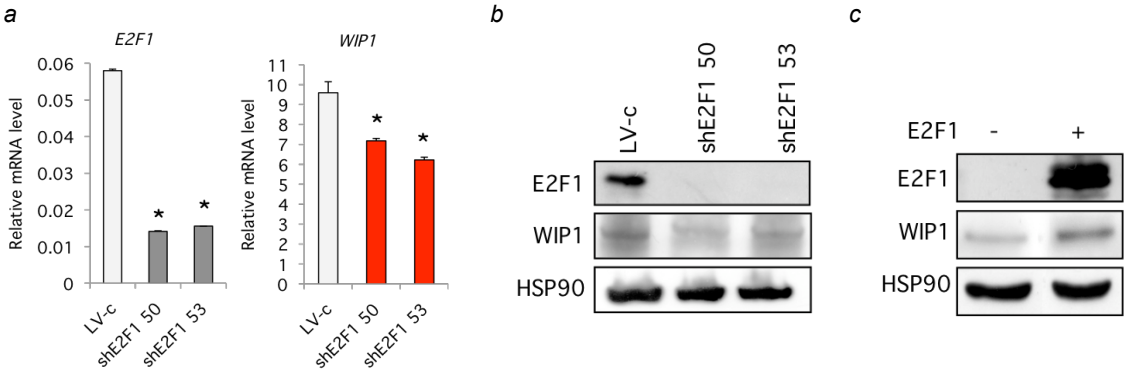


Figure 24 *E2F1 controls WIP1 expression in melanoma. (a) qPCR analysis of WIP1 and E2F1 in SSM2c cells transduced with lentiviruses as indicated. The y-axis represents expression ratio of gene/(GAPDH+βACTIN average). (b) Western blot analysis of SSM2c cells transduced with control (LV-c), shE2F1 50 or shE2F1 53, showing the expression of endogenous E2F1 and WIP1. HSP90 served as loading control. (b) Western blot analysis of M26c cells transfected with control or E2F1, showing the expression of exogenous E2F1 and endogenous WIP1. HSP90 served as loading control. *p<0.05*

Real-time PCR and western blot analysis showed that both shE2F1 tested strongly reduced E2F1 mRNA levels and completely abolished E2F1 protein levels in SSM2c cells (Fig.24a, b). E2F1 ablation also reduced WIP1 mRNA and protein levels compared to the control (Fig.24a, b). Consistently, E2F1 overexpression increased endogenous WIP1 protein levels in M26c cells (Fig.24c). Altogether, these results indicate that E2F1 controls WIP1 expression in melanoma.

3.2.2. HH pathway controls E2F1 expression in melanoma

Recent data indicate that SHH induces the E2F1-mediated expression of fatty acid synthase (FASN) in medulloblastoma (Bhatia, B. *et al.*, 2011), suggesting that HH pathway might control E2F1 expression. To investigate whether modulation of HH pathway might affect E2F1 expression in melanoma we activated HH pathway in MCF7 cells by PTCH1 silencing, achieved by transducing the cells with a replication incompetent lentivirus specifically targeting *PTCH1* (*shPTCH1*).

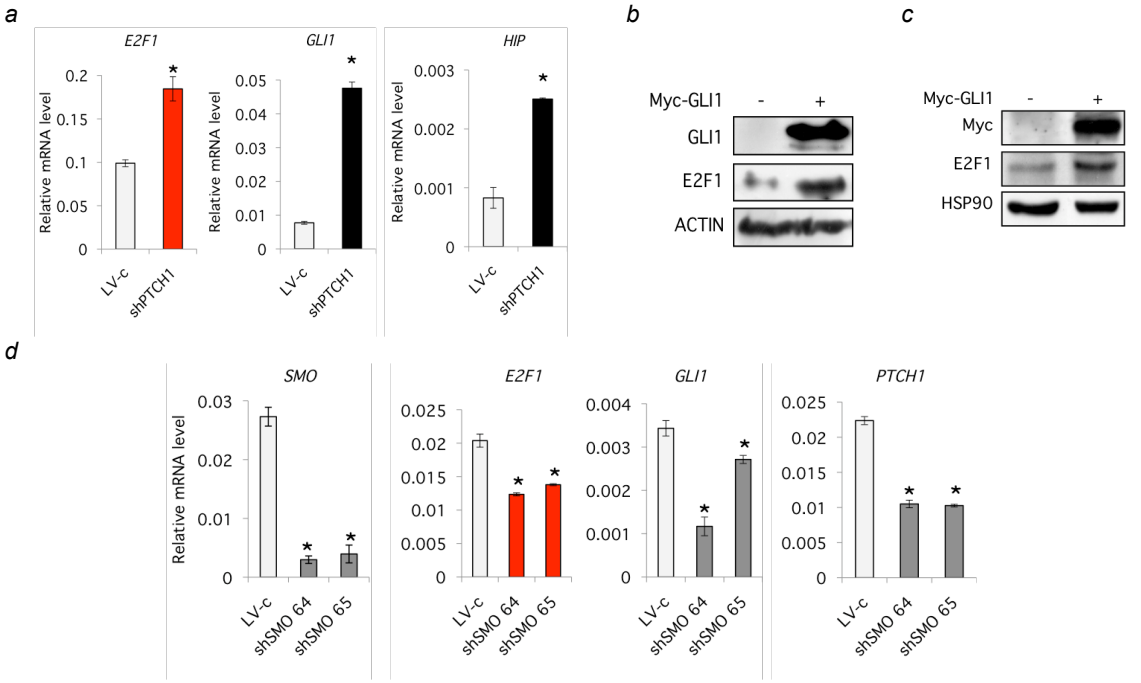


Figure 25 *HH pathway controls E2F1 expression. (a-c) HH pathway activation induces E2F1 expression. (a) qPCR analysis of E2F1, GLI1 and HIP in MCF7 cells transduced with lentiviruses as indicated. The y-axis represents expression ratio of gene/(GAPDH+βACTIN average). (b, c) Western blot analysis of SSM2c cells (b) and M26c (c) transfected with Myc-tagged-GLI1, showing the expression of exogenous GLI1 and endogenous E2F1. ACTIN and HSP90 served as loading control. (d) HH pathway inhibition reduces E2F1 expression. qPCR analysis of SMO, E2F1, GLI1 and PTCH1 in SSM2c cells transduced with lentiviruses as indicated. The y-axis represents expression ratio of gene/(GAPDH+βACTIN average). *p<0.05*

PTCH1 ablation resulted in an increase of HH targets *GLI1* and *HIP*, as well as a strong increase of *E2F1* mRNA level (Fig.25a). Similar results were obtained in SSM2c and M26c cells, where activation of HH pathway by transfection of GLI1 led to an increase of endogenous E2F1 protein (Fig.25b, c). To confirm our results, we inhibited HH pathway by genetic ablation of the transmembrane receptor SMO, by transducing SSM2c cells cells with two replication incompetent lentiviruses expressing shRNA specifically targeting SMO (*shSMO* 64, targeting exon 9, and *shSMO* 65, targeting exon 12 of SMO mRNA). Real-time PCR analysis showed that both the *shSMO* tested strongly reduced the mRNA levels of *SMO*, as well as of the two HH targets *GLI1* and *PTCH1*, confirming the inhibition of HH signaling (Fig.25d). SMO silencing also resulted in a significant decrease of *E2F1* mRNA levels compared to the control (Fig.25d). Altogether these results indicate that HH pathway modulates E2F1 expression in melanoma.

3.2.3. GLI1 directly binds to E2F1 promoter

The final effectors of the HH pathway are the GLI transcription factors, with GLI1 being the stronger transcriptional activator. To test whether the induction of E2F1 was directly mediated by GLI1, we performed chromatin precipitation assay in HEK-293T cell line and in SSM2c and M26c patient-derived melanoma cells. We transfected the cells with low amount of Myc-tagged GLI1 or the corresponding empty vector as negative control and we immunoprecipitated the exogenous GLI1 by an anti-Myc antibody. We then recovered the immunoprecipitated DNA and we performed real-time PCR (qPCR) using primers specific for the promoters of known direct transcriptional targets of GLI1 or of E2F1. qPCR analysis showed that GLI1 was able to bind the regulatory region of its targets *PTCH1* or *HIP*, but not that of *GAPDH*, used as negative control. Interestingly, GLI1 was able to efficiently bind to *E2F1* promoter (Fig.26), suggesting that GLI1 transcriptionally induces E2F1 expression by directly binding to its promoter.

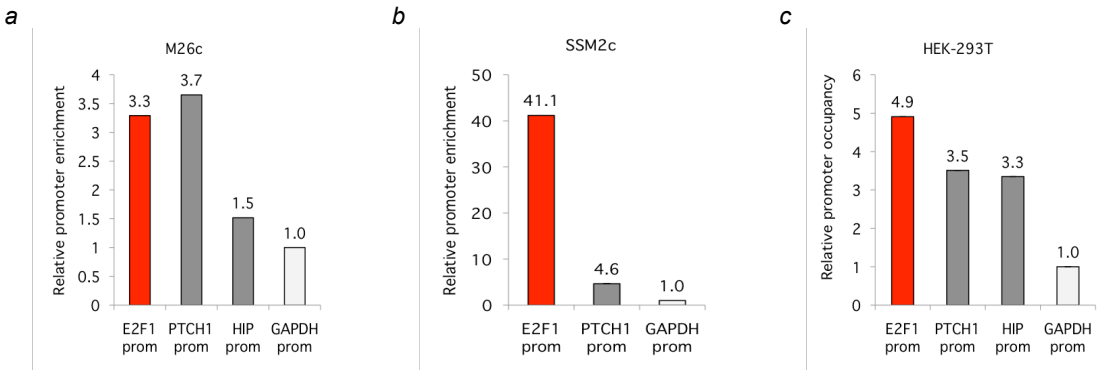


Figure 26 *GLI1 binds to E2F1 promoter. M26c (a), SSM2c (b) and HEK-293T (c) were transfected with Myc-tagged GLI1 and subjected to chromatin immunoprecipitation. qPCR analysis with primers specific for the promoters indicated showed that GLI1 binds to E2F1 promoter as well as to the promoters of known HH targets, as indicated. The y-axis represents the ratio of ChIP/IM DNA, with the ratio for GAPDH promoter (negative control) equated to 1.*

3.2.4. GLI1 and GLI2 activate E2F1 promoter by binding to a region between -132bp and -269bp

Bioinformatic analysis did not identify any canonical GLI consensus sequence in E2F1 promoter. However, a recent report indicated that GLI transcription factors can bind variant GLI binding sites with relatively low affinity, still leading to strong transcriptional activation (Winklmayr, M. *et al.*, 2010). Therefore E2F1 promoter might contain a degenerate GLI consensus sequence which drives E2F1 expression upon HH stimulation. To identify the region of E2F1 promoter responsible for the modulation by GLI transcription factors, we cloned three different fragments of E2F1 promoter upstream the luciferase gene (132bp, 269bp and 656bp) (Fig.27a). We transfected the patient-derived melanoma cells SSM2c and M26c with the reporter vector along with GLI1, GLI2 or E2F1, that we used as a positive control, because the E2F1 promoter contains an E2F responsive element just upstream the transcription start site (Johnson, D.G. *et al.*, 1994). The assay showed that GLI1 was able to activate the reporter driven by the -269bp ($p<0.0001$ in SSM2c; $p=0.0183$ in M26c) and -656bp ($p=0.0214$ in SSM2c; $p=0.0007$ in M26c), but not by the -132bp fragment ($p=0.8068$ in SSM2c; $p=0.3559$ in M26c). As expected, E2F1 was able to transactivate all three regions ($p<0.04$ in all cases) (Fig.27b, c).

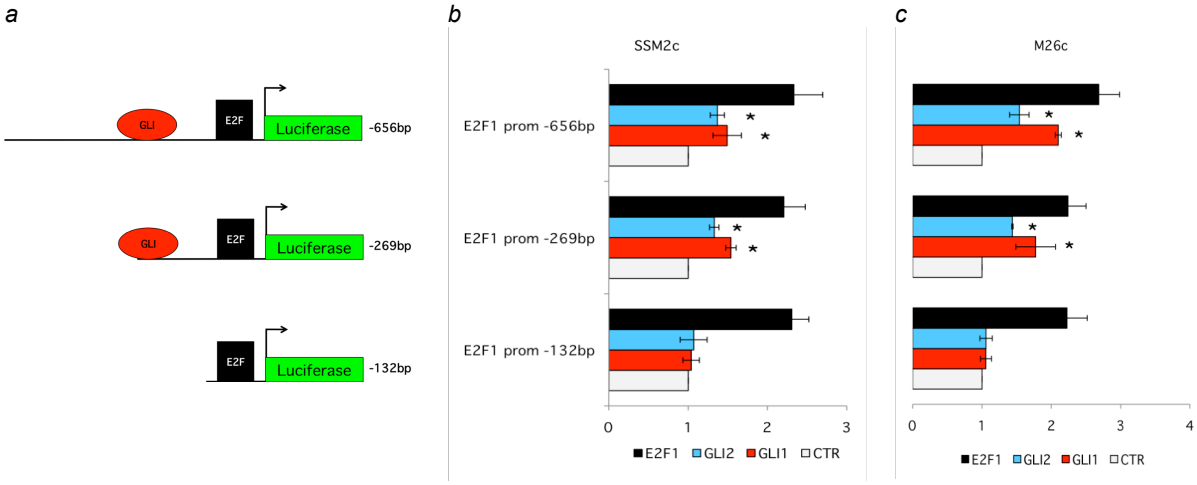


Figure 27 *GLI1 and GLI2 transactivate E2F1 promoter. (a) Scheme of the E2F1 endogenous promoter and of the three fragments cloned for the reporter assay. (b, c) Luciferase reporter assay in SSM2c (b) and M26c (c) cells showing that GLI1 and GLI2 transactivate the -656bp and the -269bp fragments. E2F1 was used as positive control. *p<0.05*

Because both GLI1 and GLI2 bind to the same region on the DNA, we expected that GLI2 as well would transactivate E2F1 promoter. Indeed, luciferase reporter assay showed that GLI2 transactivated the reporter driven by the -269bp ($p<0.0001$ in SSM2c; $p=0.0006$ in M26c) and -656bp ($p=0.0013$ in SSM2c; $p=0.0182$ in M26c) fragments, although at a lesser extent compared to GLI1. Like GLI1, GLI2 did not transactivate the reporter driven by the -132bp fragment ($p=0.4753$ in SSM2c; $p=0.3965$ in M26c) (Fig.27b, c). These results indicates that E2F1 promoter contains one (or more) GLI responsive element(s) between -132bp and -269bp upstream the transcription start site (Fig.27).

