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HEDGEHOG/GLI-E2F1-iASPP: a novel axis involved in melanoma cell growth

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LIST OF ABBREVIATIONS

HH= Hedgehog

PTCH= Patched

SMO= Smoothened

TFs= Transcription factors

GLI= glioma-associated oncogene

PI3K= phosphoinositide-3 kinase

WT= wild-type

iASPP= inhibitor of apoptosis-stimulating protein p53

PAX3= paired box 3

SOX10= sex-determining region Y-box10

HES1= hairy/enhancer of split

MITF= microphthalmia-associated transcription factor

UVR= ultraviolet radiation

SSM= superficial spreading melanoma

LMM= lentigo malignant melanoma

ALM= acral lentiginous melanoma

NM= nodular melanoma

RGP= radial growth phase

VGP= vertical growth phase

AJCC= American Joint Committee on Cancer

TNM= Tumor-Node-Metastasis

UICC= Union for International Cancer Control

TGF-β= Transforming growth factor-β

BMPs= bone morphogenetic proteins

DHH= Desert Hedgehog

SHH= Sonic Hedegehog

IHH= Indian Hedgehog

Twhh= Tiggy-Winkle hedgehog

Ehh= Echidna hedgehog

Qhh= Qiqihar hedgehog

HIP= HH-interacting protein

GLI-TFs= GLI transcription factors

Cos2= Costal 2

Fu= Fused

SuFu= Suppressor of Fused

GLI^A= GLI activator

GLI^R= GLI repressor

PKA= protein kinase A

CK1= casein kinase 1

GSK3= glycogen synthase kinase 3

PDD= processing determinant domain

miRNAs= microRNAs

GCPs= cerebellum granule cell progenitors

MIM= actin-binding protein missing in metastasis

BCNS= basal cell nevus syndrome

ERK= extracellular signal-regulated kinase

EGFR= epidermal growth factor receptor

PKCδ= protein kinase C δ

aPKC ι/λ= Atypical Protein Kinase C ι/λ

PCAF= p300/CBP-associated factor

E1A= early region 1 A

pRB= retinoblastoma protein

DP1= DRTF-1 Polypeptide 1

MB= Marked Box

NLS= nuclear localization sequence

NES= nuclear export sequence

PcG= polycomb complex

E2F1= E2-factor 1

MDM2= Murine Double Minute 2

ASPP1/2= Apoptosis Stimulating Proteins of p53

JMY= Junction Mediating and Regulatory Protein

TP53INP1= Tumor Protein p53 Inducible Nuclear Protein 1

Apaf-1= protease-activating factor 1

Smac/DIABLO= Second Mitochondria-derived Activator of Caspases/Direct IAP-Binding protein with Low PI

ASK1= apoptosis signal-regulating kinase 1

MKKs= MAPK Kinases

HDACs= histone deacetylases

HP1= heterochromatin protein 1

BRCT= BRCA carboxy-terminal

UTR= untranslated region

CLL= chronic lymphocytic leukemia

SIRT1= Sirtuin 1

ATM= Ataxia Telangiectasia Mutated

ATR= ATM and Rad3 related

CHK2= checkpoint kinase 2

SETD7= SET domain-containing protein 7

LSD1= lysine demethylase 1

PRMT5= protein arginine methyltransferase 5

NEDD8= neural precursor cell-expressed developmentally down-regulated 8

p45SKP2= S-phase kinase-associated protein 2, p45

SCF= SKP/cullin/F-box protein complex

CGNPs= cerebellum granule neuron precursor

PCa= prostate cancer

GLI-BS= GLI-Binding Site

TSS= Transcription Start Site

ABSTRACT

Melanoma is a highly aggressive form of skin cancer that originates from the malignant transformation of melanocytes; it has high metastatic propensity and it is refractory to most traditional chemotherapeutic drugs.

The Hedgehog-GLI (HH-GLI) signaling is an evolutionarily conserved pathway, which plays an important role in embryonic development; in the adult it regulates stem cell maintenance, tissue repair and regeneration. In the canonical HH signaling, in absence of HH ligands, the transmembrane receptor Patched (PTCH) inhibits the transmembrane protein Smoothened (SMO), blocking signal transduction. Upon ligand binding, PTCH inhibition of SMO is relieved, leading to the activation of three zinc-finger transcription factors (TFs) GLI1, GLI2 and GLI3. HH pathway is aberrantly activated in many human cancers, including melanoma, where it sustains growth and survival of cancer stem cells. Aberrant activation of HH signaling can result not only from loss of function of the negative regulator PTCH1 or from constitutive activation of SMO, but also from activation of the downstream GLIs transcription factors by oncogenic inputs, such as RAS, MEK, phosphoinositide-3 kinase (PI3K), AKT and the oncogenic phosphatase WIP1. Previous data showed a reciprocal crosstalk between HH signaling and p53. More than 80% of melanomas retain wild-type (wt) p53, but its tumor-suppressor function is impaired by several mechanisms, such as overexpression of MDM2 and MDMX or deletion of the CDKN2A locus. Activation of HH signaling contributes to reduce p53 function, by increasing MDM2 levels. In turn, p53 inhibits GLI1 transcriptional activity in glioblastoma, and enhances its degradation by the induction of PCAF upon DNA damage.

Recently, another mechanism of p53 inactivation has been proposed, in which the inhibitor of apoptosis-stimulating protein p53 (iASPP) might play an important role. iASPP is often upregulated in human cancers, and melanomas that harbour wt p53, present high levels of phosphorylated iASPP.

Reactivation of p53 activity is a strategy of target therapy to suppress melanoma growth and recent evidence indicate E2F1 as a putative biomarker for anti-cancer therapies based on inhibitors of MDM2/p53 interaction. The transcription factor E2F1 is a crucial regulator of cell cycle and it is also able to induce apoptosis in response to DNA damage. Abnormalities in E2F1 expression are present in different cancer types, including malignant melanoma and are frequently associated with poor patient survival. Recent data suggested that HH signaling might increase E2F1 levels, but no clear evidence indicates that the GLIs transcription factors directly control E2F1 expression.

In this study we show that both GLI1 and GLI2 directly regulate E2F1 expression in melanoma cells, by binding to a functional non-canonical GLI consensus sequence. Consistently, the analysis of a public microarray data set, shows a significant correlation between *E2F1* and *PTCH1*, *GLI1* and *GLI2* expression in human metastatic melanomas. Functionally, we investigated the role of E2F1 in mediating the effects of HH pathway activation in melanoma, and we find that E2F1 is an important mediator of HH signaling and it is required for melanoma cell growth induced by activation of HH pathway, both *in vitro* and *in vivo*.

Moreover, we present evidence that the HH/GLI-E2F1 axis positively regulates iASPP expression in melanoma cells. HH pathway activation increases mRNA and protein levels of iASPP and this induction is directly mediated by E2F1, which binds to *iASPP* promoter. In addition, we also find that HH pathway, not only induces iASPP expression through E2F1, but also contributes to iASPP activation. Indeed, HH signaling controls Cyclin B1 and CDK1 levels, which form a complex responsible of iASPP phosphorylation.

Our data indicate that E2F1 plays a crucial role in determining melanoma cell fate, proliferation and apoptosis, in response to HH signaling activation through modulation of iASPP expression and activation. These findings suggest the presence of a novel HH/GLI-E2F1-iASPP axis involved in melanoma cell growth regulation, and provides a further mechanism through which activation of HH signaling impairs p53 function.

1. INTRODUCTION

1.1 Melanoma: the malignant transformation of melanocytes

Cutaneous melanomas originate from the malignant transformation of epidermal melanocytic cells. Melanocytes are the pigmented-producing cells of the epidermis, which are located principally in the basal epidermal layer in human skin, but also in the ears, gastrointestinal tract, eyes, oral and genital mucosa and leptomeninges. Melanocytes are present in about 1-2% of epidermis cells, whereas over the 95% of the total epidermal cell population is represented by keratinocytes (Yaar and Gilchrest, 2001). Melanocytes derive from unpigmented precursor cells, called melanoblasts that migrate from the neural crest to the epidermis and hair follicles, the final destination where they differentiate and become mature melanocytes. During embryogenesis, several different signaling pathways contribute to regulate the survival and migration of melanocytes. They include the Wingless signaling (WNT)/β-catenin (Dorsky et al., 1998, Dunn et al., 2000), the endothelin B receptor and its ligand endothelin-3 (Baynash et al., 1994), the receptor tyrosin kinase KIT (Giebel et al., 1991; Karafiat et al., 2007) and its ligand KIT-ligand/SCF (stem cell factor) (Hirobe et al., 2010), and NOCTH (Moriyama et al., 2006, Schouwey et al., 2007). In melanocyte development is also important the activity of transcription factors, such as paired box gene (PAX3) (Hornyak et al., 2001, Lang et al., 2005), sex-determining region Y-box10 (SOX10) (Honoré et al., 2003, Aoki et al., 2003), hairy/enhancer of split (HES1) (Moriyama et al., 2006), and microphthalmia-associated transcription factor (MITF) (Hornyak et al., 2001, Shibahara et al., 2000). The melanocytes have the physiological role to produce melanin pigments that are synthetized in specific cytoplasmic organelles called melanosomes and transferred to neighbouring keratinocytes. The melanin pigments have a protective role, ensuring skin protection against the effects of ultraviolet radiation (UVR) (Kobayashi et al., 1998) and reducing the UVR-induced cellular DNA damage and genomic instability. An intense and frequent exposure to UV radiation

sunlight is the principal environmental risk factor for melanoma development (Kanavy et al., 2011).

Cutaneous melanoma is the most aggressive form of skin cancer that develops from the malignant transformation of melanocytes. Melanoma has high metastatic propensity and it is refractory to most traditional chemotherapeutic drugs and most patients develop resistance to current therapies (Chudnovsky et al., 2005; Walia et al., 2012). The incidence of malignant melanoma has been increased in white population during the past several decades (Hall et al., 1999; Van Der Rhee et al., 1999; Toender et al., 2014). Melanoma is a cancer with a relatively good prognosis when diagnosed at early-stage, and can be treated through surgical excision of the primary lesion; conversely, prognosis is worse at late-stage because melanoma has spread from primary site to distant organs. Metastatic melanomas have a median survival rate comprised from six to ten months (Balch et al., 2009). The transformation process of normal melanocytes into melanoma cells is considered a multistep and multi-factorial process, in which, environmental, genetic and host factors contribute to its development.

Melanoma is currently classified into four different clinical subtypes (Clark et al., 1969, Reed et al., 1985; Whiteman et al., 2011; Schoenewolf et al., 2014), primary based on anatomic location and patterns of growth: superficial spreading melanoma (SSM), lentigo malignant melanoma (LMM), nodular melanoma (NM), and acral lentiginous melanoma (ALM). SSM is the most common form (nearly 70%) of melanoma that occurs at any site and at any age (Langholz et al., 2000). LMM presents a slowing grow and covers a largest surface area and may be confused with SSM (Cohen et al., 1995); lentigo melanoma constitutes the 10-15% of cutaneous melanoma. NM is the second most common type of melanoma that represents the 15-35% of all melanomas; it may originate at any site, but is common on exposed areas of the head and neck (Langholz et al., 2000). ALM (5-10% of melanoma cases) is characterized by the site of origin, such as palm, sole or subungal. There is another type of melanoma, the mucosal melanoma (1.3%-1.4% of all melanomas) (Batsakis et al., 2000), that occurs at any mucosal site, for

example in oral cavity, male and female genital tract, anorectum, and follow the histological features of ALM.

Melanoma presents different steps in tumor progression, based on clinical and histo-morphological features. The majority of melanoma subtypes progress through two distinct histological phases, radial and vertical, and this is the first step toward the invasive phenotype. Melanomas progress from the radial growth phase (RGP) to the vertical growth phase (VGP) (Meier et al., 1998). In the radial growth phase, melanoma cells lack the capacity to invade the dermis and metastasize. In vertical growth phase melanomas invades and goes deep into the dermis and has propensity to metastasize (Meier et al., 1998; Miller and Mihm, 2006; Whiteman et al., 2011).

1.1.1 Melanoma staging and classification

Melanoma can be classified into clinical stages on the base of significant prognostic factors. This staging system was revised by the American Joint Committee on Cancer (AJCC) (Balch et al., 2009) and it is continuously evolving. Three main classes of adverse prognostic factors play an important role in determining prognosis in melanoma: pathological, clinical, and genetic alterations. The pathological class includes lesion thickness (Breslow thickness), presence and extent of ulceration (Balch et al., 1980; In 't Hout et al., 2012), mitotic index (Barnhill et al., 2005; Thompson et al., 2011), level of invasion (Clark level), tumor-infiltrating lymphocytes (Mihm et al., 1996; Clemente et al., 1996; Burton et al., 2011), presence and location of metastases, tumour vascularity (Kashani-Sabetet al., 2002; Nagore et al., 2005), microsatellites (Nagore et al., 2005), lymphovascular invasion (Dadras et al., 2003, Egger et al., 2011), and elevated levels of serum lactic dehydrogenase (Balch et al., 2009). Clinical adverse factors include age (Austin et al., 1994), sex (Leiter et al., 2004), location of the lesion, and metastasis. Clark's level and Breslow's thickness are used for microscopic staging of primary cutaneous melanoma. Clark levels consider the anatomic location, epidermis, dermis and fat, to classify melanoma (Clark et al., 1969, McGovern et al., 1970, Mihm et

al., 1971); this classification system correlates anatomic level of invasion, coupled with mitotic index, with prognosis. Breslow thickness measures the absolute thickness of the tumor, from the granular layer, the most superficial nucleates layer of the epidermis, to the deepest layer of lesion invasion (Breslow et al., 1970). The increase in thickness of the tumor and the presence of ulceration are both inversely correlated with survival.

The AJCC and Union for International Cancer Control (UICC) staging system is based TNM (Tumor-Node-Metastasis) classification system (Sobin et al., 2001). TNM is the most used system and describes the anatomic extent of cancer. This system takes into account the size of local primary tumor (T), the presence or the absence of regional lymphatic metastases (N), and the presence of distant metastases (M). The principal prognostic factors for the evaluation of primary tumor are represented by Breslow's thickness and ulceration; instead, the number of involved lymph nodes is the second major prognostic significant factor. To evaluate the presence of metastasis, the metastatic site and the lactate dehydrogenase concentrations represent the most significant prognostic factors. A simplified overview of the TNM staging system, classifies cutaneous melanoma in five main stages:

- ✓ Stage 0: melanoma involves the epidermis but has no reached the underlying dermis. Stage 0 is also called melanoma *in situ*.
- ✓ Stage I (A and B) and II (A, B and C): melanoma is characterized by tumour thickness and ulceration status. There are no evidence of regional lymph node or distant metastases.
- ✓ Stage III (A, B and C): melanoma is characterized by lymph node metastases, but there are no evidence of distant metastases.
- ✓ Stage IV: melanoma is characterized by the presence of distant metastases and increased level of lactate dehydrogenase.

Each stage presents subgroups, depending to the absence/presence of ulceration, lymph-node involvement, and metastasis.

1.1.2 Melanoma disease: the role of molecular biology

Melanoma is a genetically and phenotypically heterogeneous tumor, as shown by recent studies (Curtin et al., 2005). The initiation and progression of melanoma result from genetic and/or epigenetic alteration in key genes that control processes such as proliferation, apoptosis, senescence and response to DNA-damage. These changes lead to the activation of oncogenes or inactivation of tumor suppressor genes or DNA-repair genes (Hodis et al., 2012), resulting in alteration of normal biological behaviour in cells that can accelerate tumor progression (Figure 1.1). Identification of predisposing genes and pathway implicated in the acquisition of malignant melanoma phenotype is crucial to better understand this disease and improve its treatment (Mourah and Lebbé, 2014).