3.2.5. E2F1 mediates the effects of HH pathway activation on cell growth and self renewal

Our data suggest that HH pathway activation induces E2F1 expression. E2F1 has been shown to behave as either oncogene, by driving cell proliferation, or as tumor suppressor, by inducing apoptosis, depending on the cellular context. On the other hand, activation of HH pathway in melanoma has been shown to increase cell proliferation and self renewal of cancer stem cells (Stecca, B. *et al.*, 2007; Santini, R. *et al.*, 2012). To investigate whether E2F1 is a downstream mediator of the HH signaling,

we mimicked HH pathway activation by silencing *PTCH1* (Stecca, B. *et al.*, 2009) and knocked-down *E2F1* in M26c and SSM2c cells. The silencing was achieved by transducing the cells with replication incompetent lentivectors specifically targeting *PTCH1* (*shPTCH1*) or *E2F1* (*shE2F1*). Cells transduced with LV-c (control lentivector), *shPTCH1*, *shE2F1* or both (*shPTCH1/shE2F1*) were seeded and allowed to grow for 7 days. In both M26c and SSM2c cells *shPTCH1* increased cell number compared to LV-c (p=0.0002 in M26c, p=0.0018 in SSM2c), whereas *shE2F1* reduced it (p=0.0041 in M26c, p=0.044 in SSM2c).

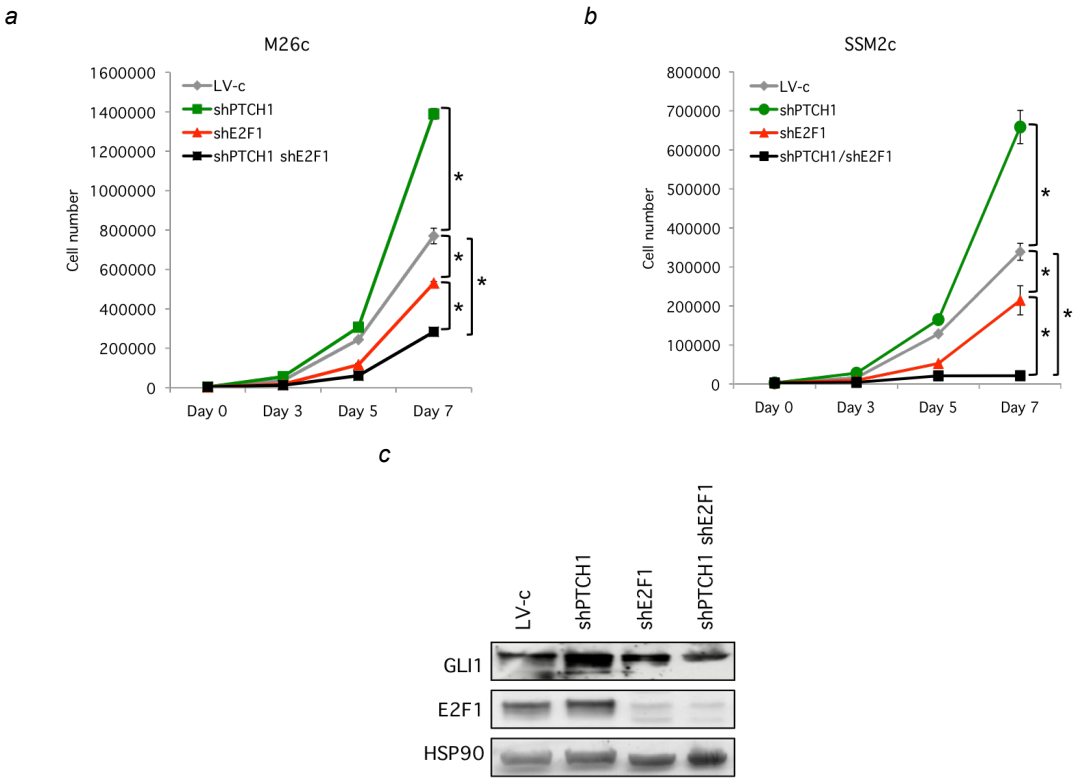


Figure 28 *E2F1 is required for melanoma cell. (a) Growth assay in M26c (a) and SSM2c cells (b) transduced with LV-c, shPTCH1, shE2F1 or shPTCH1/shE2F1 lentivectors, showing that E2F1 silencing reduced melanoma cell proliferation at day 7. *p<0.05 (c) Western blot analysis of M26c cells transduced with LV-c, shPTCH1, shE2F1 or shPTCH1/shE2F1 lentivectors, showing the expression of GLI1 and E2F1. HSP90 served as loading control.*

Interestingly, cells transduced with both *shPTCH1* and *shE2F1* showed a reduction of cell growth even bigger than cells transduced with *shE2F1* alone (LV-c versus *shPTCH1/shE2F1*, p=0.0003 in M26c, p=0.0001 in SSM2c; *shE2F1* versus *shPTCH1/shE2F1*, p=0.0001in M26c, p=0.0066 in SSM2c) (Fig.28a, b).

To confirm that these differences were dependent on the interplay between E2F1 and HH pathway, we measured the expression of GLI1 and E2F1 in the M26c cells transduced with LV-c, *shPTCH1*, *shE2F1* or *shPTCH1/shE2F1*. Western blot analysis showed that the increased proliferation observed upon PTCH1 silencing was associated to increased E2F1 protein levels. Similarly, E2F1 levels in M26c cells transduced with *shE2F1* and *shPTCH1/shE2F1* correlated with their growth. (Fig.28c). These data indicate that E2F1 is critical to maintain cancer cell growth induced by activation of the HH signaling and suggest that activation of HH pathway, which increases E2F1 expression (Fig.25a), renders the cells more dependent on E2F1 function.

As already mentioned, the HH signaling regulates cancer stem cell (CSC) self-renewal in several instances (e.g. Eberl, M. *et al.*, 2012; Clement, V. *et al.*, 2007; Peacock, C.D. *et al.*, 2007; Varnat, F. *et al.*, 2009; Santini, R. *et al.*, 2012; Liu, S. *et al.*, 2006). However, at present there is no clear evidence describing that E2F1 might control the self-renewal of CSC, even though its pivotal role in the control of cell cycle suggests that it might play a role in this cellular process. To test for a possible role of E2F1 in controlling HH-mediated CSC self-renewal, we used CSC cultures from M26c patient-derived melanoma cells (melanomaspheres) transduced with *shE2F1* and/or *shPTCH1*. As a measure of self-renewal, we quantified the ability of dissociated, single cells (plated at limiting dilution) to generate secondary spheres. Silencing of *PTCH1* increased by almost 2-fold the number of melanomaspheres ($p=0.0009$) (Fig.29) compared to LV-c, whereas silencing of *E2F1* significantly decreased the number of melanomaspheres ($p=0.0004$).

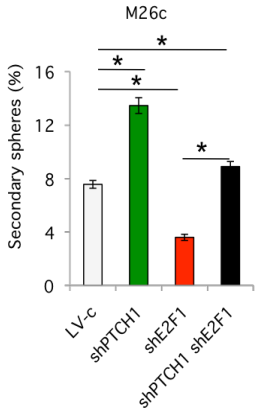


Figure 29 *E2F1 silencing reduces melanoma cancer stem cell self renewal. Quantification of the self-renewal assay in M26c cells transduced with LV-c, shPTCH1, shE2F1 or shPTCH1/shE2F1. The y-axis represents the percentage of secondary spheres formed over the number of cells seeded at 1cell/μl. The data represent mean±SEM values of 3 independent experiments. *p=0.05*

In contrast with the results obtained in the growth assay, *PTCH1* silencing increased the self renewal of both control CSC and of CSC depleted of *E2F1* (*shE2F1* versus *shPTCH1/shE2F1*, p=0.0003), suggesting that HH pathway controls self-renewal independently from E2F1 (Fig.29). Altogether, these data suggest that both HH pathway and E2F1 control melanoma CSC self-renewal, but that the two pathways act independently.

4. MATERIAL AND METHODS

4.1. Cell lines and patient samples

MCF7, HEK-293T, p53wt and p53ko HCT116, A375 and NIH3T3 cells were obtained from ATCC. Human melanoma samples were obtained after approved protocols. Fifteen human melanoma (Table 1). After mechanical disruption, tumors were incubated for 1 hour at 37°C with 1mg/ml collagenase A and 20µg/ml DNase I (Roche Applied Science, Basel, Switzerland) in DMEM/F12 (Euroclone, Milan, Italy). After dissociation and filtration in 70 µm cell strainers, cells were grown in DMEM/F12 with 10% FBS and epidermal growth factor (EGF) (5ng/ml) (Invitrogen, Carlsbad, CA). SSM2c, and M26c cultures were cloned from the original metastases (SSM2 and M26, respectively) by plating one cell per well. Patient-derived melanomas were passaged one to two times prior to RNA extraction and *in vitro* experiments. The identity of melanoma cells was verified by immunocytochemistry using primary antibodies specific for melanoma: anti-Melan A (A103), anti-S100 (Dako, Glostrup, DK) and anti-Vimentin (V9) (Santa Cruz Biotechnology). Drugs used for treatments were puromycin (2µg/ml), CCT007093 (CCT, 10µM), cycloheximide (CHX, 80µg/ml), tomatidine (Tom, 2.5µM) (Sigma-Aldrich, St. Louis, MO, USA), cyclopamine (Cyc, 2.5µM, TRC, Toronto, Canada) and SAG (100nM for 48hrs, Enzo Life Sciences, Farmingdale, NY, USA).

4.2. Plasmids, mutagenesis and transfections

Vectors used to overexpress human E2F1 and Myc-tagged GLI proteins were: pCMV-E2F1 (kind gift from M. Chiariello) (Melillo, R.M. *et al.*, 1994), pCS2+MT-GLI1 (kind gift from A. Ruiz i Altaba) (Stecca, B. *et al.*, 2009), pCS2+MT-GLI2 (Addgene, Cambridge, MA) (Roessler, E. *et al.*, 2005), pCS2+MT-GLI3 (kind gift from A. Ruiz i Altaba) (Stecca, B. *et al.*, 2009). WIP1 and p53 cDNAs were PCR amplified with Platinum Pfx DNA polymerase (Life Technologies, Grand Island, NY, USA) and cloned

into pCAG vector (Life Technologies) using the following primers: WIP1clon-F, 5'-TCATGAGCTCTGATCAATGGCGGGGCTGTACTCGCTG-3'; WIP1clon-R, 5'-TGCCT AGGGTCGACTCAGCAAACACAAACAGTTTTCC-3'; p53clon-F, 5'-TGATCAATGGAG GAGCCGCAGTCA-3'; p53clon-R, 5'-GTCGACTCAGTCTGAGTC AGGCCCTT-3'. WIP1D314A cDNA was subcloned into pCAG from pcDNA4/TO-WIP1D314A-FlagNT (kind gift from R.H. Medema) (Lindqvist, A. *et al.*, 2009). Mutation in pCAG-p53 (S175H) was introduced using QuikChange II (Agilent Technologies, Santa Clara, CA, USA). Control empty vectors used were pCS2+MT (Addgene) or pCAG. The identity of the vectors used was verified by direct sequencing. All transfections were performed in OptiMEM (Life technologies) using X-tremeGENE 9 transfection reagent (Roche Applied Science) according to manifacturer's protocol.

4.3. Lentiviral vectors, virus production and transductions

Replication incompetent lentiviruses were produced in HEK-293T cells by cotransfecting the cells with the lentiviral vector of interest, the packaging vector pCMV-dR8.74 and the envelope vector pMD2.G in a ratio 4:3:1. The supernatant containing the lentiviral particles was harvested after 48 hrs and 72 hrs from transfection, syringe-filtered with a 0.45µm PVDF filter and stored at -80°C until use. Transduction was performed on cells seeded at low density with a MOI=500; 8µg/ml hexadimethrine bromide (Polybrene®, Sigma-Aldrich) was used to increase transduction efficiency. Lentiviral vectors used were: pLV-CTH (LV-c), pLV-CTH-shPTCH1 (*shPTCH1*), (targeting sequence 5′-GCACTATGCTCCTTTCCTC-3', exon 18), pWPXL-shp53 (*shp53*) (kind gift from A. Ruiz i Altaba) (Stecca, B. *et al.*, 2009), pLKO.1-puro (LV-c), pLKO.1-puro-shWIP1 39 (*shWIP1* 39) (targeting sequence 5'-CC CTTCTCGTGTTTGCTTAAA-3', 3'UTR), pLKO.1-puro-shWIP1 40 (*shWIP1* 40) (targeting sequence 5'- CGAGAGAATGTCCAAGGTGTA-3', exon 6), pLKO.1-puro-shSMO 64 (*shSMO* 64) (targeting sequence 5'- GTGGAGAAGATCAACCTGTTT -3', exon 9), pLKO.1-puro-shSMO 65 (*shSMO* 65) (targeting sequence 5'-

CCTGATGGACACAGAACTCAT-3', exon 12), pLKO.1-puro-shE2F1 50 (*shE2F1* 50) (targeting sequence 5'- GACCTCTTCGACTGTGACTTT-3', exon 7), pLKO.1-puro-shE2F1 53 (*shE2F1* 53) (targeting sequence 5'-ACCTCTTCGACTGTGACTTTG-3', exon 7) were from Open Biosystem (Lafayette, CO, USA). Most of the experiments were done with shWIP1 40 and shE2F1 50.

4.4. Luciferase reporter assays

To measure GLI transcriptional activity we used a GLI-responsive luciferase reporter (p8x3GLI-BS, GLI-BS) which contains 8 direct repeats of the GLI consensus sequence GACCACCCA cloned upstream the luciferase gene (kind gift from H. Sasaki) (Sasaki, H. *et al.*, 1997). To confirm the specificity of our assay, we also used its corresponding mutant (p8x3GLI-BSmut) (kind gift from H. Sasaki) (Sasaki, H. *et al.*, 1997). To measure the transcriptional activity of p53 and its variants we used a p53-responsive (p21-Luc) luciferase reporter (WWP-Luc) (Addgene) (el-Deiry, W.S. *et al.*, 1993). To measure the ability of GLI1, GLI2 and E2F1 to activate the E2F1 promoter, three fragments of E2F1 promoter (-132bp, -269bp, -656bp) were cloned upstream the luciferase gene in pGL3Basic vector using the following primers: E2F1prom-132bp-F, 5'-ACGCTAGCGCGCGTTAAAGCCAATAGG-3'; E2F1prom-269bp-F, 5'-ACGCTAGCATGTTCCGGTGTCCCCAC-3'; E2F1prom-656bp-F, 5'-ACGCTAGCACTGGACTGTGAGCTCCTTAGG-3'; E2F1prom_clon-R 5'-ACCTCGAGATCCTTTTTGCCGCGAAA-3'. The identity of the reporters was verified by direct sequencing. All reporters were used in a dual-reporter assay in combination with Renilla pRL-TK vector (Promega, Madison, WI, USA) (ratio 10:1) to normalize luciferase activities; pGL3Basic vector (Promega) was used to equal DNA amounts. The luciferases signals were measured using the Dual-Glo® Luciferase Assay System (Promega) and the GloMax® 20/20 Luminometer (Promega).