Epidemiological evidence link solar UV irradiation, to primary cause of melanoma; up to 65% of malignant melanomas are sun-related (Katsambas et al., 1996). The first gene found to be mutated in melanoma was NRAS which belong to RAS family (Ball et al., 1994, Goel et al., 2006). This family consists of three isoforms HRAS, NRAS and KRAS each encoding a membrane-localized small GTPase. In response to cellular stimuli, RAS assumes an activated state, leading downstream cytoplasmic and/or nuclear events, that include the recruitment of RAF. The serine/threonine RAF kinases family is composed of three isoforms ARAF, BRAF, CRAF (RAF-1) activated by the small GTPase RAS. Melanomas harbour activating mutations of *NRAS* in 15-30% of cases; the most common mutations are substitutions of glutamine at position 61 by lysine or proline (Q61K, Q61R) (Goydos et al., 2005). BRAF was identified mutated in nearly 50-70% of melanomas; the most common *BRAF* mutation (80% of BRAF mutations) is a substitution of valine at position 600 by glutamic acid (V600E) that leads to a strong increase in BRAF kinase activity (Davies et al., 2002). NRAS and BRAF mutations are mutually exclusive (Sensi et al., 2006). The V600E mutation induces a constitutively activation of MAPK pathway, independent of a previous activation by RAS oncogene and extracellular stimulus, inducing an increase of ERK activation, and causing a melanoma cell proliferation. Mutation V600E also promotes

melanoma survival by regulating expression and function of pro-apoptotic and anti-apoptotic proteins such as Bcl-2 family member (BIM, BAD) (Paraiso et al., 2011; Boisvert-Adamo et al., 2008). Oncogenic RAS activates not only RAF, but also plays a role in PI3K/AKT cascade, which is frequently altered in several cancers, including melanoma. Alterations in PI3K/AKT pathways is principally due to aberrant expression of the tumor suppressor PTEN, often lost or reduced, as a consequence of somatic point mutations and deletion of PTEN (30-50% of melanomas) (Guldberg et al., 1997; Tsao et al., 2003).

Melanoma development is also strongly associated with inactivation of CDKN2A locus, which encodes p16^{INK4} and p14^{ARF}, two tumor suppressors involved in cell cycle entry at the G_1 checkpoint and in stabilization of p53 expression. The CDKN2A locus is mutated through homozygous deletion or mutation in approximately 25-50% of melanomas (Flores et al., 1996, Cachia et al., 2000). Several studies have identified additional candidate genes, involved in melanoma development, such as c-KIT (Rivera et al., 2008; Torres-Cabala et al., 2009), BAP1 (Abdel-Rahman et al., 2011; Carbone et al.; 2012), RAC1 (Krauthammer et al., 2012), PREX2 (Berger et al., 2012), MITF (Garraway et al., 2005), and candidate gene family, such as tyrosine kinase family (Prickett et al., 2009), tyrosine phosphatases (Solomon et al., 2008), MAPK effectors (Nikolaev et al., 2011; Stark et al., 2011), proteases (metalloproteinases, disintegrin and metalloproteinases, and disintegrin and metalloproteinases with trombospondin domain) (Palavalli et al., 2009), and members of glutamate signaling pathway (Wei et al., 2011).
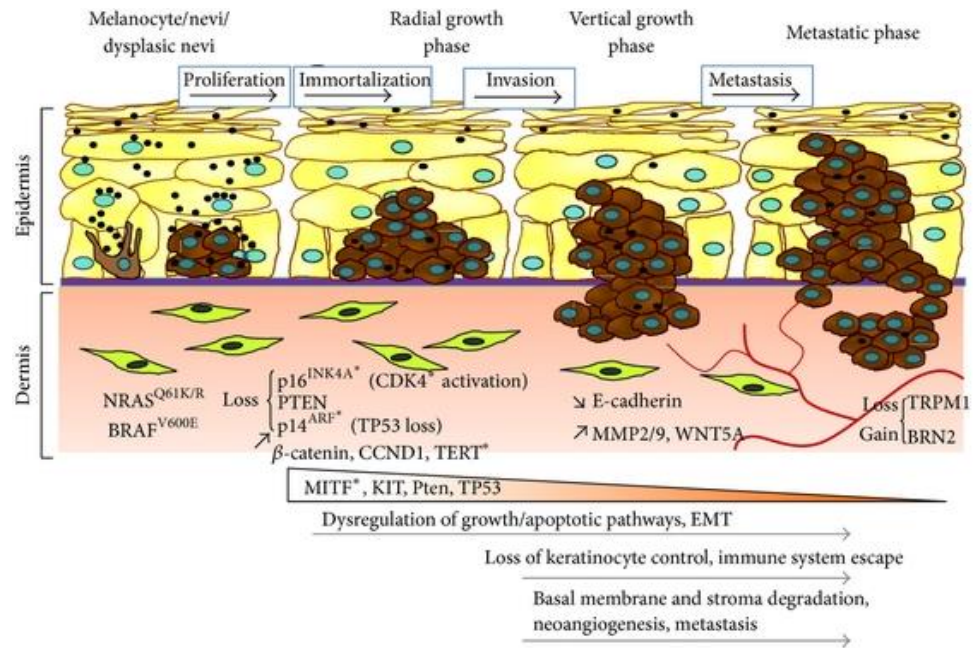
Figure 1.1. Hypothetical model of melanoma development. Melanoma development is a multistep process regulated by a key set of genes. Here are represented the major genes that are often mutated during the different phases of melanoma progression. Asterisks (*) indicate genes mutated in the germline.

Several genetic pathways regulate various steps in melanocytes development, and they have been found altered in melanoma. These pathways include: NOTCH, WNT, TGF-β, and Hedgehog signaling. The NOTCH pathway is highly conserved in most organisms and responsible for cell fate determination in the embryonic development and adult life. This pathway is frequently aberrant, most commonly by over-activation, in many types of cancer, including melanoma (Murtas et al., 2014; Aydin et al., 2014), and confers a survival advantage on tumor (Balint et al., 2005). Expression levels of Notch receptors are low in mature melanocytes, whereas in melanoma lesions and melanoma cell lines there is an increased expression of Notch receptors (Balint et al., 2005). The WNT signaling pathway is an ancient system, highly conserved from *Drosophila* to human. It is involved in several cellular functions and has a crucial role in embryonic development, adult homeostasis and tumor progression (Klaus and Birchmeier, 2008). The function of WNT/β-catenin signaling in melanoma development is interesting because it has been shown to play dual roles in both enhancing and preventing

melanoma progression. The consequences of Wnt/β-catenin cascade activation are complex and likely context-dependent (Chien et al., 2009). Among Wnt ligands, Wnt5a is the best characterized in melanoma where it has been shown to promotes cell mobility and invasion (Dissanayake et al., 2007, Weeraratna et al., 2002). Elevated expression of Wnt5a is frequently correlated with poor survival in melanoma patients (Da Forno et al., 2008). Transforming growth factor-β (TGF-β) family is a group of structurally related growth factors, which includes TGF-β, activin, nodal, bone morphogenetic proteins (BMPs), and others. These growth factors play crucial roles in regulating several biological processes during embryonic development and adult tissue homeostasis; its deregulation has been associated with many human diseases, including cancer. In melanoma TGF-β signaling controls its tumorigenesis and metastasis formation (Albino et al., 1991). An important Sonic Hedgehog mediator, GLI2, has been identified as a direct transcriptional target of TGFβ/SMAD pathway in melanoma cells (Dennler et al., 2007). Hedgehog pathway is another important pathway in melanoma, as described below. TGF-β2 expression was found to correlate with tumor thickness and TGF-β is associated with invasive signature (Perrot et al., 2013).

1.2 Hedgehog-GLI pathway

The Hedgehog (Hh) signaling pathway was initially identified in *Drosophila* as a crucial mediator of segmental patterning during embryonic development; the *Hh* gene was discovered in 1980 by Christiane Nusslein-Volhard and Eric F. Wieschaus during a screening of mutations that disrupt the *Drosophila* larval body plan (Nusslein-Volhard and Wieschaus, 1980). The name Hedgehog derives from the short and 'spiked' phenotype of the cuticle presented by *hh* mutant Drosophila larvae, which were similar to the spikes of a hedgehog (Varjosalo and Taipale, 2008; Ingham and McMahon, 2001). HH signaling is a conserved pathway, essential in embryonic development, in particular within the neural tube and skeleton (Dorus et al., 2006). During development, inactivation of HH signaling results in severe abnormalities in mice and humans (Chiang et al., 1996, Belloni et al., 1996, Roessler et al.,

2005). However, in normal adult tissue, Hh signaling is critical for maintaining tissue polarity in vertebrate and invertebrate embryos, and it is involved in stem cell maintenance, tissue homeostasis, repair and regeneration after injury (Beachy et al., 2004, Clement et al., 2007). Aberrant activation of HH signaling pathway in the adult is also linked to human cancer (Teglund et al., 2010). The discovery of loss-of-function mutations of human *PTCH1* (a negative regulator of HH pathway) was the initial link with human cancers. The HH family protein regulates several cellular processes, such as cell growth, migration, survival, differentiation and tissue patterning. HH signaling has different roles in different contexts: it can act as a morphogen in a dose-dependent manner controlling multiple different developmental processes, or as mitogen regulating cell proliferation or inducing factors that control the form of developing organs (Ingham and McMahon, 2001).