4.5. Immunofluorescence

Cells were transfected with equimolar amounts of the indicated plasmids. After 48 hrs cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 in phosphate buffered saline (PBS), blocked with 10% goat serum in PBS-0.1% Triton X-100, and incubated overnight with mouse anti-Myc (9E10) or rabbit anti-WIP1 (H-300) (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies. Secondary antibodies were anti-mouse Rhodamine Red-conjugated and anti-rabbit FITC-conjugated (Life Technologies). Nuclei were counterstained with 4',6-diamidin-2-fenilindolo (DAPI) and slides were mounted with p phenylenediamine (pPDA)-containing mounting medium. Immunofluorescence was visualized using a Nikon Eclipse TE2000-E confocal microscope. For acetylated tubulin staining, NIH3T3 cells were grown to 60% confluency, serum starved to force cilia formation and treated with CCT (10μM) for 16hrs. Other antibodies used were: mouse anti-acetylated tubulin (6-11B-1) (Sigma-Aldrich), rabbit anti-p-Chk2 and anti-γH2AX antibodies (Cell Signaling Technology). The identity of melanoma cells on primary melanoma cultures was verified by immunocytochemistry. Patient-derived melanoma adherent cells were fixed with 4% PFA, blocked with 10% goat serum in PBS-Tween 0.01% and incubated with rabbit anti-S100 (Dako), mouse anti-MelanA/MART-1 (clone A103, Dako) and mouse anti-Vimentin (clone V9, Santa Cruz). Secondary antibodies were anti-mouse Rhodamine Red-conjugated and anti-rabbit FITC-conjugated (Invitrogen) (Santini, R. *et al.*, 2012).

4.6. Protein extraction, western blot (WB), co-immunoprecipitation (IP) assay and cell fractionation

Cells were transfected with equimolar amounts of the plasmids indicated and lysed in ice in RIPA buffer (1% NP-40, 150mM NaCl, 5mM EDTA, 0.25% NaDOC, 50mM Tris-HCl pH 7.5) added with 1X Complete EDTA-free Protease Inhibitor Cocktail (Roche Applied Science) and phosphatase inhibitors for 20min. After centrifugation for 20min at 14000rpm the supernatant containing the whole cell extract (WCE) was

recovered and quantified with Coomassie Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). For WB 80µg of proteins were resolved on a sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Membranes were blocked in 6% Non-fat dry milk in PBS-Tween buffer (PBS and 0.05% Tween 20) (PBS-T) for 1h and incubated with the primary antibody of interest overnight at 4°C. After incubation with HRP-conjugated secondary antibody (Cell Signaling Technology, Beverly, MA, USA), bands were visualized by chemiluminescent detection. Ponceau staining was used as control for proper transfer.

For IP experiments 300µg of WCE were diluted with IP buffer (0.5% NP-40, 100mM NaCl, 5mM EDTA, 10% glycerol, 50mM Tris-HCl pH 7.5) added with 1X Complete EDTA-free Protease Inhibitor Cocktail and phosphatase inhibitors and incubated overnight at 4°C in continuous rotation with Dynabeads Protein G (Life Technologies) pre-conjugated with the antibody of interest. Beads were washed three times with IP buffer, proteins were eluted with Laemmli buffer, resolved on SDS-PAGE and transferred onto a nitrocellulose membrane for blotting. For cell fractionation, cells were lysed in 20mM Hepes buffer, 10mM KCl, 1mM EDTA, 0.2% NP-40, 10% Glycerol added with 1X Complete EDTA-free Protease Inhibitor Cocktail and phosphatase inhibitors. This preparation was centrifuged at high speed and the cytoplasmic extract was collected in the resulting supernatant, whereas the pellet (nuclei and membrane) was dissolved in 20mM Hepes, 420mM NaCl, 20% Glycerol, 10mM KCl, 1mM EDTA added with 1X Complete EDTA-free Protease Inhibitor Cocktail and phosphatase inhibitors. The sample was centrifuged and the nuclear protein extract collected from the supernatant.

The following antibodies were used: rabbit anti-GLI1 (Ab49314), mouse anti-Myc (9E10), rabbit anti-WIP1 (H-300), mouse anti-HSP90 (F-8), goat anti-GAPDH

(V18), goat anti-Fibrillarin (D14), mouse anti-p53 (DO-1), goat anti-p21 (C-19) (Santa Cruz Biotechnology), rabbit anti-E2F1, mouse anti-Gli1 (L42B10) (Cell Signaling Technology), goat anti-GLI2 (AF3635), goat anti GLI3 (AF3690) (R&D, Minneapolis, MN, USA), mouse anti-β-ACTIN (AC15) (Sigma-Aldrich). Blots were imaged using ChemiDoc XRS (Bio-Rad) and relative band densities were determined by Quantity One software (Bio-Rad).

4.7. Quantitative real time PCR (RT-PCR)

Total RNA was isolated with TriPure Isolation Reagent (Roche Applied Science). Twenty micrograms of total RNA were subjected to DNase I treatment (Roche Applied Science) for 20min at 30°C and purified by phenol:chloroform extraction; the quality of the DNase I treated RNA was assessed by gel electrophoresis Three micrograms of DNase I treated RNA was subjected to reverse transcription with High Capacity cDNA Reverse Transcription Kit (Life Technologies). Real-time quantitative PCR amplifications (qPCR) were carried out at 60°C using Power SYBR Green PCR Master Mix (Life Technologies). Calculations were done using the delta-delta Ct method. Primer sequences were the following: GLI1-F, 5'-CCCAGTACATGCTGGTGGTT-3' and GLI1-R, 5'-GCTTTACTGCAGCCCTCGT-3'; GLI2-F, 5'-CACCGCTGCTCAAAGAGAA-3' and GLI2-R, 5'-TCTCCACGCCACTGTCATT-3'; WIP1-F, 5'-CGCTGGAGGCAGCGTATG-3' and WIP1-R, 5'-CCTTGGCCATGGATCCTCC-3'; PTCH1-F, 5'-GGCAGCGGTAGTAGTGGTGTTC-3' and PTCH1-R, 5'-TGTAGCGGGTATTGTCGTGTGTG-3'; FOXM1-F, 5'-ACCCAAACCAGCTATGATGC-3' and FOXM1-R, 5'-GAAGCCACTGGATGTTGGAT-3'; SMO-F, 5'-GGGAGGCTACTTCCTCATCC-3' and SMO-R, 5'-GGCAGCTGAAGGTAATGAGC-3'; SNAI1-F, 5'-GCGAGCTGCAGGACTCTAA-3' and SNAI1-R, 5'-GACAGAGTCCCAGATGAGC-3'; BMI1-F, 5'-ATGCAGCTCATCCTTCTGCT-3' and BMI1-R, 5'-CCGATCCATCTGTTCTGGT-3'; MITF-M-F, 5'-CTCGAGCTCATGGACTTTCC-3' and MITF-M-R, 5'-CCAGTTCCGAGGTTGTTGTT-3'; E2F1-F, 5'-GCTGAGCCACTCGGCTGACG-3';

E2F1-R, 5'-CCACTGTGGTGTGGCTGCCC-3'. Primers for human reference genes were: GAPDH-F, 5'-GACGCTGGGGCTGGCATTG-3' and GAPDH-R, 5'-GCTGGTGGT CCAGGGGTC-3'; β-ACTIN-F, 5'-GAAAATCTGGCACCACACCT-3' and β-ACTIN-R, 5'-TAGCACAGCCTGGATAGCAA-3'.

4.8. p53 sequencing

Sequencing of p53 in melanoma cell lines was performed by using the following primers (5' to 3'): p53-F, 5'-ATGGAGGAGCCGCAGTCAGAT-3'; p53-R, 5'-AAACCGTA GCTGCCCTGGTAGG-3'; p53-R2, 5'-TTCCGTCCCAGTAGATTACC-3'.

4.9. Chromatin immunoprecipitation

Cells were transfected with pCS2+MT (negative control) or pCS2+MT-GLI1 vectors. Fourty eight hours after transfection cells were fixed with 1% formaldehyde for 10min at RT; formaldehyde was neutralized by incubation with 125mM glycine for 5min at RT. Fixed cells were collected by scraping in PBS containing 1X Complete EDTA-free Protease Inhibitor Cocktail (Roche Applied Science) and were washed once with PBS. The pellets from $4x10^6$ cells were resuspended in 300µl SDS Lysis Buffer (0.5% SDS, 50mM Tris-HCl, pH 8) containing 1X Complete EDTA-free Protease Inhibitor Cocktail and were incubated for 15min in ice. The DNA was sonicated to an average size of 300-500bp using a SONOPULS mini20 sonicator (Bandelin) equipped with a cuphorn. The remaining insoluble material was removed by centrifugation at maximum speed for 15min at 4°C. The supernatants were diluted 5 times with ChIP Dilution Buffer (1.8% Triton X-100, 2mM EDTA, 300mM NaCl) containing 1X Complete EDTA-free Protease Inhibitor Cocktail; 75µl (5%) were collected as an Input Material (IM) and stored at -20°C. For each sample 20µl of Dynabeads Protein G (Life Technologies) were conjugated with 3µg of anti-Myc (9E10) (Santa Cruz Biotechnology) for 30min at room temperature. Beads were washed to remove the unbound antibody and resuspended in 75µl 100mg/ml BSA and 7.5µl 20mg/ml glycogen (Roche Applied

Science) prior to be added to the sonicated chromatin and to be incubated overnight at 4°C with gentle rotation. The immunocomplexes were then washed once with Low Salt Wash Buffer (20mM Tris-HCl, pH 8, 2mM EDTA, 150mM NaCl, 0.1% SDS, 1% Triton X-100), High Salt Wash Buffer (20mM Tris-HCl, pH 8, 2mM EDTA, 500mM NaCl, 0.1% SDS, 1% Triton X-100) and LiCl Wash Buffer (250mM LiCl, 1% NP-40, 1mM EDTA, 10mM Tris-HCl, pH 8, 1% sodium deoxycholate), and twice with TE (1mM EDTA, 10mM Tris-HCl, pH 8). The beads were then resuspended in 200µl 1% SDS, incubated at 85°C for 10min and briefly vortexed to elute the protein-DNA complexes from the antibody. NaCl was added to a final concentration of 200mM to the eluates and to the IMs, and the cross-links were reversed by incubation at 65°C overnight. The samples were then incubated with 4µg RNaseA (Life Technologies) at 37°C for 30min and with 20µg Proteinase K (Roche Applied Science) at 60°C for 2h. The immunoprecipitated DNA (ChIP DNA) and the IM were then purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA) and eluted in 50µl of 10mM Tris-HCl, pH 8. Real-time quantitative PCR amplifications (qPCR) were carried out at 60°C using Power SYBR Green PCR Master Mix (Life Technologies) and 2.5µl of ChIP DNA and of IM diluted 1:50. Primers used were: E2F1prom-F, 5'-ACGCGCCAAATCCTTTTTGCCG-3' and E2F1prom-R, 5'-AATAGGAACCGCCGCCGTTG-3'; PTCH1prom-F, 5'-ACACACTGGG TTGCCTACC-3' and PTCH1prom-R, 5'-CTGTCAGATGGCTTGGGTTT-3'; HIPprom-F, 5'-GGTGACTTATTTTTGCTGCCC-3' and HIPprom-R, 5'-GAGAAACTGTGCCTCCAA GC-3'; GAPDHprom-F, 5'-CCGTCCTTGACTCCCTAGT-3' and GAPDHprom-R, 5'-CCTACTTTCTCCCCGCTTTT-3'.

4.10. Colony formation, growth curve and self-renewal assays

For colony formation assay 800 cells/well were plated in 6-well plates. After 15 days cells were fixed with 100% methanol for 20min at -20°C and stained with 0.1% crystal violet in 10% ethanol for 10min at room temperature. Cells were washed with distilled water and colonies were counted. For growth curve 3000 (SSM2c and M26c)

or 5000 (MCF7) cells/well were plated in 12-well plates and counted on days 3-5-7. For Cyc and CCT treatments 9000 (MCF7) or 20000 (SSM2c, M26c and M21) cells/well were plated in 12-well plates in medium containing 2.5% FBS and viable cell count by trypan blue exclusion was performed at 72hrs (SSM2c, M26c and M21) or 120hrs (MCF7) after treatment.

For self-renewal assay in SSM2c cells, 5 cells/µl were plated in DMEM/F12 added with 20µg/ml insulin, 0.6% glucose, 1X N2, 10ng/ml bFGF, 10ng/ml EGF (Life Technologies) (Santini, R. *et al.*, 2012). For self-renewal assay in M26c cells, 5 cells/µl were plated in a medium constituted for 2/3 of DMEM/F12 and for 1/3 of stem cell medium (SCM) (70% mouse embryo fibroblast (MEF) conditioned medium, 24% DMEM/F12 added with 6% knock-out serum replacer, 0.6mM Glutamine, 0.7% β-mercaptoethanol) and added with 4ng/ml bFGF (all reagents were from Life Technologies). For self-renewal assay in MCF7 cells, 5 cells/µl were plated in DMEM/F12 added with 5µg/ml insulin, 1X B27, 10ng/ml bFGF, 10ng/ml EGF (Dontu, G. *et al.*, 2003). At day 7, p0 spheres formed were dissociated and plated in 96-well plates at 1cell/well or in 12 well plates at 1cell/µl dilutions. For self renewal the cells were seeded in the same medium used to form primary spheres added with one third of their filtered conditioned medium. After 10-15 days the number of secondary spheres formed was counted and the size was measured.

4.11. Annexin V/7-AAD staining and flow cytometry analysis

Annexin V/7-AAD staining was performed according to manufacturer's protocol (Becton Dickinson, Franklin Lakes, NJ). For flow cytometry analysis transduced cells were harvested and resuspended in 50µg/ml Propidium iodide, 0.1% Triton X-100 and 0.1% sodium citrate before cytometric analysis with FACSCanto II (Becton Dickinson).

4.12. Cell sorting, nude mice and xenografts

SSM2c melanoma cells were transduced with either pLV-CTH (LV-c) or pLV-CTH-shPTCH1 (*shPTCH1*) lentiviruses. GFP+ cells were sorted with the BD FACSAria cell sorter (Becton Dickinson, Franklin Lakes, NJ) and transduced with either pLKO.1-puro (LV-c) or pLKO.1-puro-shWIP1 40 (*shWIP1*) lentiviruses. Cells were selected with 2µg/ml puromycin for at least one week. On the day on injection, transduced cells were resuspended in Matrigel (Becton Dickinson)/DMEM (1/1) and inoculated subcutaneously into adult female athymic-nude mice (Harlan Laboratories, Indianapolis, IN, USA). 1000 cells/injection were inoculated. Tumor size was measured three times a week by a caliper. The tumor volume was calculated by using the formula: $L^2xWx0.5$.

4.13. Statistical analysis

The data represent mean±SEM values and are calculated on at least 3-4 independent experiments. *P* values were calculated using Student's *t*-test. A two-tailed value of $P<0.05$ was considered statistically significant. The effect of cyclopamine and CCT combined treatments was measured by the Excess over Bliss Additivism score.