1.2.1 Hedgehog ligands: processing and release

The *Drosophila* genome encodes a single *hh* gene, while the vertebrate genome duplication resulted in expansion of *HH* genes (Wada and Makabe, 2006), which can be classified in three subgroups: *Desert Hedgehog* (*DHH*), *Indian Hedgehog* (*IHH*) and *Sonic Hedgehog* (*SHH*) in birds and mammals (Echelard et al., 1993). In *Zebrafish* there are three extra *hh* homolog, one in the *Shh* subgroup (*Tiggy-Winkle hedgehog, Twhh*) and two others in the *Ihh* subgroup (*Echidna hedgehog* and *Qiqihar hedgehog*: *Ehh* and *Qhh*, respectively), also in this case, as consequence of duplication and further rearrangements (Jaillon et al., 2004). *Dhh* is closest to *Drosophila hh*, while *Ihh* and *Shh* are more closely related to each other than *Dhh*. Mammalian Hh proteins have different roles during the development, resulting from diverse pattern of expression (McMahon et al., 200; Sagai et al., 2005). *Shh* is expressed in the developing nervous system during the early vertebrate embryogenesis, while in the late stage of development, during organogenesis, it is expressed in many epithelial tissues (Meyer and Roelink, 2003; Watanabe et al., 2000). *Ihh* is specifically expressed in a limited number of tissues, such as primitive endoderm (Dyer et al., 2001) and gut (van den Brink, 2007); it also

acts in bone development (St-Jacques et al., 1999). *Dhh* expression is restricted to the peripheral nervous system and reproductive organs, including sertoli cells of testis and granulosa cells of ovaries (Wijgerde et al., 2005; Varjosalo and Taipale, 2008). All Hh ligands undergo similar multiple events before signaling. HH protein is synthesized as a precursor molecule that is translocated into the endoplasmic reticulum (ER) lumen. In the ER the precursor undergoes cholesterol-dependent autocatalytic cleavage, originating an amino-terminal (N-terminal) and a carboxy-terminal (C-terminal) fragments (Lee et al., 1994; Porter et al., 1995; Porter et al 1996a, b; Chen, et al., 2011; Ryan and Chiang, 2012). In this process, the C-terminal domain plays an active role and acts as an intramolecular cholesterol transferase. HH processing and cholesterol modification are essential for proper signaling activity and tissue distribution. The cholesterol modified N-terminal fragment is subjected to a second covalent modifications by attachment of palmitate to its N-terminus (Chamoun et al., 2001; Buglino et al., 2008), generating the fully active form of Hh, which is released from the cells and is responsible for all the signaling effects of Hh pathway.

1.2.2 Canonical Hedgehog signal transduction

In human, the signaling cascade of HH pathway is initiated by binding of the HH ligand to its membrane receptor PATCHED (PTCH). There are two PTCH homolog genes in vertebrates, called *PTCH1* and *PTCH2* (Motoyama et al., 1998; Carpenter et al., 1998); mouse deficient in *ptch2* are viable, but develop alopecia and epidermal hypoplasia and have increased tumor incidence in the presence of *ptch* mutation (Lee et al., 2007; Nieuwenhuis et al., 2006). Instead, loss of *PTCH1* results in a complete activation of HH pathway and this suggests that PTCH1 is the functional ortholog of *Drosophila* ptc. PTCH is a 12-span transmembrane receptor, which in absence of ligands catalytically inhibits the activity of the 7-span transmembrane receptor, SMOOTHENED (SMO), by alterating its localization on the cell surface and inactivating HH target genes expression. PTCH is a negative regulator of HH signaling, while SMO is a positive regulator. The binding of HH ligands to PTCH results in the

loss of PTCH inhibition on SMO, which is activated and it can transduce the HH signal to the cytoplasm (Taipale et al., 2002). Loss of inhibition of SMO triggers the activation of the downstream SHH effectors, the glioma-associated (GLI) family of transcription factors.

The *GLI* transcription factors (*GLI1*, *GLI2*, *GLI3* in vertebrates and *Ci* in *Drosophila*) are the final effectors of HH pathway, controlling the expression of SHH target genes. GLI2 and GLI3 are the primary mediators of HH pathway, while GLI1 is itself a target of HH signaling, which is part of a positive feedback to reinforce the GLI activity (Bai et al., 2002). Several molecules are engaged in the reception of HH ligands with PTCH. The HH-interacting protein (HIP) encodes a membrane glycoprotein that binds all three mammalian Hedgehog proteins and competes with PTCH to bind HH, acting as a negative regulator of HH signaling (Chuang and McMahon, 1999). On the other hand, the HH-binding proteins CDO and BOC, and GAS1 act cooperatively with PTCH1 for HH binding and enhance signaling activity (Martinelli et al., 2007; Allen et al., 2007; Seppala et al., 2007; Tenzen et al., 2006). Glypican-3 (GPC3) competes with PTCH for HH binding, and acts as a negative regulator of HH signaling during development (Capurro et al., 2008). How PTCH regulates SMO activity is not completely clear; the current model involves trafficking of PTCH and SMO in and out of the cilium, a cell organelle present on the most mammalian cells, that is a crucial event in SMO activity regulation (Rohatgi et al., 2007; Corbit et al., 2005; Wang et al., 2009). In this model PTCH and SMO do not interact physically, but PTCH is localized at the bases of the primary cilium and, upon binding of Hh ligand, the receptor/ligand complex is translocated out of the primary cilium and internalized in endosomal vesicles. This event leads to mobilization of SMO into the primary cilium (Figure 1.2).
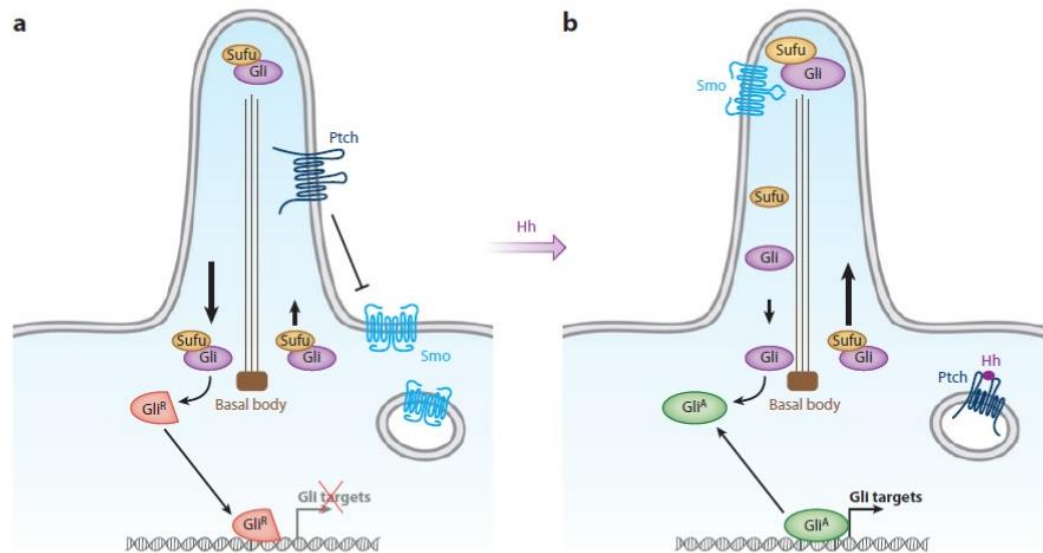
Figure 1.2. Hedgehog signaling pathway. This model briefly describes HH pathway activation and its components. (A) In absence of HH ligands, PTCH1 is located in the cilium and inhibits SMO. GLI transcription factors are present in repressor forms, preventing the activation of target genes. (B) HH ligands bind PTCH1 receptor, which moves out of the primary cilium. SMO is derepressed and moves into the primary cilium where it promotes the formation of the activator forms of the GLI transcription factors, through dissociation of Suppressor of Fused (SuFu)/GLI complex. Activators GLI-TFs translocate in the nucleus, where they induce the transcription of HH target genes.

In *Drosophila*, several molecules have been identified to act downstream of SMO signaling, including the atypical kinesin-like protein Costal 2 (Cos2), serine/threonine kinase Fused (Fu) and the Suppressor of Fused (SuFu), which is not required for pathway activity. In vertebrate Cos2 and Fu are not conserved, although SuFu is an important negative regulator of all mammalian *GLI* transcription factors activity, controlling their nuclear translocation and degradation (Kogerman et al., 1999; Ding et al., 1999; Merchant et al., 2004; Dunaeva et al., 2003). Intriguingly, PTCH, HIP, GAS1 and GLI1 are components, but also transcriptional targets of HH pathway, suggesting the presence of a feedback regulatory loop as part of mechanisms to maintain the level of HH signaling and modulate the response of HH signaling (Allen et al.,

2007; Cohen et al., 2010). On one hand, PTCH and HIP provide a negative feedback regulation, whereas GLI1 forms a positive auto-regulatory loop, extending the duration of signaling. On the other hand, HH pathway down-regulates GAS1 but, at the same time, it acts as a positive regulator for HH signaling. In addition the timing and the strength of signaling is regulated by the timing of ligand action (Ruiz I Altaba et al., 2007).

1.2.3 GLI transcription factors

The final effectors of mammalian HH signaling are the glioma-associated oncogene family member (GLI) zinc-finger transcription factors GLI1, GLI2 and GLI3 (Rubin and de Sauvage, 2006; Zhu and Lo, 2010). The roles of GLI transcription factors (GLI-TFs) are regulated by phosphorylation and proteolytic processing that convert some GLI proteins from a full-length form, which acts as a transcriptional activator (GLI^A), into a C-terminus truncated repressor (GLI^R). In frog, fish, mice and human, GLI1 acts as a strong transcriptional activator, indeed it contains only a C-terminal transcriptional activation domain; GLI2 acts both activator and repressor (Ruiz I Altaba, 1999) whereas GLI3 has mainly repressor functions, although it can also be a positive modulator inducing target genes transcription. Consistent with this, GLI2 and GLI3 possess both C-terminal activation and N-terminal repression domains (Ruiz Altaba et al., 1999; Nguyen et al., 2005; Sasaki et al., 1999). The C-terminal region contains a transactivator domain, comprised between amino acids 1020-1091, that is required for the GLI-induced transcriptional activation (Yoon et al., 1998). GLI3 processing requires the sequential phosphorylation of multiple serine residues at the C-terminal region, by protein kinase A (PKA), casein kinase 1 (CK1), and glycogen synthase kinase 3 (GSK3) (Tempé et al., 2006; Price et al., 2002). GLI3 processing is also dependent on Slimb/βTrCP, a substrate-specific receptor of the SCF-type E3 ubiquitin ligase complex (Jia et al., 2005; Smelkinson et al., 2006). The initial phosphorylation is a prerequisite for βTrCP action (Wang et al., 2006). Slimb/βTrCP can directly bind only phosphorylated GLI3 protein *in vitro* and *in vivo*; GLI3 is polyubiquitinated in the cell and this processing is carried out by the proteasome (Wang et al.,

2006). PKA, CK1 and GSK3 also phosphorylate GLI2, which shares with GLI3 44% sequence identity and it has conserved PKA, CK1 and GSK3 phosphorylation sites (Niewiadomski et al., 2014). Despite that, phosphorylation of GLI2 induces its complete degradation by the proteasome, and only a tiny fraction of GLI2^R is formed (Pan et al., 2006; Pan and Wang, 2007; Pan et al., 2009), consistent with the potential dual functions of GLI2. Pan and Wang in 2007 showed that the low efficiency of GLI2 processing is determined by the processing determinant domain (PDD). This specific region contains the first 197 amino acid residues of GLI2/GLI3 C-termini and functions as a signal for protein processing by proteasome (Pan and Wang, 2007). Consistent with this model, GLI1 does not show GLI^R activity because lacks a PDD.

In absence of HH ligands, *GLI1* is transcriptionally repressed, whereas *GLI2* and *GLI3* can be expressed and act as transcriptional repressors silencing HH-GLI targets (Bai et al., 2004; Stamataki et al., 2005). Otherwise, in response to HH activation, GLI2 is the main activator, inducing the expression of GLI1 and additional GLI target genes (Lee et al., 1997; Sasaki et al., 1999; Ikram et al., 2004). GLI1 is a transcriptional target of HH signaling and acts as a transcriptional activator to reinforce GLI^A function (Jiang et al., 2008).

The three GLI-TFs behave differently and have context-dependent repressor and activator functions. The function of GLI proteins is articulated: they have partially redundant and partially distinct functions and induce distinct target genes (Ruiz I Altaba et al., 2002; Aberger and Ruiz I Altaba, 2014). For example, GLI1 and GLI2 induce motor neurons in frog spinal cord, whereas GLI3 has an opposite function (Ruiz I Altaba et al., 1998). In contrast, in the same species, Gli1 is the principal mediator of SHH signaling, inducing floor plate differentiation, whereas both Gli2 and Gli3 repress this function. (Lee et al., 1997). However, in mice, Gli2 is primarily involved in floor-plate development (Matise et al., 1998). Indeed, *Gli2* deficient mice exhibit severe developmental defects, such as the lacking of a floor plate, and they are compromised in the development of other SHH-dependent structures (Mo et al., 1997; Matise et al., 1998; Ding et al., 1998). In contrast, functional

disruption of *Gli1* gene did not result in developmental defects, suggesting that probably does not act as primary transducer of SHH signaling (Park et al., 2000).

Originally, the *GLI1* gene was the first gene of GLI family described in 1987; it was identified in malignant glioma as amplified gene (Kinzler et al., 1987). *GLI1* maps on chromosome 12q13.3-14.1 and encodes for a transcription factor of 1106 amino acids. In 2008, was discovery a GLI1 splice variant lacking of 128 amino acids at N-terminus, called GLI1ΔN (Shimokawa et al., 2008). This variant has a reduced ability to translocate in the nucleus and activates the transcription of target genes. GLI1ΔN is present in both normal and cancer cell lines (Shimokawa et al., 2008). There is another isoform of GLI1, discovered in 2009, called tGLI1 which lacks of 41 amino acids (Lo et al., 2009). This isoform maintains all functional domains that are present in GLI1 full length, and preserves the ability to translocate in the nucleus and induce GLI target genes (Lo et al., 2009). tGLI1 expression is tumor-specific (Lo et al., 2009). The transcription factor *GLI2* maps on chromosome 2q14, and encodes a protein of 1586 amino acids. In 2005 Roessler discovery an isoform of GLI2, which lacks the N-terminus region, called GLI2ΔN. The amino-terminal repressor domain was essential for the dominant negative activity of GLI2, so the isoform GLI2ΔN results more active that the full length GLI2 (Roessler et al., 2005). The last GLI-TF is *GLI3*, located on chromosome 7p13. *GLI3* encodes a protein of 1580 amino acids, and there is no variant described for this gene.