5. DISCUSSION

HH signaling was discovered as an important pathway in development. However, its aberrant activation in different types of human cancer makes it an important player in tumorigenesis and a possible target for cancer therapy. In fact, it sustains the growth of tumor bulk and the self renewal of cancer stem cells, which are responsible for tumor relapse. Beside the ligand-mediated activation of HH pathway, several studies highlighted the importance of non-HH oncogenic inputs in controlling the activity of the GLI transcription factors, the final effectors of HH signaling. GLI1, in particular, has emerged as a crucial regulator of cancer and stemness in different contexts (Stecca, B. *et al.*, 2010). GLI1 is a transcription factor and exerts its oncogenic function by inducing a variety of targets that, directly or indirectly, control cancer cell growth, survival and stemness. At present, however, only few GLI1 direct transcriptional targets have been identified in cancer and the molecular mechanisms by which GLI1 acts as an oncogene have not been clarified.

In this study we provide evidence of a positive feedback loop involving the oncogenic phosphatase WIP1 and the transcription factor E2F1, that regulates HH signaling in melanoma. We identify WIP1 as a novel positive modulator of GLI1 protein and we demonstrate that GLI1 directly controls the expression of E2F1, an important player in melanoma (Alla, V. *et al.*, 2010). Because E2F1 directly controls WIP1 expression (Hershko, T. *et al.*, 2006), the activation of HH pathway in tumors expressing WIP1 and E2F1 is sustained by a positive regulatory loop that fuel itself. Our data also support the concept of combination treatment with SMO and WIP1 inhibitors as efficient therapeutic option for tumors expressing WIP1 and with activated HH pathway.

Protein phosphatases have been shown to modulate the GLI proteins. PP2A

positively regulates the GLI homologue Ci in *Drosophila* (Jia, H. *et al.*, 2009) and negatively controls subcellular localization and activity of GLI3 (Krauss, S. *et al.*, 2008, 2009). Moreover, the B56 subunit of PP2A regulates Gli1 function in frog embryos (Rorick, A.M. *et al.*, 2007) and, indirectly, negatively controls the stability of the Gli proteins (Jin, Z. *et al.*, 2011). WIP1 is emerging as an important regulator of tumorigenesis (Lu, X. *et al.*, 2008). Wip1 deletion impairs spontaneous and oncogene-induced tumorigenesis (Nannenga, B. *et al.*, 2006; Bulavin, D.V. *et al.*, 2004; Demidov, O.N. *et al.*, 2007). In addition, Wip1 cooperates with known oncogenes (Erb2, H-Ras, Wnt1) to transform mouse embryonic fibroblasts and to accelerate breast cancer formation (Bulavin, D.V. *et al.*, 2002, 2004; Demidov, O.N. *et al.*, 2007). Here we identify a novel mechanism for WIP1 in promoting tumorigenesis, by showing that it is critically involved in modulating the HH pathway. Our study also provides the molecular basis to understand why WIP1 enhances Sonic hedgehog-dependent medulloblastoma formation (Doucette, T.A. *et al.*, 2012). We found that WIP1 increases GLI1 transcriptional activity, nuclear localization and protein stability. However, WIP1 does not appear to significantly affect activity and processing of GLI2 and GLI3. The co-localization and interaction of WIP1 and GLI1 suggest that WIP1 might stabilize GLI1 by retaining it into the nucleus, thus preventing its cytoplasmic export and consequent proteasome degradation.

We found that the modulation of GLI1 transcriptional activity depends on WIP1 phosphatase activity, suggesting a direct dephosphorylation. However, we were unable to document a clear shift in GLI1 band mobility in presence of WIP1 or CCT treatment. Therefore, at present it remains unclear whether WIP1 directly dephosphorylates GLI1 or a third protein which, in turn, directly modifies GLI1. The effect of WIP1 on GLI1 could be mediated by one of the known WIP1 dephosphorylation targets, such as p53, or ATM, Chk1, Chk2 or p38 MAPK (Lu, X. *et al.*, 2008). Here we have tested the involvement of p53 as a potential mediator, because WIP1 directly dephosphorylates

p53 and the above-mentioned stress-induced kinases, resulting ultimately in the attenuation of p53 function. Our data indicate that WIP1 modulates GLI1 transcriptional activity in a p53-independent manner. However, we can not exclude that the effect of WIP1 silencing on cell growth are the result of a complex autoregulatory loop involving WIP1/GLI1 and p53 functions. Indeed, WIP1 enhances GLI1 function (this work) and keeps p53 in an inactivated state (Lu, X. *et al.*, 2005), thus attenuating the inhibition of GLI1 by p53 (Stecca, B. *et al.*, 2009). At the same time, HH pathway activation itself down-regulates p53 by activating Mdm2 (Abe, Y. *et al.*, 2008). We can thus speculate that during tumorigenesis the imbalance in favor of WIP1 and HH would override p53-mediated tumor suppression and further enhance proliferation and self-renewal.

The predominant effect of WIP1 appears to be on GLI1. However, our data also suggest that WIP1 might positively influence the HH signaling pathway upstream of GLI1. In fact, WIP1 silencing reduces the expression of endogenous HH targets *PTCH1* and *GLI1* and it reverses the increase in cancer cell growth induced by activation of the HH pathway. In addition, we show that WIP1 silencing reduces the increase in breast and melanoma CSC self-renewal induced by activation of the HH pathway, although *shWIP1* by itself shows only modest effects. It is therefore tempting to speculate that during tumorigenesis WIP1 over-expression/amplification might contribute to increase proliferative and self-renewing activities of GLI1, therefore enabling to an expansion of CSC and derived progenitors that sustain tumor growth. This hypothesis is supported by *(i)* the documented role of the HH-GLI signaling and, in part, of WIP1 in regulating normal and cancer stem cells (e.g. Zhu, Y.H. *et al.*, 2009; Lai, K. *et al.*, 2003; Clement, V. *et al.*, 2007; Peacock, C.D. *et al.*, 2007; Varnat, F. *et al.*, 2009; Santini, R. *et al.*, 2012; Liu, S. *et al.*, 2006); *(ii)* the finding that WIP1 is often over-expressed in human cancers with abnormal activation of the HH signaling, such as medulloblastomas and gliomas (Castellino, R.C. *et al.*, 2008; Liang, C. *et al.*, 2012); *(iii)* the finding of a positive correlation between the expression of *WIP1*, *GLI1* and

PTCH1 in melanomas (this study).

Notably, our data point to the relevance of the HH signaling and WIP1 in the context of melanoma, the most lethal skin cancer (Chin, L. *et al.*, 2006; Gray-Schopfer, V. *et al.*, 2007). First, we show that activation of the HH signaling increases human melanoma cell proliferation and orthotopic melanoma xenograft growth, suggesting that enhanced HH pathway might promote melanoma progression. These results are consistent with the high GLI1 expression reported in human melanoma metastases (Das, S. *et al.*, 2009) and complement our previous findings on the requirement of HH signaling for melanoma xenograft growth (Stecca, B. *et al.*, 2007). Second, we find that WIP1 inhibition reduces melanoma cell proliferation and xenograft growth, highlighting an unprecedented role of WIP1 in melanoma. Our findings suggest that WIP1 might contribute to the progression of melanomas with activated HH pathway. Besides, WIP1 might also keep p53 in an inactivated state, directly or indirectly, explaining the low p53 mutational rate in melanoma (Castresana, J.S. *et al.*, 1993).

The observation that WIP1 and GLI1 contribute to melanoma proliferation and self renewal does not explain the molecular mechanism underlying the increased growth observed upon HH pathway activation. However, the identification of E2F1 as a direct transcriptional target of GLI1 identifies a major player mediating the effects of HH signaling. Previous reports indicated that the ligand-dependent activation of HH pathway resulted in induction of E2F1 in medulloblastoma (Bhatia, B. *et al.*, 2011). However, at present there is no evidence for a direct transcriptional regulation of E2F1 by GLI transcription factors. Our work shows that GLI1 directly binds to E2F1 promoter by chromatin immunoprecipitation (Fig.26) and we identified the region between -132bp and -269bp upstream the transcription start site as the one required for the induction of E2F1 by exogenous GLI1 and GLI2 (Fig.27). We were not able to identify a canonical GLI binding site in the region of E2F1 promoter responsive to GLI1 and

GLI2. However, according to a recent biochemical study (Winklmayr, M. *et al.*, 2010), several non-canonical GLI binding sites might be identified in E2F1 promoter and they might still drive an effective GLI-dependent transcriptional response. The fact that also GLI2, although at a lesser extent, is able to transactivate E2F1 promoter, is expected, because both GLI1 and GLI2 recognize the same consensus sequence on the DNA. However, we hypothesize that GLI1 is the major player in HH-driven melanoma tumorigenesis. In fact, amplification of WIP1 or its E2F1-dependent induction during tumor progression might contribute to specifically enhance GLI1 activity, thus fueling a positive autoregulatory loop involving WIP1-GLI1-E2F1 that sustains HH pathway activation (Stecca, B. *et al.*, 2009; Lai, K. *et al.*, 2004).

E2F1 is a master regulator of either cell proliferation or cell death, depending on the cellular context. In melanoma it is highly expressed and controls genes involved in cell cycle control and invasiveness (Nelson, M.A. *et al.*, 2006; Alla, V. *et al.*, 2010; Dar, A.A. *et al.*, 2011; Halaban, R. *et al.*, 2000). The fact that E2F1 silencing reduces *in vitro* tumor growth confirms its role as oncogene in this type of human cancer. The observation that the activation of HH pathway, along with E2F1 silencing, results in an inhibition of cell growth stronger than the one produced by E2F1 silencing alone suggests that, in presence of an increased HH stimulation, the cell relies mostly on the proliferative function of E2F1. Indeed, PTCH1 silencing produces an increase of the endogenous E2F1 levels and the knock-down of E2F1 in that context results in a more dramatic drop of E2F1 levels in the cell (Fig.28c). Our data also highlight an unprecedented role for E2F1 in controlling melanoma CSC self renewal, because E2F1 silencing strongly reduces the number of secondary melanomaspheres (Fig.29). Nevertheless, the discrepancy between the results of the growth curve and the self renewal assay suggests that E2F1 acts as a main effector of HH pathway in controlling cell proliferation, but it is not that crucial in mediating the HH-induced increase of CSC self renewal. This is not surprising, because, although both cell proliferation and stem

cell self renewal depend on cell division, the two processes are not the overlapping. Some of the pathways that control stem cell self renewal also control cell proliferation, but a number of these mechanisms preferentially regulate stem cell self renewal (Nishino, J. *et al.*, 2008; Molofsky, A.V. *et al.*, 2003).

This work identifies a positive regulatory loop that fuels HH pathway activation in melanoma (Fig.30). The oncogenic phosphatase WIP1 enhances GLI1 transcriptional activity at post-translational level, by increasing its nuclear localization and stabilizing the protein. Activated GLI1, then, directly binds to E2F1 promoter and induces its expression. E2F1, in turn, mediates the HH-induced proliferative effects and, at the same time, induces WIP1 expression, thus closing the loop that fuels HH pathway activation.

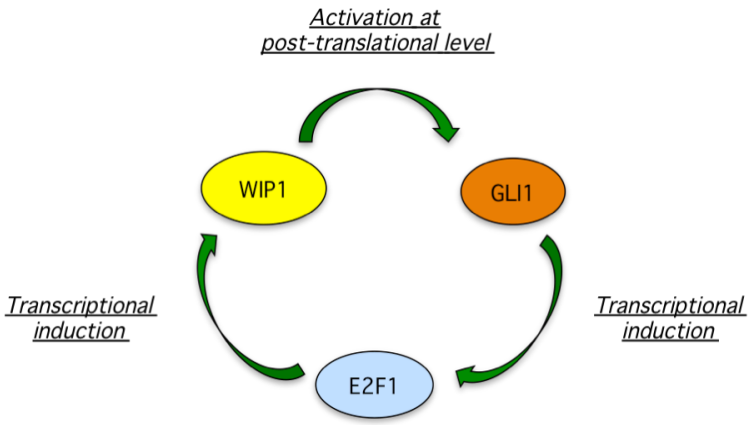


Figure 30 *Model of the positive regulatory loop involving WIP1, GLI1 and E2F1. WIP1 enhances GLI1 function at post-translational level. Activated GLI1 directly induces E2F1 expression, which, in turn, increases WIP1 expression.*

From a therapeutic point of view, a relevant finding of this work is that combined inhibition of WIP1 and of HH signaling at the level of SMO acts synergistically to decrease cancer cell proliferation. HH pathway inhibitors have already appeared on the horizon of human cancer therapy (Low, J.A. *et al.*, 2010) and WIP1 itself has been proposed as a target for cancer drug development (Yamaguchi, H. *et al.*, 2006 Hayashi, R. *et al.*, 2011), although at present no drugs are available for clinical treatment. Our data suggest a possible novel therapeutic approach for a subset of

melanomas and other cancer types expressing high levels of WIP1 and with activated HH pathway (Liang, C. *et al.*, 2012; Castellino, R.C. *et al.*, 2008). Indeed, targeting the HH signaling at the level of SMO, with a SMO antagonist, and at the same time at the level of GLI1, through WIP1 inhibition, should result in a more effective cancer growth inhibition. On the other hand, targeting WIP1 in tumors with wt p53 would lead not only to restoration of p53 tumor suppressor activity, which in turn might inhibit GLI1 (Stecca, B. *et al.*, 2009), but also to a direct attenuation of GLI1 function (this study), resulting in a stronger inhibition of the HH pathway. Most importantly, combinatory treatments with SMO and WIP1 inhibitors would block at two different steps the positive regulatory loop WIP1-GLI1-E2F1. Finally, because this regulation controls cancer cell proliferation, as well as self renewal, this therapeutic approach might inhibit not only the growth of melanoma bulk, but also that of putative melanoma CSC, which are responsible for tumor relapse.

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7. APPENDIX

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ORIGINAL ARTICLE

WIP1 phosphatase modulates the Hedgehog signaling by enhancing GLI1 function

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The Hedgehog-GLI (HH-GLI) signaling plays a critical role in controlling growth and tissue patterning during embryogenesis and is implicated in a variety of human malignancies, including those of the skin. Phosphorylation events have been shown to regulate the activity of the GLI transcription factors, the final effectors of the HH-GLI signaling pathway. Here, we show that WIP1 (or PPM1D), an oncogenic phosphatase amplified/overexpressed in several types of human cancer, is a positive modulator of the HH signaling. Mechanistically, WIP1 enhances the function of GLI1 by increasing its transcriptional activity, nuclear localization and protein stability, but not of GLI2 nor GLI3. We also find that WIP1 and GLI1 are in a complex. Modulation of the transcriptional activity of GLI1 by WIP1 depends on the latter's phosphatase activity and, remarkably, does not require p53, a known WIP1 target. Functionally, we find that WIP1 is required for melanoma and breast cancer cell proliferation and self-renewal *in vitro* and melanoma xenograft growth induced by activation of the HH signaling. Pharmacological blockade of the HH pathway with the SMOOTHENED antagonist cyclopamine acts synergistically with inhibition of WIP1 in reducing growth of melanoma and breast cancer cells *in vitro*. Overall, our data uncover a role for WIP1 in modulating the activity of GLI1 and in sustaining cancer cell growth and cancer stem cell self-renewal induced by activation of the HH pathway. These findings open a novel therapeutic approach for human melanomas and, possibly, other cancer types expressing WIP1 and with activated HH pathway.