The effects of the GLI code, the sum of GLI positive and negative functions, are tightly regulated by the HH signaling and orchestrate an arrays of different cellular functions. Regulation of GLI proteins involves the function of many factors, such as SuFu, Zic proteins and microRNAs (miRNA). SUFU is a conserved negative regulator of GLI signaling that may affect their nuclear-cytoplasmic shuttling or their activity in the nucleus, modulating cellular responses (Kogerman et al., 1999). During HH pathway activation, the inhibitory role of SuFu must be suspended to allow the induction of a positive GLI code (Svärd et al., 2006). In addition, SuFu degradation by the ubiquitin-

proteasome system is promoted by HH signaling itself (Yue et al., 2009). GLI and ZIC proteins physically interact through their zinc finger domains and regulate each other's subcellular localization and transcriptional activity in a context-dependent manner (Koyabu et al., 2001; Nguyen et al., 2005; Chan et al., 2011). Moreover, recent studies show that GLI-TFs physically interact with the mammalian homologue of Drosophila Costal2, KIF7, which controls their proteolysis and stability, and acts both positively and negatively in HH signaling (Cheung et al., 2009; Liem et al., 2009; Endoh-Yamagami et al., 2009; Li et al., 2012). ULK3, a serine/threonine kinase that shares homology with *Drosophila* Fused protein, is involved in the SHH pathway as a positive regulator of GLI proteins. ULK3 enhances GLI1 and GLI2 transcriptional activity, alters subcellular localization of GLI1, and phosphorylates GLI proteins *in vitro* (Maloverjan et al., 2010). Furthermore, HH signaling can be regulated through specific miRNA, such as miRNA-125b, miRNA-324-5p and miRNA-326 that functionally suppress SMO. miRNA-324-5p also targets GLI1 in cerebellum granule cell progenitors (GCPs) and human medulloblastoma cell lines (Ferretti et al., 2008). In addition, miRNA-17/92 cluster has been shown to synergize with SHH signaling in GCPs and medulloblastoma and it is a positive effector of Shh-mediated proliferation (Northcott et al., 2009, Uziel et al., 2009). Additional proteins that have been shown to affect HH signaling are: REN (KCTD11), a negative regulator which prevents GLI translocation to the nucleus (Di Marcotullio et al., 2004), similarly to SuFu; DYRK1, the dual specificity YAK1-related kinase that has been shown to enhance GLI1-dependent gene transcription by retaining GLI1 in the nucleus and by enhancing its transcriptional activity (Mao et al., 2002); the actin-binding protein missing in metastasis (MIM) that has been identified as a modulator of HH signaling by regulating the activity of GLIs in skin development and in tumourigenesis (Callahan et al., 2004).

1.2.4 Transcriptional targets of HH signaling

The GLI family has a highly conserved DNA-binding domain comprising five sequential C_2-H_2 zinc finger domains (Pavletich et al., 1993). All GLI proteins recognize the consensus sequence 5'-GACCACCCA-3' in the

promoter and enhancer regions of target genes (Kinzler and Volgestein, 1990; Hallikas et al., 2006). The two cytosine in 4° and 6° position in the consensus sequence are crucial for binding, whereas the other positions allow a certain grade of variation (Winklmayr et al., 2010; Peterson et al., 2012). HH activation induces several genes, such as *PTCH1, HIP1* and *GLI1*, which can trigger positive or negative effects on the pathway. Activation of HH-GLI pathway increases the expression of key regulators of G1/S and G2/M phase of cell cycle and of genes involving in differentiation process, such as *Cyclin D1* and *D2*, *N-Myc*, *E2F1, Wnts*, *Pdgrα*, *Igf2*, *FoxM1*, *Hes1* (Mullor et al., 2001; Teh et al., 2002; Kenney et al., 2003; Bhatia et al., 2011; Ingram et al., 2008; Shi et al., 2010). GLI-TFs play also a role in promoting cell survival; indeed, GLI1 and GLI2 induce the expression of the anti-apoptotic factor *BCL2* in epidermal cells (Regl et al., 2004; Bigelow et al., 2004). In addition, HH-GLI signaling has been implicated in the control of genes involved in regulation of invasiveness (Osteopontin) (Das et al., 2009), epithelial-mesenchymal transition (*Snail1*, *Sip1*, *Elk1* and *Msx2*) (Li et al., 2006; Varnat et al., 2009; Ohta et al., 2009), angiogenesis (*Vegf*) (Pola et al., 2001), and self-renewal (*Bmi1*, *Nanog, Sox2*) (Leung et al., 2004; Stecca and Ruiz I Altaba, 2009; Po et al., 2010; Santini et al., 2014).

1.2.5 HH pathway in cancer

Aberrant HH signaling is associated with the development and progression of a wide range of human malignancies (Scales and de Sauvage, 2009). The first evidence of a link between HH signaling and human cancers was made in 1970 from the identification of somatic *PTCH1* mutations in patients with Gorlin syndrome and basal cell nevus syndrome (BCNS) (Gorlin, 1995). These individuals are highly predisposed to developing cancers such as BCC (Epstein et al., 2008), medulloblastoma and rhabdomyosarcoma. Additional evidence of HH signaling implication in human cancer derive from discovery that 90% of sporadic BCCs have inactivating mutations in *PTCH1* gene (loss of function mutation) (Gailani et al., 1996; Johnson et al., 1996; Hahn et al., 1996; Santos et al., 2011) and about 10% have activating mutation in *SMO* (gain of function

mutation) (Xie et al., 1998; Couvé-Privat et al., 2002). Other genetic alterations in HH pathway components include SUFU mutations (Taylor et al., 2002) in medulloblastoma, GLI1 and GLI3 mutations in pancreatic adenocarcinoma and GLI1 and GLI2 amplifications in glioblastoma and medulloblastoma, respectively (Kinzler et al., 1987; Jones et al., 2008; Cho et al., 2011, Nobusawa et al., 2010). The aberrant activation of the signaling caused by mutations in HH pathway genes is ligand-independent.

Aberrant activation of HH signaling plays a role in other cancers that generally do not harbor mutations in HH pathway components. This is the case of lung (Watkins et al., 2003; Yuan et al., 2007), pancreatic (Thayer et al., 2003; Feldmann et al., 2007), gastrointestinal tract (Berman et al., 2003) and prostate cancers (Sanchez et al., 2004; Sheng et al., 2004), gliomas (Clement et al., 2007; Ehtesham et al., 2007), melanomas (Stecca et al., 2007) and colon cancers (Varnat et al., 2009). In these human cancers, the abnormal activation of HH pathway is ligand-dependent. They are characterized by up-regulation of the expression of HH ligands, which appear to function in an autocrine or paracrine manner or in a combination of both. Two models have been proposed to explain how HH ligands promote tumor growth: the first model proposes an autocrine mechanism in which HH ligands produced by cancer cells, their stromal environment, or both maintain stem cell in the tumor in an undifferentiated and proliferative state (Jiang and Hui, 2008). The second model proposes that HH ligands secreted by the tumor act in a paracrine manner, resulting in pathway activation in the stromal microenvironment, which in turn produces factors that indirectly promote tumor growth (Yauch et al., 2008; Tian et al., 2009). HH signaling regulates proliferation and survival of human melanomas both *in vitro* and *in vivo* (Stecca et al., 2007) and it drives self-renewal and tumorigenicity of melanoma-initiating cells (Santini et al., 2012). A systemic interference of HH function prevents melanoma growth and metastasis formation *in vivo* (Stecca et al., 2007; Jalili et al., 2013; O'Reilly et al., 2013).

1.2.6 Activation of GLI transcription factors by oncogenes in cancer

Classical HH signaling is activated by HH ligands which bind PTCH leading to the final activation of GLI-TFs. In contrast, the "non-canonical" HH signaling is SMO-independent. Several lines of evidence suggest that activation of GLI proteins is induced by various pathways that are frequently altered in human malignancies, and not only exclusively by HH signaling itself (Pandolfi and Stecca, 2015). Several pathways are involved in the modulation of GLI activity, these include phosphinositide-3 kinase (PI3K)/AKT signaling (Riobo et al., 2006), RAS/RAF/MEK signaling (Stecca et al, 2007), extracellular signal-regulated kinase (ERK) (Riobò et al., 2006), Epidermal growth factor receptor (EGFR) signaling (Schnidar et al., 2009), protein kinase C δ (PKCδ) (Cai et al., 2009) and transforming growth factor β/SMAD (Dennler et al., 2007).

The PI3K/AKT pathway has an important role in regulating HH signaling, indeed AKT protects GLI2 and GLI3 from their proteolytic degradation through PKA/CK1/GSK3β (Riobo et al., 2006). AKT1 also enhances GLI1 transcriptional activity and nuclear localization in melanoma cells (Stecca et al., 2007). In contrast, in neuroblastoma, PI3K/AKT2 pathway negative regulates GLI1 transcriptionally activity, reducing its nuclear accumulation (Paul et al., 2013).

In melanoma, glioma and prostate-cancer cells, oncogenic GLI1 activity is potentiate by H-RAS or N-RAS, AKT1 or MEK1, enhancing its nuclear localization and transcriptional activity, and counteracting GLI1 cytoplasmic retention by SUFU (Stecca et al., 2007). Similarly, in pancreatic cancer cells, HH-GLI signaling is up-regulated through the oncogenic KRAS, which suppresses GLI1 degradation (Ji et al., 2007). In addition, K-RAS-MEK-ERK pathway has a positive effect in regulating GLI transcriptional activity in gastric cancer (Seto et al., 2009). The interplay between HH-GLI and RAS-RAF-MEK signaling has been described in mouse melanomas induced by oncogenic N-RAS that show an active HH pathway and require HH-GLI function (Stecca et al., 2007).