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Keywords: Hedgehog; GLI1; WIP1; melanoma; xenograft; self-renewal; cancer stem cells

INTRODUCTION

The Hedgehog-GLI (HH-GLI) signaling is critical for growth and patterning of numerous tissues during embryogenesis and early development,[1,2] but it is mostly quiescent in the adult, where it mainly regulates stem cell behavior.[3] Aberrant activation of the HH pathway has been implicated in several types of cancer, including those of the skin.[4,5] Canonical HH pathway activation is initiated by the binding of HH ligands to the transmembrane protein Patched 1 (PTCH1), which becomes internalized and relieves its inhibition on the transmembrane protein Smoothened (SMO). Consequently, active SMO triggers an intracellular signaling cascade leading to the formation of activating forms of the GLI zinc finger transcription factors GLI2 and GLI3, which directly activate GLI1.[6,7] GLI1 and GLI2 act as the main mediators of the HH signaling in cancer by controlling the expression of target genes involved in proliferation, survival, stemness and metastasis.[8]

Tumorigenic activation of the HH pathway can take place through loss of the inhibitory function of PTCH1[9,10] or by activating mutations in SMO.[11] Recent reports suggest that in several types of cancer, GLI proteins can be modulated by proliferative and oncogenic inputs, in addition or independent of upstream HH signaling.[8,12–17] We have previously shown that p53 negatively regulates GLI1 function.[18] We thus hypothesized that WIP1, a phosphatase specifically inhibiting p53 function, might be involved in the modulation of the HH signaling.

WIP1 (*PPM1D*) is a nuclear Ser/Thr phosphatase expressed at low levels in most normal tissues. Its expression is controlled by E2F1[19] and it is increased in a p53-dependent manner in response to genotoxic stress.[20] Wip1 null mice have revealed a role for Wip1 in aging, lymphoid cell function, fertility and adult neurogenesis[21,22] and in pathological conditions, such as obesity and atherosclerosis.[23] In recent years, WIP1 has emerged as an important player in tumorigenesis. Indeed, it is amplified/overexpressed in several types of human cancer[24–27] and deletion of Wip1 confers tumor-resistant phenotype in mice.[28–30] Wip1 on its own does not transform cells, but it accelerates tumor formation in cancer-prone mouse models.[24,31] The oncogenic properties of WIP1 depend on its ability to dephosphorylate specific targets such as p53, ATM, Chk1, Chk2, p38MAPK, Mdm2 and the histone γ-H2AX,[32–37] resulting in reduced stress response and p53 inactivation.

Here, we investigated a possible functional interaction between WIP1 and the HH signaling. Using gain and loss of function approaches, we demonstrated that WIP1 enhances GLI1 activity and it is required to maintain tumor growth and cancer stem cell (CSC) self-renewal induced by activation of the HH pathway. Finally, we present evidence that inhibition of the HH pathway acts synergistically with blockade of WIP1 in reducing cancer cell growth. These findings identify WIP1 as novel positive modulator of GLI1 and suggest combined targeting of HH signaling and WIP1 as a novel anti-cancer approach.

RESULTS

WIP1 positively modulates GLI1 activity

To examine the effect of WIP1 silencing on GLI function, we co-transfected low amount of GLI1, GLI2 and GLI3 expression

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constructs with the GLI-BS luciferase reporter in HEK-293T cells, patient-derived melanoma cells SSM2c and in the breast cancer cell line MCF7, which all express high levels of WIP1. Knockdown of WIP1 significantly reduced GLI1 transcriptional activity in a dose-dependent manner (Figures 1a–c; Supplementary Figure S1a), but did not significantly affect GLI2 nor GLI3 (Figures 1a–c; Supplementary Figure S1b). WIP1 silencing was achieved by two independent short hairpin RNA (shRNA) expressed from replication incompetent lentivectors (*shWIP1*). Both of them reduced WIP1 protein level in MCF7 cells (Figure 1d), which harbor *WIP1* gene amplification.[24,38] Overexpression of WIP1 in the colon cancer cell line HCT116, which expresses low level of WIP1, induced GLI1 transcriptional activity of $>80\%$ and failed to increase the activity of GLI2 and GLI3 (Figure 1e). WIP1 did not change GLI1 transcriptional activity in the presence of a mutated GLI-dependent reporter (GLI-BS mut) (Supplementary Figure S1c). To investigate the requirement of WIP1 phosphatase activity for GLI1 modulation, we performed GLI-BS luciferase reporter assay with catalytically inactive WIP1 (D314A)[34] or with the specific WIP1 inhibitor CCT007093 (CCT).[39] WIP1 D314A mutant did not change GLI1 transcriptional activity in HCT116 cells (Figure 1e). Consistently, treatment of MCF7 cells with CCT reduced by $>60\%$ GLI1 transcriptional activity (Figure 1f), mimicking the effect of *shWIP1*. Altogether, these results indicate that WIP1 positively regulates the transcriptional activity of GLI1 and that modulation of GLI1 by WIP1 depends on its phosphatase activity.

WIP1 modulates the endogenous HH signaling

To investigate the effect of WIP1 on the endogenous HH pathway, we tested the protein level and transcriptional activity of endogenous GLI1 protein after WIP1 silencing or inhibition. Western blot (WB) analysis showed that endogenous GLI1 was greatly decreased upon WIP1 knockdown (Figure 2a). Co-transfection of WIP1 expression vector increased endogenous reporter activity of 40–50%, whereas chemical (CCT) or genetic (*shWIP1*) inhibition of WIP1 decreased by 50–60% endogenous reporter activity in both MCF7 and SSM2c cells (Figures 2b and c). WIP1 silencing significantly reduced endogenous mRNA levels of *GLI1*, *PTCH1*, *FOXM1* and *SNAI1* (two HH targets), in both MCF7 and SSM2c cells (Figures 2d and e), confirming the downregulation of the endogenous pathway. Consistently, WIP1 overexpression increased GLI1 mRNA (Figure 2f). To investigate the function of Wip1 on the Hh-Gli pathway under physiological conditions, we used the Hh-competent murine NIH3T3 cells transfected with the GLI-BS luciferase reporter treated with the Smo agonist SAG.[40] Wip1 inhibitor CCT suppressed Hh signaling in a dose-dependent manner (Supplementary Figure S2a), it slightly reduced the levels of endogenous Gli1 but did not change Gli2 and Gli3 nor their processing (Supplementary Figure S2b). Primary cilia, which play an important role in physiological Hh pathway signal transduction,[41,42] were not disturbed in morphology and frequency in CCT exposed NIH3T3 cells stained for acetylated tubulin (Supplementary Figure S2c).

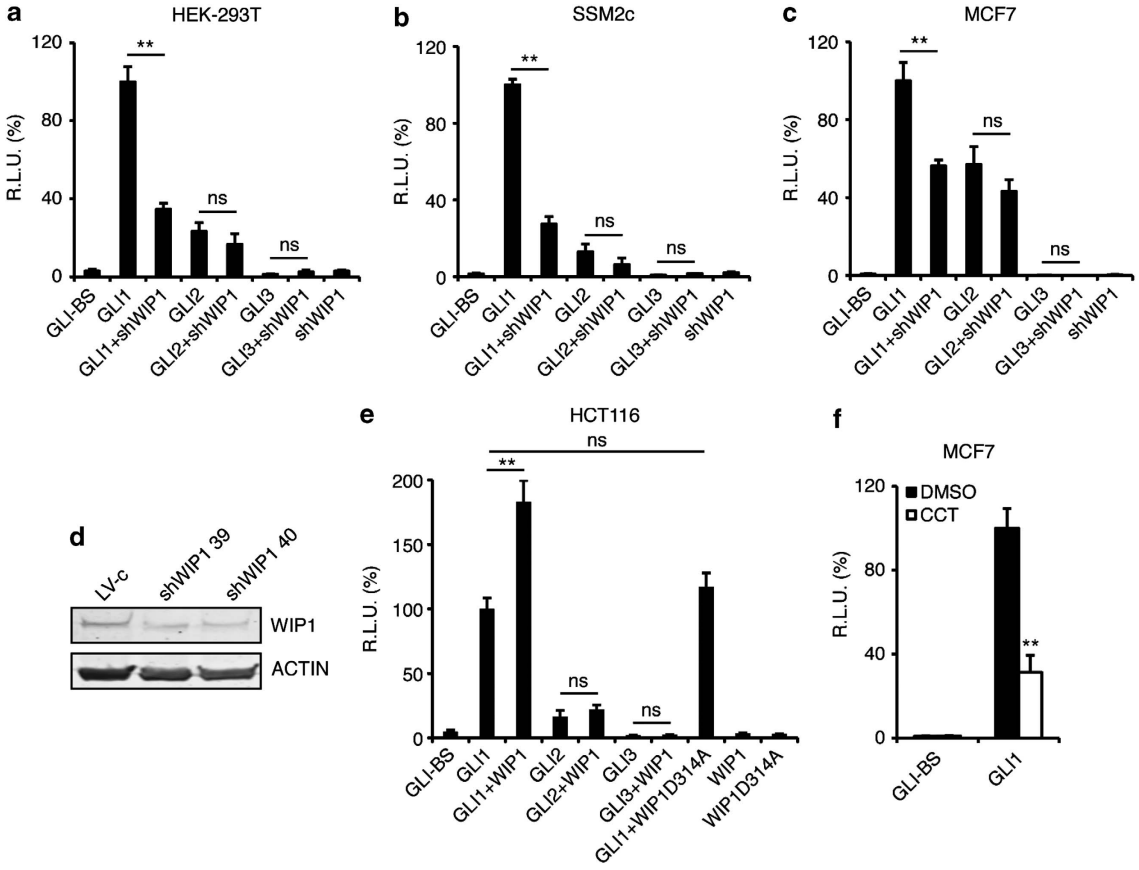


Figure 1. Regulation of GLI transcriptional activity by WIP1. (**a**–**c**, **e**, **f**) Quantification of GLI-dependent luciferase reporter assays. Relative luciferase units (R.L.U.) were GLI-dependent reporter firefly/renilla control ratios, with the level induced by GLI1 equated to 100%. (**a**–**c**) WIP1 silencing (*shWIP1*) reduced transcriptional activity of GLI1 in HEK-293T ($P=0.0001$), SSM2c ($P=0.0001$) and MCF7 ($P=0.0002$) cells, but not that of GLI2 and GLI3. (**d**) shRNA-mediated knockdown of WIP1. WB analysis of control (LV-c) and shWIP1 transduced MCF7 cells shows reduced WIP1 expression with two independent shRNA. β-Actin served as loading control. (**e**) WIP1 increased transcriptional activity of GLI1 ($P<0.0002$), but not that of GLI2 and GLI3 in HCT116 cells. Catalytically inactive WIP1 (D314A) did not enhance GLI1 activity. (**f**) Treatment of MCF7 cells with WIP1 inhibitor CCT007093 (CCT, 10 μM) (white bar) reduced GLI1 transcriptional activity ($P<0.0001$). The data represent mean ± s.e.m. of at least three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$.

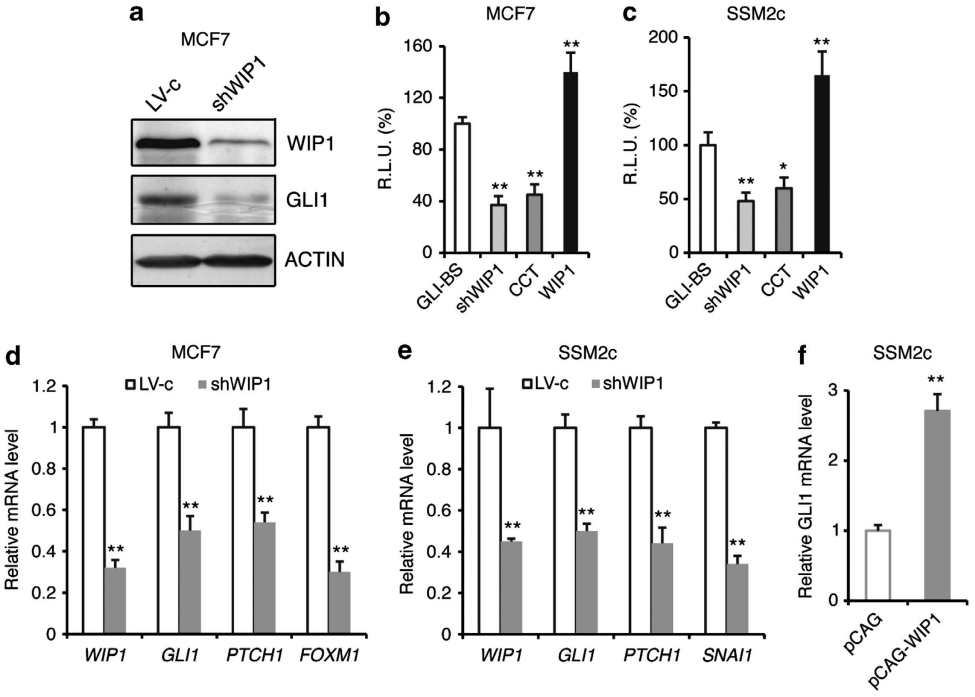


Figure 2. WIP1 modulates the activity of the endogenous HH pathway in cancer cells. (**a**) WB analysis showing endogenous GLI1 and WIP1 proteins after WIP1 silencing in MCF7 cells. β-Actin served as loading control. (**b**, **c**) Quantification of endogenous GLI-dependent luciferase reporter assay in MCF7 (**b**) and SSM2c (**c**) cells after WIP1 inhibition with *shWIP1* or CCT (10 μM, 16 h) and transfection with WIP1 expressing vector. (**d**, **e**) Expression of *WIP1* and of HH pathway components in MCF7 (**d**) and SSM2c (**e**) cells transduced with LV-c or *shWIP1,* measured by qPCR. (**f**) Endogenous *GLI1* expression in SSM2c cells transfected with pCAG or pCAG-WIP1. The controls (LV-c and pCAG) were set to 1. The *y* axis represents expression ratio of gene/(*GAPDH* + β*ACTIN* average). The data represent mean ± s.e.m. of three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$.

Modulation of GLI1 transcriptional activity by WIP1 does not require p53

We have previously shown that p53 negatively regulates GLI1.[18] This finding, along with the ability of WIP1 to dephosphorylate p53 at Ser15,[32] and thus to decrease its activity, suggested that p53 might mediate the modulation of GLI1 by WIP1. To investigate this possibility, we performed a GLI-dependent luciferase reporter assay silencing either p53, WIP1 or both. p53 silencing increased GLI1 transcriptional activity, as expected,[18] but it did not reverse the effect of WIP1 silencing on GLI1 in HEK-293T and M26c patient-derived melanoma cells (Figures 3a and b). Consistently, WIP1 enhanced and shWIP1 decreased GLI1 transcriptional activity in both HCT116 p53wt and p53ko isogenic cell lines (Figure 3c), suggesting that the modulation of GLI1 by WIP1 did not require p53. To further confirm our results, we overexpressed wild-type (wt) or mutant p53 in combination with shWIP1. p53 wt efficiently reduced GLI1 activity (by 52%), whereas R175H (conformational mutant) did not have any effect, indicating that functional p53 is required to inhibit GLI1 (Figure 3d). WIP1 silencing, however, still inhibited GLI1 activity in the presence of R175H p53 mutant (by 60%) (Figure 3d). To confirm the efficiency of shp53 and the activities of the p53 variants, we used a p53-inducible luciferase reporter driven by $p21^{WAF1/CIP1}$ promoter (p21-Luc) (Figures 3e and f). This revealed an endogenous p53 transcriptional activity in M26c (p53 wt) and HEK-293T (p53 wt), despite the presence of SV40 large T-antigen in the latter. Altogether, these data suggest that modulation of GLI1 transcriptional activity by WIP1 does not require p53.

WIP1 increases stability and nuclear localization of GLI1 and interacts with it

GLI2 and GLI3, but not GLI1, are regulated by proteolytic cleavage to convert them from full-length transcriptional activators to cleaved repressor forms in the absence of HH ligands.[43] We then asked whether WIP1 might affect the processing and levels of GLI proteins. Co-transfection of WIP1 and Myc-tagged GLI1, GLI2 and GLI3 showed that WIP1 induced by twofold GLI1 protein level, did not change GLI2 and slightly increased levels of GLI3-FL and GLI3-R proteins without changing the ratio between full-length and repressor forms (Figure 4a). Stabilization of GLI1 protein is a key event for HH signaling in cancer,[44] therefore we tested GLI1 protein stability in presence of WIP1. Treatment with cycloheximide (CHX) to block *de novo* protein synthesis indicated that co-expression of WIP1 significantly increased GLI1 stability (Figures 4b and c).

WIP1 is a nuclear phosphatase,[20] whereas GLI1 shuttles between nucleus and cytoplasm.[45] Thus, we tested whether WIP1 might affect intracellular trafficking of the GLI1, GLI2 and GLI3 proteins. Epitope-tagged GLI1 was nuclear and cytoplasmic in HEK-293T and HCT116 cells, whereas co-expression of WIP1 greatly enhanced nuclear localization of GLI1 (Figures 4d and e; Supplementary Figure S3). GLI2 and GLI3 localization was not affected by WIP1 (Figure 4e). Catalytically inactive WIP1 D314A mutant or treatment with WIP1 inhibitor CCT slightly increased the cytosolic fraction of GLI1 (Figures 4d and e), suggesting that WIP1 phosphatase activity is required for GLI1 nuclear localization. We corroborated these findings by performing cell fractionation in HEK-293T cells transfected with low doses of GLI1 in combination with WIP1 or CCT treatment. Overexpression of GLI1 in combination with WIP1 resulted in the disappearance of GLI1 in the cytoplasm and increase of GLI1 level in the nucleus (Figure 4f). Consistently, CCT treatment reduced GLI1 protein levels in both nuclear and cytoplasmic fractions.

Co-localization of WIP1 and GLI1 in the nucleus suggested that WIP1 might interact with GLI1. Thus, we tested whether WIP1 and GLI1 were physically associated. When Myc-tagged GLI1 and WIP1 were overexpressed in HEK-293T cells, GLI1 was

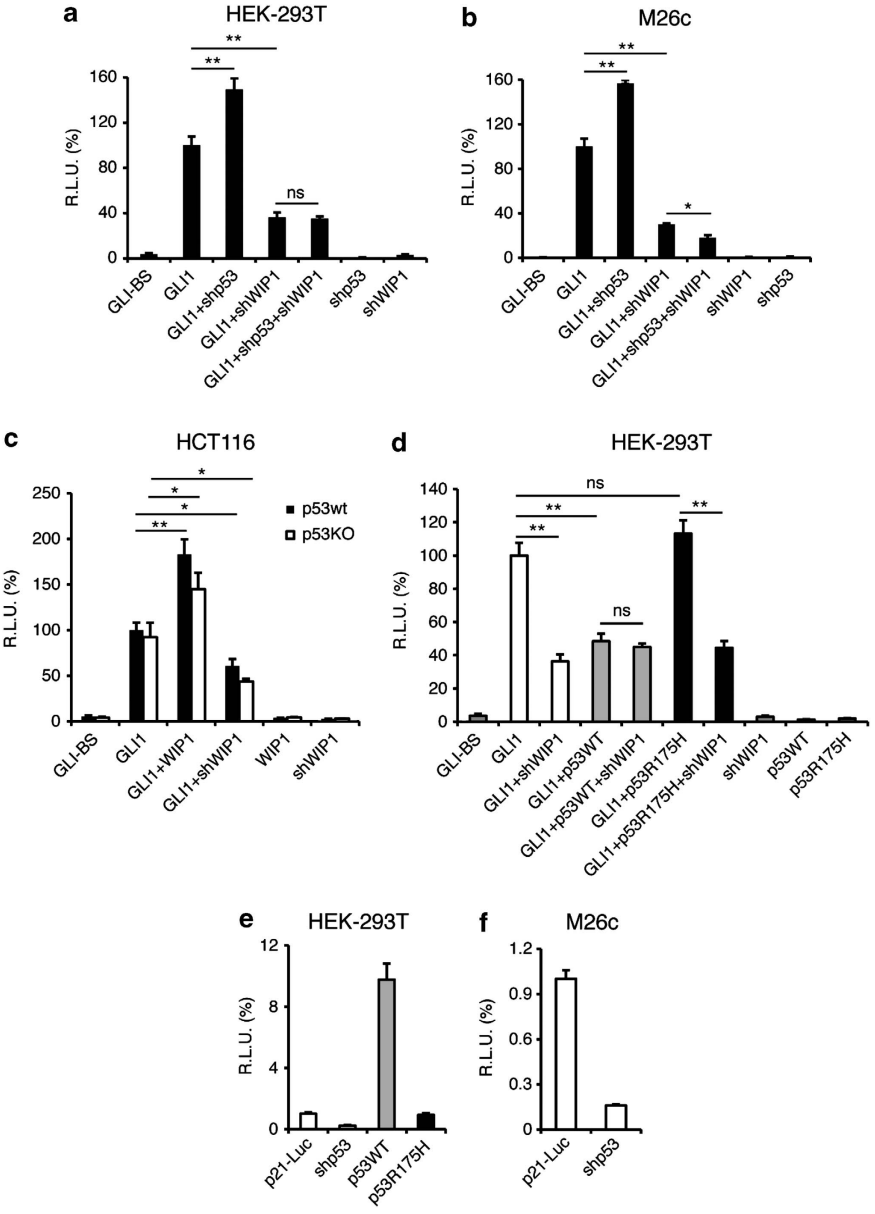


Figure 3. Enhancement of GLI1 transcriptional activity by WIP1 does not require p53. (**a**–**d**) Quantification of GLI-dependent luciferase reporter assays. (**a**, **b**) p53 silencing in HEK-293T and M26c cells increased GLI1 transcriptional activity ($P = 0.0001$ in both cells), but it did not reverse the effect of WIP1 silencing. (**c**) WIP1 overexpression increased and WIP1 silencing reduced GLI1 transcriptional activity in both p53wt (black) and p53ko (white) isogenic HCT116 cells ($P = 0.0002$). (**d**) Overexpression of p53 wt (gray) (GLI1 versus GLI1 + p53wt, $P < 0.0001$), but not mutant p53R175H (black), reduced GLI1 transcriptional activity in HEK-293T cells. WIP1 silencing reduced GLI1 transcriptional activity in presence of mutant p53R175H ($P < 0.001$, but not in presence of p53 wt.). p53/GLI1 DNA ratios were 0.5/2 in all cases. (**e**, **f**) Quantification of p53-dependent luciferase reporter assay (p21-Luc) to test the efficacy of shp53 ($P = 0.001$ in both cell types) and the transcriptional activity of p53 wt and the mutant p53R175H. The data represent mean ± s.e.m. of three independent experiments. *$P < 0.05$, **$P < 0.01$.

immunoprecipitated by an anti-WIP1 antibody (Figure 4g). Reciprocal experiments showed that WIP1 was immunoprecipitated by GLI1 (Figure 4g), indicating that WIP1 and GLI1 are in a complex.

WIP1 is required for HH-induced cancer cell growth and CSC self-renewal

Our data suggest that WIP1 positively regulates the HH pathway by enhancing GLI1 function. To understand the potential role of WIP1 in regulating HH-induced cell growth, we activated HH pathway and silenced *WIP1* in MCF7 and SSM2c cells. MCF7 cells were transduced with *shPTCH1*[18] to mimic HH pathway activation, or with *shWIP1*, alone or in combination, and allowed to form colonies. *shPTCH1* led to a 60% increase in the number of colonies compared with LV-c. *shWIP1* significantly reduced the number of colonies compared with LV-c, and, surprisingly, it drastically diminished the effect induced by HH pathway activation (Figures 5a and b). As a complementary approach, we assessed proliferation by viable cell count. In both MCF7 and SSM2c cells, *shPTCH1* increased cell number compared with LV-c, and *shWIP1* reduced it. Consistently with the results obtained in colony assays, *shWIP1* suppressed the effect of *shPTCH1* in both cell types (Figures 5c and d). To confirm that these differences were

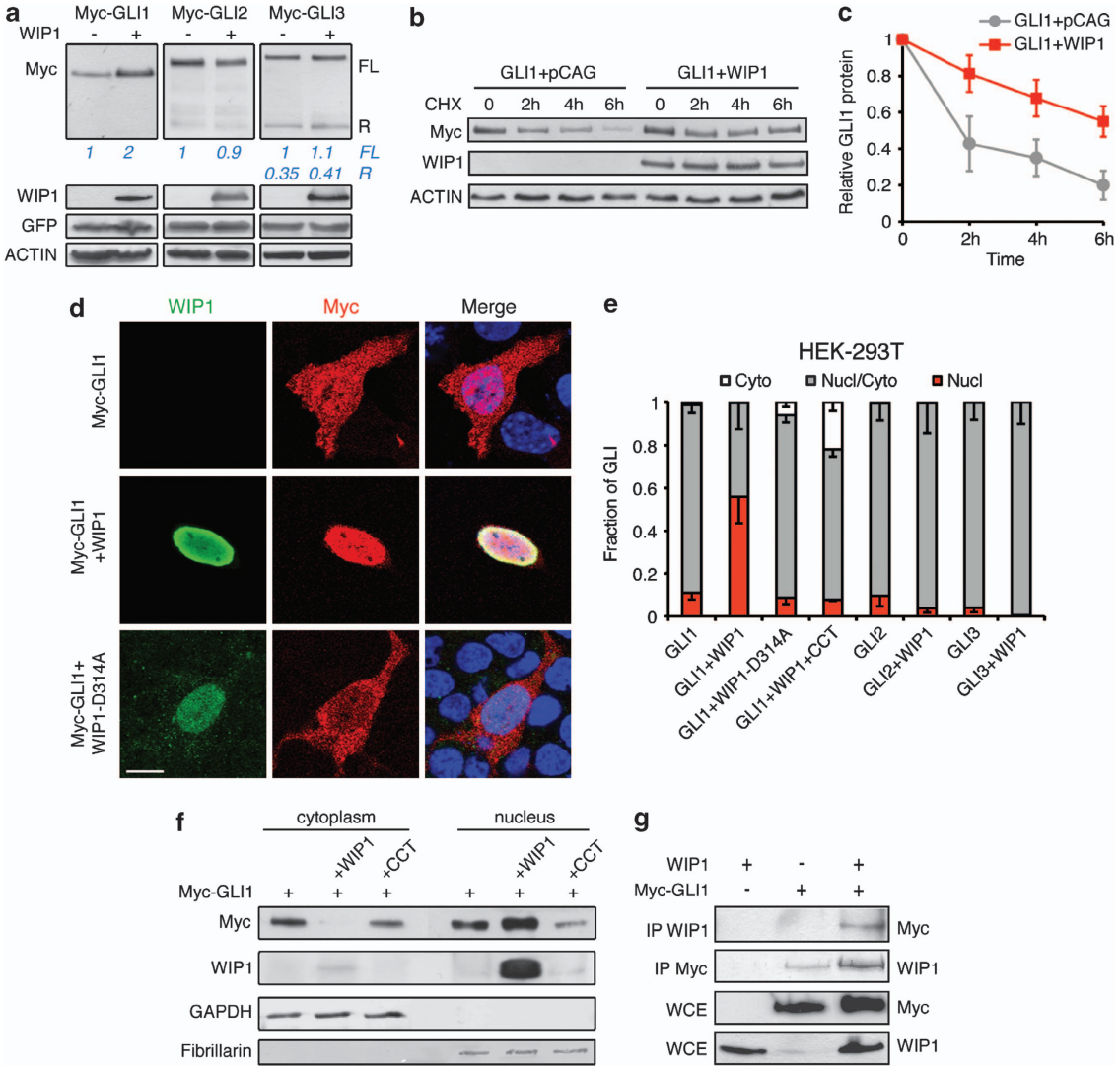
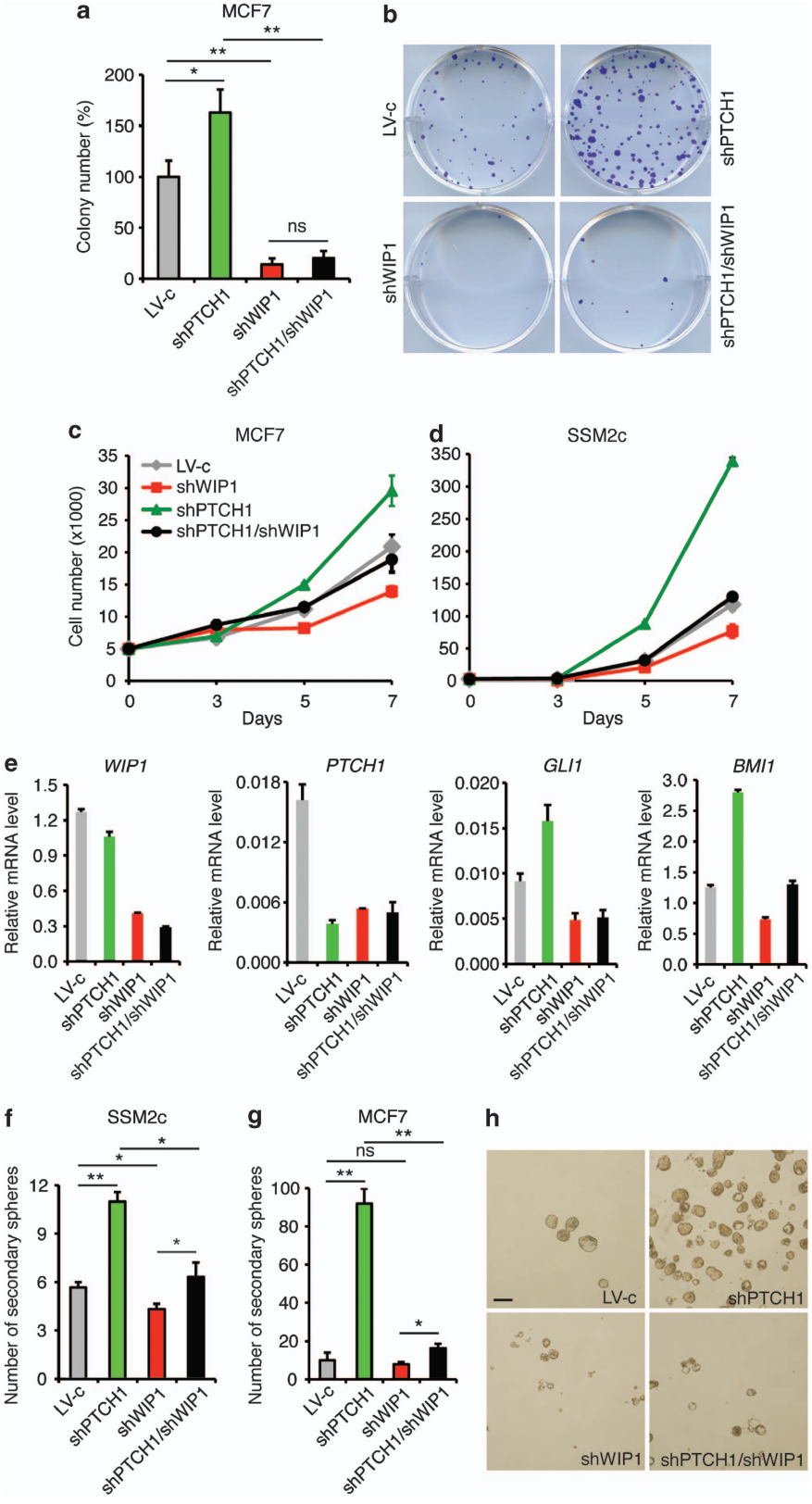