Several lines of evidence have indicated a link between HH-GLI and epidermal growth factor receptor (EGFR) pathway. In human keratinocytes, EGFR signaling cooperates with GLI1 and GLI2 in transcriptionally modulating a subset of HH-GLI target genes via activation of RAS/RAF/MEK/ERK pathway (Kasper et al., 2006). Schnidar et al. demonstrated that HH-GLI and EGFR pathway interaction can induce oncogenic transformation and cancer development through the activation of RAS/RAF/MEK/ERK pathway leading to JUN/activator protein 1 activation. JUN/activator protein 1 cooperates with GLI1 and GLI2 (Schnidar et al., 2009), which, in turn directly regulate its expression (Laner-Plamberger et al., 2009).

TGF-β is another pathway that modulates the expression of the transcription factors GLI1 and GLI2, enhancing, or prolonging HH signals. TGF-β and HH pathway interact downstream of SMO (Dennler et al., 2007). TGF-β stimulation triggers GLI2 expression through SMAD3, resulting in an increase of GLI1 levels (Dennler et al., 2009). TGF-β can increase GLI1 protein also through the induction of Kindlin-2, which promotes GLI1 expression by inhibiting GSK3β. On the other hand, GLI1 transcriptionally represses Kindlin-2 originating a feedback loop (Gao et al., 2013).

Recent findings have shown that HH signaling is also modulated by protein phosphatases. For instance, the oncogenic wild-type p53-induced phosphatase 1 (WIP1) increases tumor formation in SHH-dependent medulloblastoma (Doucette et al., 2012). Our group has shown that WIP1 enhances specifically the activity and stability of GLI1, but not that of GLI2 nor GLI3 in melanoma cells. In addition, we demonstrated that WIP1 function is required for activation of the HH pathway. In fact, WIP1 silencing reduces HH-dependent increase in self-renewal and tumorigenicity of melanoma cells (Pandolfi et al., 2013).

HH signaling can be regulated by several members of the PKC family. Atypical Protein Kinase C ι/λ (aPKC ι/λ) has been identified as a modulator of HH-dependent processes, by phosphorylating and activating GLI1. *Prkci*, the

gene encoding for aPKC ι/λ protein, is also a HH target gene, indicating the presence of a feedback loop that contributes to HH activation in basal cell carcinoma (Atwood et al., 2013). GLI1 activity is also positive regulated by PKCα, which increases its transcriptional activity via MEK/ERK signaling (Cai et al., 2009). In contrast, PKCδ negative regulates GLI1 function, affecting its nuclear localization and transcriptional activity (Cai et al., 2009).

1.2.7 Crosstalk between Hedgehog/GLI pathway and p53

The major tumor suppressor, p53, is the most common target of genetic alterations in human cancer. p53 is the main sensor of stress and it controls the expression of several genes involved in diverse biological functions, such as cell cycle arrest, apoptosis, and senescence (Vousden et al., 2002; Oren et al., 2003; Vogelstein et al.,2000). During the early steps of tumorigenesis, aberrant activation of oncogenes gives rise to oncogenic stress, which leads to a p53-mediated response that has as final effect cellular apoptosis or senescence (Bartkova et al., 2006; Di Micco et al., 2006).

Recent data suggest a crosstalk between Hedgehog-GLI signaling and p53 in cancer. First of all, activation of the HH pathway inhibits p53 function, through phosphorylation and activation of the p53 inhibitor MDM2 (Murine Double Minute 2) (Abe et al., 2008), which enhances p53 degradation. In turn, p53 has a negative role in modulation of GLI1 function in neural stem cells and human cancer cells, such as glioblastoma, inhibiting GLI1 activity, nuclear localization and protein levels (Stecca and Ruiz I Altaba, 2009). Thus, the balance between p53 and GLI1 activity seems to be a critical point: HH signaling inhibits the tumor-suppressor function of p53 (Abe et al., 2008), which is no longer able to inhibiting GLI1 activity (Stecca and Ruiz I Altaba, 2009), leading to tumor progression. Recently it has been shown that upon DNA damage, p53 inhibits the function of GLI1, inducing its ubiquitin-dependent degradation (Mazzà et al., 2013). This effect is mediated by p53, which stimulates the induction of acetyltransferase p300/CBP-associated factor (PCAF), a novel E3 ubiquitin ligase of GLI1 (Mazzà et al., 2013).

1.3 The E2F transcription factors family

The E2F (E2 promoter binding factor) was originally identified as a cellular component that is required for the early region 1 A (E1A) transforming protein of the adenovirus (Kovesdi et al., 1986). E2F is a DNA binding protein which binds specific region of the adenoviral E2 promoter (Yee et al., 1989). The regions of E1A responsible of the increase of E2F activity are the same regions involved in the binding with pRB, p107 and p130; therefore it is believed that E1A binds the retinoblastoma protein (pRB), promoting the release of free E2F, followed from the induction of the transcription of its target genes (Bagchi et al., 1990; Bandara et al.,1991; Fattaey et al., 1993).

E2F family includes a number of transcription factors that are critical regulators of several genes involved in a wide range of cellular processes, including cell-cycle progression, DNA replication, DNA repair, differentiation, and apoptosis. The E2F family consists of eight genes in mammals (E2F1-E2F8) (Iaquinta et al., 2007; Polager and Ginsberg, 2008) and three related DP genes (DP1, DP2/3 and DP4) (Jooss et al., 1995; Milton et al., 2006). The protein products from these two groups heterodimerize and give rise to functional E2F activity (Bandara et al., 1993; Magae et al., 1996; Wu et al., 1995). The first evidence of the heterodimeric nature of E2F complexes was reported by Girling et al., who isolated DRTF-1 Polypeptide 1 (DP-1) (Girlin et al., 1993). DP and E2Fs proteins have significant homology, sharing the dimerization and DNA binding domains.

The E2F family members can be classified in activators or repressors of transcription on the basis of their functional properties and structural features (Trimarchi and Lees, 2002). E2F1, E2F2 and E2F3a, coupled with DP proteins, are potent transcriptional activators of several target genes (Wu et al., 2001). E2F3b, E2F4 and E2F5 represent the repressor group, as their main role is the repression of the transcription of target genes by recruiting the pocket proteins (Sardet et al., 1995; Vaishnav et al., 1998). E2F3b is a second product of the E2F3 locus, which lacks of the N-terminal domain characteristic of the activating E2Fs. E2F6, E2F7 and E2F8 also act as transcriptional repressors of

E2F target genes, via different mechanisms than the other E2Fs repressor because they lack the sequence required for transactivation and pocket protein-binding (Cartwright et al., 1998; Trimarchi et al., 1998; Di Stefano et al., 2003; Logan et al., 2005; Logan et al., 2004). All E2Fs contain a DNA-binding domain (Figure 1.3).
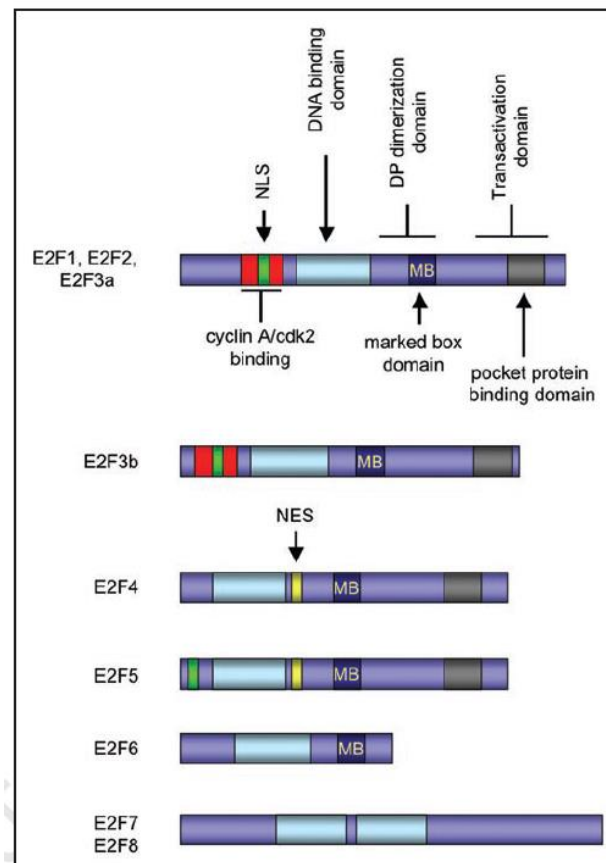
McClellan K.A and Slack R.S., Cell Cycle 2007

Figure 1.3. E2F family members. This scheme shows structure and composition of E2F proteins. Each factor contains a DNA-binding domain, a DP dimerization domain and E2F1-5 also contain a C-terminal pocket protein binding domain. In addition, E2F1, E2F2 and E2F3 have a CyclinA/CDK2-binding site (in red) in their N-terminal. E2F7 and E2F8 present two DNA-binding domain. E2F1-6 each contain a Marked Box domain (MB). In green are indicated the nuclear localization sequence (NLS); in yellow are indicated the nuclear export sequence (NES).