Figure 4. WIP1 positively modulates GLI1 protein and interacts with it. (**a**) WB analysis showing GLI1, GLI2 and GLI3 proteins after WIP1 overexpression in HEK-293T cells. GFP served as control for transfection and β-actin as loading control. The quantification of full-length (FL) and repressor (R) forms is shown in blue. (**b**) WB analysis shows increase in GLI1 protein stability in presence of WIP1 after cycloheximide treatment (CHX). (**c**) Densitometric quantification of the data in (**b**). The *y* axis represents GLI1 protein levels normalized on β-actin. The *x* axis represents hours of CHX treatment. Shown is the mean ± s.e.m. of three independent experiments. The half-lives of GLI1 alone or with WIP1 co-expression are ~2 and 6 h, respectively. (**d**, **e**) Representative images (**d**) and quantification (**e**) of GLI1, GLI2 and GLI3 subcellular localization after co-transfection with WIP1 in HEK-293T cells. Immunolocalization was with anti-Myc antibody for Myc-tagged GLI1, GLI2 and GLI3 (red) and anti-WIP1 antibody for WIP1 (green). Shown is the mean ± s.e.m. of at least three independent experiments. Nuclei were counterstained with DAPI. Over 500 cells were counted in each case. (**f**) HEK-293T cells were transfected with low doses of GLI1 in combination with WIP1 or CCT treatment (10 μM, 16 h). Cell fractionation was performed and lysates were subjected to WB with the antibodies anti-Myc (for GLI1), anti-WIP1, anti-GAPDH (control for cytoplasmic proteins) and anti-fibrillarin (control for nuclear proteins). (**g**) Reciprocal co-IP experiments showing that exogenous WIP1 and GLI1 are in a complex. GLI1 and WIP1 expression in WCE was determined by WB. Scale bar = 15 μM.

dependent on the modulation of HH pathway, we measured the expression of HH components by qPCR and we found that WIP1 knockdown reduced the expression of GLI1 and the target gene BMI1[14] in MCF7 cells (Figure 5e). We ruled out the possibility that WIP1 silencing by itself would trigger a DNA damage response and apoptosis. In fact, *shWIP1* did not increase phosphorylated Chk2 or γ-H2AX and did not induce changes in the amount of apoptotic cells in both SSM2c and MCF7 cell types, consistently with a previous study.[46] However, *shWIP1* produced a slight reduction of cells in S phase (MCF7) and an increase of cells in G1 phase (SSM2c) (Supplementary Figures S4 and S5). These data indicate that WIP1 silencing reversed the effect of increased proliferation obtained by HH pathway activation, supporting the hypothesis that endogenous WIP1 is critical to maintain cancer cell growth induced by activation of the HH signaling.

The HH signaling regulates CSC self-renewal in several instances.[16,47–51] Recent data indicate that WIP1 inhibition suppresses self-renewal and growth of mouse mammary CSC.[52] To test for a possible role of WIP1 in controlling HH-mediated CSC self-renewal, we used melanoma and breast CSC cultures (melanomaspheres and mammospheres) from, respectively, SSM2c and MCF7 cells, transduced with *shWIP1* and/or *shPTCH1*. These cells, seeded in non-adherent culture conditions form spheres enriched in stem and progenitor cells able to self-renew. As a measure of self-renewal, we quantified the ability of dissociated, single cells (plated at limiting dilution) to generate

secondary spheres. Silencing of *PTCH1* increased by twofold the number of melanomaspheres (Figure 5f) and by ninefold the number of mammospheres (Figures 5g and h) compared with LV-c. Silencing of *WIP1* slightly decreased the number of melanomaspheres but did not change the number of MCF7-derived mammospheres; however, it reversed the increase in self-renewal induced by *shPTCH1* in both cell types (Figures 5f–h). These data suggest that endogenous WIP1 is required for the maintenance of CSC self-renewal induced by activation of the HH signaling.



WIP1 correlates with the expression of HH pathway components in human melanomas

Recent data indicate that WIP1 enhances Sonic hedgehog-dependent medulloblastoma formation[53] and that human melanomas require an active HH pathway.[14] To investigate the significance of HH modulation by WIP1 in melanoma, we evaluated the expression of *WIP1* mRNA and of components of the HH pathway in a panel of 15 patient-derived short-term melanoma cultures (1 from a primary and 14 from metastatic melanomas) (Supplementary Table S1).[51] qPCR analysis revealed a positive correlation between the expression of *WIP1* and *GLI1* ($R^2 = 0.807$), *PTCH1* ($R^2 = 0.867$) and *SMO* ($R^2 = 0.787$) (Figure 6a; Supplementary Figure S6). No correlation was found between the expression of *WIP1* and *GLI2*, nor between *WIP1* and microphtalmia-associated transcription factor (*MITF-M*), a melanoma marker (Supplementary Figure S7).

WIP1 is required for melanoma xenograft growth induced by activation of the HH pathway

To test for a role of WIP1 in regulating HH-induced melanoma xenograft growth *in vivo*, 10^3 SSM2c cells stably transduced with LV-c, *shPTCH1* and/or *shWIP1* were engrafted subcutaneously into athymic-nude mice. FACS-sorted GFP+ SSM2c cells expressing *shPTCH1* yielded more than twofold larger xenografts than control GFP+ cells (LV-c) (Figure 6b and c). Combined with the results of growth curve assays (Figure 5d), these data suggest that activation of the HH pathway increases melanoma cancer cell growth *in vitro* and in a xenograft model. WIP1 silencing decreased melanoma xenograft growth compared with LV-c and drastically reduced tumor growth induced by *shPTCH1* to levels comparable to LV-c (Figures 6b and c), consistent with *in vitro* data (Figure 5d). These results suggest that endogenous WIP1 is critical in regulating melanoma xenograft growth induced by activation of the HH pathway.

Because WIP1 enhances GLI1 activity, we considered the possibility that combined inhibition of WIP1 and HH signaling might produce a more potent pathway inhibition, resulting in reduced cancer cell growth. To test this hypothesis, we treated patient-derived melanoma cells SSM2c, M21 and M26c, and MCF7 cells with low doses of cyclopamine (Cyc; 2.5 μM), a SMO inhibitor,[54] or of CCT (10 μM), a WIP1 inhibitor, or both. Single treatments were of limited efficacy (Figure 6d); however, Cyc and CCT combined treatments produced synergistic inhibition of cell growth in both melanoma and breast cancer cells (Figure 6d, Excess over Bliss Additivism > 8), consistent with a convergent action of the two agents.

DISCUSSION

Previous studies have pointed to a role of non-HH oncogenic inputs in controlling the activity of the GLI transcription factors, the terminal effectors of the HH signaling. GLI1, in particular, has emerged as a crucial regulator of cancer and stemness in different contexts.[8] Here, we provide evidence for a function of the oncogenic WIP1 phosphatase in regulating the HH signaling. Our results identify WIP1 as a novel positive modulator of GLI1 and support the concept of combination treatment with SMO and WIP1 inhibitors as efficient therapeutic option for tumors expressing WIP1 and with activated HH pathway. Protein phosphatases have been shown to modulate the GLI proteins. PP2A positively regulates the GLI homolog Ci in *Drosophila*[55] and negatively controls subcellular localization and activity of GLI3.[56,57] Moreover, the B56 subunit of PP2A regulates Gli1 function in frog embryos[58] and, indirectly, negatively controls the stability of the Gli proteins.[59]

WIP1 is emerging as an important regulator of tumorigenesis.[60] Wip1 deletion impairs spontaneous and oncogene-induced tumorigenesis.[28,29,31] In addition, Wip1 cooperates with known oncogenes (Erb2, H-Ras, Wnt1) to transform mouse embryonic fibroblasts and to accelerate breast cancer formation.[24,29,31] Here, we identify a novel mechanism for WIP1 in promoting tumorigenesis, by showing that it is critically involved in modulating the HH pathway, providing the molecular basis to understand why WIP1 enhances Sonic hedgehog-dependent medulloblastoma formation.[53] Our results show that WIP1 increases GLI1 transcriptional activity, nuclear localization and protein stability. However, WIP1 does not appear to significantly affect activity and processing of GLI2 and GLI3. The co-localization and interaction of WIP1 and GLI1 suggest that WIP1 might stabilize GLI1 by retaining it into the nucleus, thus preventing its cytoplasmic export and consequent proteasome degradation.

In this study, we found that the modulation of GLI1 transcriptional activity depends on WIP1 phosphatase activity, suggesting a direct dephosphorylation. However, we were unable to document a clear shift in GLI1 band mobility in presence of WIP1 or CCT. Therefore, at present it remains unclear whether WIP1 directly dephosphorylates GLI1 or a third protein which, in turn, directly modifies GLI1. The effect of WIP1 on GLI1 could be mediated by one of the known WIP1 dephosphorylation targets, such as p53, or ATM, Chk1, Chk2 or p38 MAPK.[60] Here, we have tested the involvement of p53 as a potential mediator, because WIP1 directly dephosphorylates p53 and the above-mentioned stress-induced kinases, resulting ultimately in the attenuation of p53 function. Our data indicate that WIP1 modulates GLI1 transcriptional activity in a p53-independent manner. However, we cannot exclude that the effects of WIP1 silencing on cell growth are the result of a complex autoregulatory loop involving WIP1/GLI1 and p53 functions. Indeed, WIP1 enhances GLI1 function (this paper) and keeps p53 in an inactivated state,[32] thus attenuating the inhibition of GLI1 by p53.[18] At the same time, HH pathway activation itself downregulates p53 by activating Mdm2.[61] We can thus speculate that during tumorigenesis, the imbalance in favor of WIP1 and HH would override p53-mediated tumor suppression and further enhance proliferation and self-renewal.

Figure 5. WIP1 is required for cancer cell growth and CSC self-renewal induced by HH pathway activation. (**a**) Histogram of the quantification and (**b**) representative images of the colony assay in MCF7 cells transduced with LV-c, *shPTCH1*, *shWIP1* or *shPTCH1/shWIP1* lentivectors, showing that WIP1 silencing reduced colony number (LV-c versus *shWIP1*, $P < 0.0001$) and prevented the increase in colony number induced by *shPTCH1* (*shWIP1* versus *shPTCH1/shWIP1*, $P = 0.53$; LV-c versus *shPTCH1*, $P = 0.049$). The *y* axis represents the percentage of colony number with the number of colonies of LV-c-transduced cells equated to 100%. (**c**, **d**) Growth assay in MCF7 (**c**) and SSM2c cells (**d**) transduced with LV-c, *shPTCH1*, *shWIP1* or *shPTCH1/shWIP1* lentivectors, showing that WIP1 silencing reduced the increase in cancer cell proliferation induced by *shPTCH1* (LV-c versus *shPTCH1*, $P < 0.05$ in both cell types; LV-c versus *shWIP1*, $P < 0.05$ in both cell types; LV-c versus *shPTCH1/shWIP1*, $P = 0.41$ in MCF7, $P = 0.38$ in SSM2c) at day 7. (**e**) qPCR analysis of *WIP1* and HH pathway components in MCF7 cells transduced with lentiviruses as indicated. The *y* axis represents expression ratio of gene/(*GAPDH* + *βACTIN* average). (**f**–**h**) Self-renewal assay showing that WIP1 silencing prevented the increase in self-renewal induced by *shPTCH1* in SSM2c (LV-c versus *shPTCH1*, $P = 0.001$; LV-c versus *shWIP1*, $P = 0.03$; LV-c versus *shPTCH1/shWIP1*, $P = 0.56$) and in MCF7 cells (LV-c versus *shPTCH1*, $P < 0.0001$; LV-c versus *shWIP1*, $P = 0.21$; LV-c versus *shPTCH1/shWIP1*, $P = 0.11$) grown as spheres (**g**, **h**). The *y* axis represents the percentage of secondary spheres formed over the number of cells seeded at 1 cell/μl. (**h**) Representative images of self-renewal assay as described in (**g**). The data represent mean ± s.e.m. values of three independent experiments. $^*P < 0.05$, $^{**}P < 0.01$. Scale bar = 150 μm.

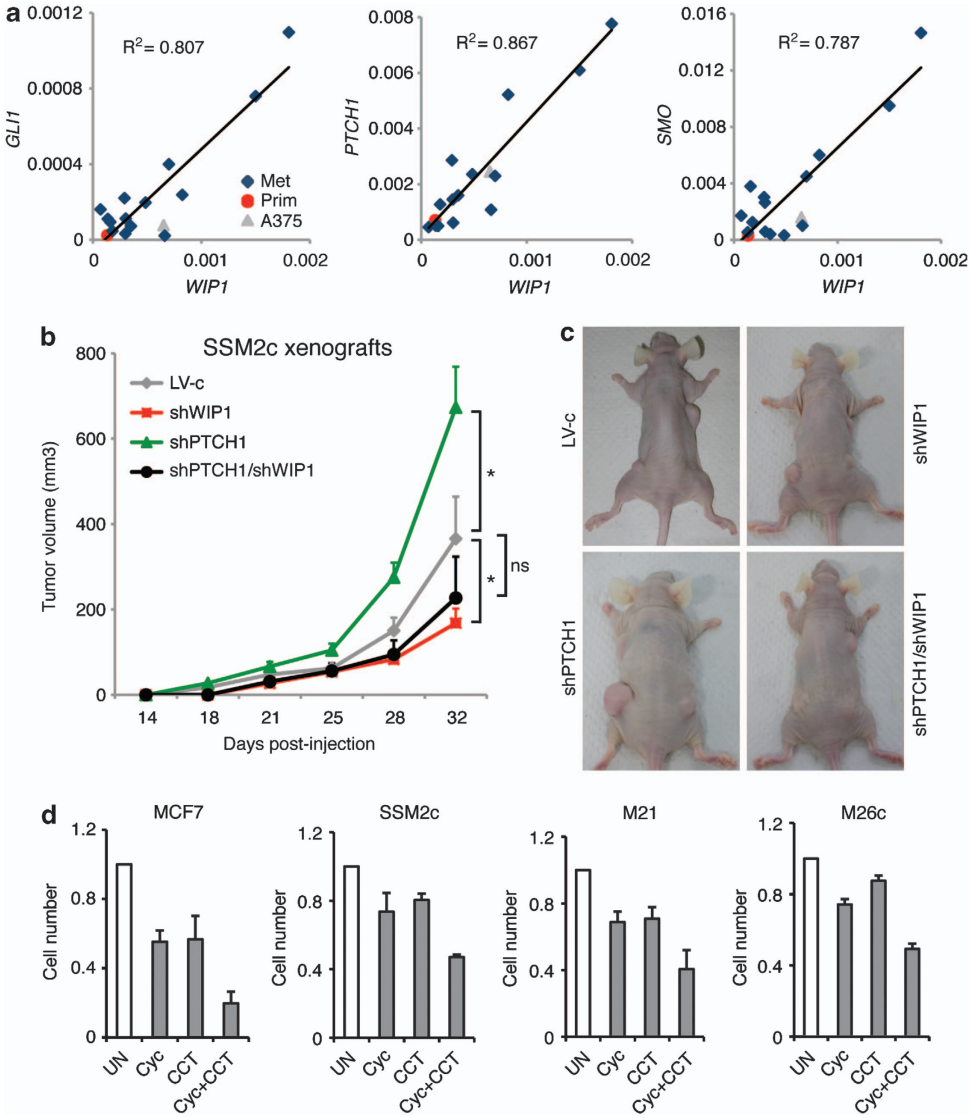


Figure 6. Interference with WIP1 prevents HH-induced melanoma xenograft growth and synergizes with SMO inhibition in reducing cancer cell growth. (**a**) Linear correlation analysis of *WIP1* with *GLI1*, *PTCH1* and *SMO* transcripts expression, measured by qPCR, in the A375 melanoma cell line and in 15 patient-derived short-term melanoma cultures, 1 of which from a primary melanoma (Prim) and 14 from metastases (Met). Each sample is represented by a dot. Axes in each graph represent expression ratio of gene/(*GAPDH* + *βACTIN* average). The extent of the correlation is indicated by R^2 coefficient. (**b**, **c**) Effect of WIP1 silencing on HH pathway activation in SSM2c melanoma xenografts. SSM2c cells were transduced with LV-c, *shPTCH1*, *shWIP1* or *shPTCH1/shWIP1* lentivectors and injected s.c. in athymic-nude mice. (**b**) Quantification of the tumor volume over time ($n = 12$/group), showing that WIP1 silencing reverted the increase in tumor growth induced by *shPTCH1* (LV-c versus *shPTCH1*, $P = 0.041$; LV-c versus *shWIP1*, $P = 0.046$; *shWIP1* versus *shPTCH1/shWIP1*, $P = 0.52$; LV-c versus *shPTCH1/shWIP1*, $P = 0.33$ at day 32) (**c**) Representative images of SSM2c xenografts growth, as indicated. (**d**) Synergistic reduction of cell number in the breast cancer cell line and in patient-derived human melanoma cells SSM2c, M21 and M26c after combined treatment with WIP1 inhibitor CCT (10 μM) and SMO antagonist Cyc (2.5 μM) for 72 h. Cyc and CCT alone modestly reduced cell growth, whereas Cyc + CCT combined treatment showed synergism (Excess over Bliss Additivism > 8).

The predominant effect of WIP1 appears to be on GLI1. However, our data also suggest that WIP1 might positively influence the HH signaling pathway upstream of GLI1. In fact, WIP1 silencing reduces the expression of endogenous HH targets *PTCH1* and *GLI1* and it reverses the increase in cancer cell growth induced by activation of the HH pathway. The HH signaling is tightly regulated by positive and negative feedback loops. As a consequence, upregulation or amplification of WIP1 during tumor progression might contribute to fuel GLI1 activity, thus potentiating a positive autoregulatory loop that sustains HH pathway activation.[18,62] In addition, we show that WIP1 silencing reduces the increase in breast and melanoma CSC self-renewal induced by activation of the HH pathway, although *shWIP1* by itself shows only modest effects. It is therefore tempting to speculate that during tumorigenesis WIP1 overexpression/amplification might contribute to increase proliferative and self-renewing activities of GLI1, therefore enabling to an expansion of CSC and derived progenitors that sustain tumor growth. This hypothesis is supported by (i) the documented role of the HH-GLI signaling and, in part, of WIP1 in regulating normal and CSCs;[22,47–51,63] (ii) the finding that WIP1 is often overexpressed in human cancers with abnormal activation of the HH signaling, such as

medulloblastomas and gliomas;[27,38] (iii) the finding of a positive correlation between the expression of *WIP1*, *GLI1* and *PTCH1* in melanomas (this study).

Notably, our data point to the relevance of the HH signaling and WIP1 in the context of melanoma, the most lethal skin cancer.[64,65] First, we show that activation of the HH signaling increases human melanoma cell proliferation and orthotopic melanoma xenograft growth, suggesting that enhanced HH pathway might promote melanoma progression. These results are consistent with the high GLI1 expression reported in human melanoma metastases[66] and complement our previous findings on the requirement of HH signaling for melanoma xenograft growth.[14] Second, we find that WIP1 inhibition reduces melanoma cell proliferation and xenograft growth, highlighting an unprecedented role of WIP1 in melanoma. Our findings suggest that WIP1 might contribute to the progression of melanomas with activated HH pathway. Besides, WIP1 might also keep p53 in an inactivated state, directly or indirectly, explaining the low p53 mutational rate in melanoma.[67]

From a therapeutic point of view, a relevant finding of this work is that combined inhibition of WIP1 and of HH signaling at the level of SMO acts synergistically to decrease cancer cell proliferation. HH pathway inhibitors have already appeared on the horizon of human cancer therapy[68] and WIP1 itself has been proposed as a target for cancer drug development,[69,70] although at present no drugs are available for clinical treatment. Our data suggest a possible novel therapeutic approach for a subset of melanomas and other cancer types expressing high levels of WIP1 and with activated HH pathway.[27,38] Indeed, targeting the HH signaling at the level of SMO, with a SMO antagonist, and at the same time at the level of GLI1, through WIP1 inhibition, should result in a more effective cancer growth inhibition. On the other hand, targeting WIP1 in tumors with wt p53 would lead not only to restoration of p53 tumor suppressor activity, which in turn might inhibit GLI1,[18] but also to a direct attenuation of GLI1 function (this study), resulting in a stronger inhibition of the HH pathway. Of note, this approach might inhibit not only the growth of melanoma bulk, but also that of putative melanoma CSC, which are responsible for tumor relapse.

MATERIALS AND METHODS

Cell lines and patient samples

MCF7, HEK-293T, p53wt and p53ko HCT116 and A375 cells were obtained from ATCC (Manassas, VA, USA). Human melanoma samples were obtained after approved protocols. Fifteen human melanoma cultures were established, 1 from a primary melanoma and 14 from metastatic melanomas (Supplementary Table S1). After mechanical disruption, tumors were incubated in Dulbecco's Modified Eagle Medium (DMEM)/F12 (Euroclone, Milan, Italy) and cells were grown in DMEM/F12 with 10% fetal bovine serum (FBS).[14] The identity of melanoma cells was verified by immunocytochemistry, as described.[51] Drugs used for treatments were puromycin (2 μg/ml), CCT007093 (CCT, 10 μM), CHX (80 μg/ml), tomatidine (Tom; 2.5 μM) (Sigma-Aldrich, St Louis, MO, USA) and Cyc (2.5 μM, TRC, Toronto, Canada).

Plasmids, mutagenesis and lentiviral vectors

Myc-tagged human GLI1, GLI3 (kind gift from A Ruiz i Altaba)[18] and GLI2 (Addgene, Cambridge, MA, USA)[71] were previously described. WIP1 and p53 cDNAs were PCR amplified with Platinum Pfx DNA polymerase (Life Technologies, Grand Island, NY, USA) and cloned into pCAG vector (Life Technologies). WIP1D314A cDNA was subcloned into pCAG from pcDNA4/TO-WIP1D314A-FlagNT (kind gift from RH Medema).[72] Mutation in pCAG-p53 (S175H) was introduced using QuikChange II (Agilent Technologies, Santa Clara, CA, USA). Lentiviruses were produced in HEK-293T cells. Lentiviral vectors pLV-CTH (LV-c), pLV-CTH-shPTCH1 (*shPTCH1*), pLV-WPXL-shp53 (*shp53*) were previously described.[18] pLKO.1-puro (LV-c), pLKO.1-puro-shWIP1 39 (targeting sequence 5′-CCCTTCTCGTGTTTGCTTAAA-3′, 3′UTR) and pLKO.1-puro-shWIP1 40 (targeting sequence 5′-CGAGAGAATGT CCAAGGTGTA-3′, exon 6) were from Open Biosystem (Lafayette, CO, USA). Most of the experiments were done with shWIP1 40.

Luciferase reporter assays

A GLI-responsive luciferase reporter (p8 × 3GLI-BS, GLI-BS) or its corresponding mutant (p8 × 3GLI-BSmut) (kind gift from H Sasaki)[73] and a p53-responsive (p21-Luc) luciferase reporter (WWP-Luc) (Addgene)[74] were used in combination with Renilla pRL-TK vector (Promega, Madison, WI, USA) (ratio 10:1) to normalize luciferase activity as already described.[18]

Immunofluorescence

Cells were transfected with equimolar amounts of plasmids. After 48 h, cells were fixed with 4% paraformaldehyde (PFA) and incubated with mouse anti-Myc or rabbit anti-WIP1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies. Secondary antibodies were anti-mouse Rhodamine Red-conjugated and anti-rabbit FITC-conjugated (Life Technologies). Immunofluorescence was visualized using a Nikon Eclipse TE2000-E confocal microscope (Melville, NY, USA).

WB, co-immunoprecipitation (IP) assay and cell fractionation

Cells were transfected with equimolar amounts of plasmids and lysed in ice in RIPA buffer (1% NP-40, 150 mM NaCl, 5 mM EDTA, 0.25% NaDOC, 50 mM Tris–HCl pH 7.5). For IP experiments, WCE (whole-cell extract) were diluted with IP buffer (0.5% NP-40, 100 mM NaCl, 5 mM EDTA, 10% glycerol, 50 mM Tris–HCl pH 7.5) and incubated with Dynabeads Protein G (Life Technologies) pre-conjugated with the antibody of interest. Beads were washed with IP buffer, and proteins were eluted with Laemmli buffer and visualized on sodium dodecyl sulfate polyacrylamide gel electrophoresis. For cell fractionation, cells were lysed in 20 mM Hepes buffer, 10 mM KCl, 1 mM EDTA, 0.2% NP-40, 10% Glycerol. This preparation was centrifuged and the cytoplasmic extract was collected in the resulting supernatant, whereas the pellet (nuclei and membranes) was dissolved in 20 mM Hepes, 420 mM NaCl, 20% Glycerol, 10 mM KCl, 1 mM EDTA. The sample was centrifuged and the nuclear protein extract collected from the supernatant. The following antibodies were used: rabbit polyclonal anti-GLI1 (Abcam, Cambridge, UK, Ab49314), mouse anti-Myc (9E10), rabbit anti-WIP1 (H-300), mouse anti-HSP90 (F-8), goat anti-GAPDH (V18), goat anti-Fibrillarin (D14) (Santa Cruz Biotechnology) and mouse anti-β-ACTIN (AC15) (Sigma-Aldrich). Chemiluminescent detection was used.

Quantitative RT–PCR

Total RNA was isolated with TriPure Isolation Reagent (Roche Diagnostics, Basel, Switzerland), subjected to DNase I treatment (Roche Diagnostics). Reverse transcription was done with High Capacity cDNA Reverse Transcription Kit (Life Technologies). Real-time quantitative PCR amplifications (qPCR) were carried out at 60 °C using Power SYBR Green PCR Master Mix (Life Technologies). Primer sequences are listed in Supplementary Materials.

Colony formation, growth curve and self-renewal assays

For colony formation assay, 800 cells/well were plated in six-well plates. After 15 days, cells were fixed with methanol, stained with Crystal Violet and colonies counted. For growth curve, 3–5000 cells/well were plated in 12-well plates and counted on days 3–5–7. For Cyc and CCT treatments, 9000–20 000 cells/well were plated in 12-well plates in medium containing 2.5% FBS and counted 96 or 120 h after treatment. For self-renewal assay in SSM2c cells, 5 cells/μl were plated in DMEM/F12 added with 20 μg/ml insulin, 0.6% glucose, 1 × N2, 10 ng/ml bFGF, 10 ng/ml epidermal growth factor (EGF) (Life Technologies).[51] For self-renewal assay in MCF7 cells, 5 cells/μl were plated in DMEM/F12 added with 5 μg/ml insulin, 1 × B27, 10 ng/ml bFGF, 10 ng/ml EGF (Life Technologies).[75] At day 7, p0 spheres formed were dissociated and plated in 96-well plates at 1 cell/well or in 12-well plates at 1 cell/μl dilutions. After 10–15 days, the number of p1 spheres formed was counted and the size was measured.

Cell sorting, nude mice and xenografts

SSM2c melanoma cells were transduced with either pLV-CTH or pLV-CTH-shPTCH1 lentiviruses. GFP + cells were sorted with the BD FACSAria cell sorter (Becton Dickinson, Franklin Lakes, NJ, USA), transduced with either pLKO.1-puro or pLKO.1-puro-shWIP1 lentiviruses, resuspended in Matrigel (Becton Dickinson)/DMEM (1/1) and inoculated s.c. into adult female athymic-nude mice (Harlan Laboratories, Indianapolis, IN, USA) (1000 cells/injection).

Statistical analysis

The data represent mean ± s.e.m. values and are calculated on at least three to four independent experiments. *P*-values were calculated using Student's *t*-test. A two-tailed value of $P < 0.05$ was considered statistically significant. The effect of Cyc and CCT combined treatments was measured by the Excess over Bliss Additivism score.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)