The transcriptional activity of E2F1 through E2F5 is mainly regulated by binding the "pocket protein" family, which includes the main member pRB and its homolog proteins p107 and p130. Generally, the pocket protein binding inhibits the transcriptional activity of E2F-DP dimers by masking the transcriptional activation domain located in the C-terminal region, and by preventing the recruitment of transcriptional activators, such as histone acetyltransferases, or by recruiting repressors to the promoter of target genes. The member E2F6 acts as a repressor interacting with member of polycomb complex (PcG) and not with the pocket proteins (Trimarchi et al., 2001). E2F7 and E2F8 also are repressors and may function in a similar manner of E2F6 (Di Stefano et al., 2003). E2F7 and E2F8 present a duplication of the DNA-binding domain; this second DNA-binding domain, probably, substitutes the function of the DP subunit in DNA binding (Logan et al., 2004; Logan et al., 2005).

The founding member of E2Fs family, E2-factor 1 (E2F1), was identified in the mid 1980s. *E2F1* gene maps on chromosome 20q11 and encodes for a transcription factor of 437aa. Structural analysis of E2F1 has identified five functional domains: at the N-terminus E2F1 presents a cyclin A-binding site (aa positions 67-108), followed by the DNA-binding domain (aa positions 128-181), a dimerization domain (aa positions 199-239) and the "marked-box" domain (aa positions 244-309). At the C-terminus E2F1 contains the transactivation domain (aa positions 369-437), necessary for the binding to the pocket protein family members.

1.3.1 E2F1: a crucial player of cell cycle progression

The transcription factor E2F1 belongs to the group of E2Fs transcriptional activator. E2F1 binds DNA cooperatively with DP proteins through the responsive site (5'-TTTC[CG]CGC-3') (Tao et al., 1997) found in the promoter region of several genes (Bieda et al., 2006). These genes are mainly involved in DNA replication, such as dihydrofolate reductase (Slansky et al., 1993), thymidine kinase, and DNA polymerase α, and cell cycle progression from G_1 to S phase, such as cyclin A and cyclin E, p107, c-Myc (Thalmeier et al., 1989;

Hiebert et al., 1989; Schulze et al., 1995; Farra et al., 2011), CDC2 and E2F1 itself. It is well established the importance of each genes in regulating cell proliferation. Their expression is tightly regulated in cell cycle with peak expression in G_1 phase (Johnson et al., 1994; Neuman et al., 1994).

During G_0 and early G_1 phase, E2F4 and E2F5 are the mainly mediators of E2F activity, which exert an inhibitory effect on cell cycle progression (Müller et al., 1997; Sardet et al., 1995; Lindeman et al., 1997). At the same time, E2F1/DP1 complex is physically associated with specifically hypophosphorylated RB1 protein (Chellappan et al., 1991; Bagchi et al., 1991; Weintraub et al., 1992; Helin et al., 1993) leading the repression of E2F target genes. Furthermore, the other two E2Fs activator (E2F2 and E2F3) are able to form dimers with the DP proteins and interact with RB1 in their inactive state. RB activity is regulated through phosphorylation by cyclin-dependent kinase (CDKs) providing a model of cell cycle control. Upon growth factor stimulation, RB1 undergoes a phosphorylation in mid-to-late G_1 phase by cyclin-D/CDK4 (Kato et al., 1993) and later by cyclin-E/CDK2 (Lundberg et al., 1998; Koff et al., 1992) complex, inducing the release of E2F from the complex and rendering E2F transcriptionally activated (Burkhart and Sage, 2008; Bates et al., 1994) (Figure 1.4). The temporal dynamics of E2F are important for a correct cell cycle progression (Zhu et al., 2005). The final result of these events is the activation of E2Fs1-3 and subsequent increased transcription of S-phase genes, including cyclin-E and cyclin-A, followed by inactivation just prior to the entry in mitosis (M) (Muller et al., 1997).

Furthermore, a genome-scale study of gene expression revealed a role for E2F in activating not only genes that encode DNA replication proteins at G_1/S phase, but also genes that are regulated at G_2 in cell cycle and encode proteins that function in mitosis (Ishida et al., 2001; Zhu et al., 2004).
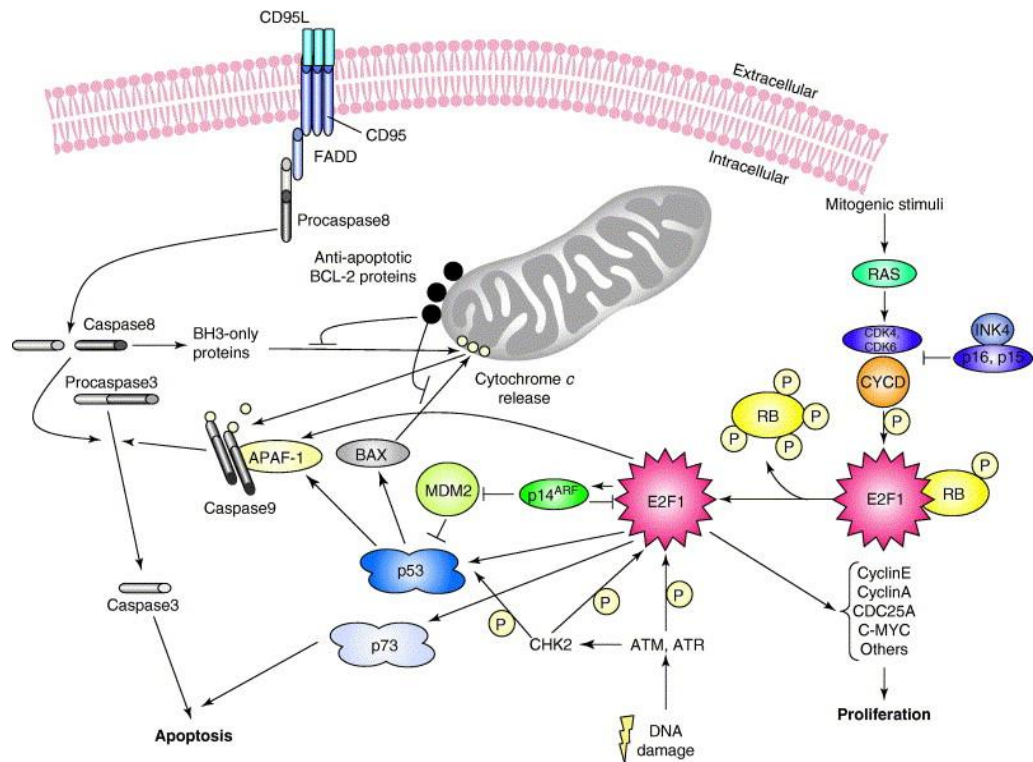
Stanelle J. and Putzer B.M., Trends Mol Med 2006

Figure 1.4. Schematic representation of E2F1 pathway. E2F1 is an important regulator of cell cycle progression (on the right) and apoptosis (on the left).

1.3.2 E2F1 and apoptosis

A feature of activator E2Fs, in particular E2F1, is the ability to induce contradictory processes, such as proliferation and apoptosis (Pan et al., 1998; DeGregori et al., 1997; O'Connor et al., 2000); therefore E2F1 has both oncogenic and tumor suppressor activities. Several evidence clearly indicates that E2F1, like p53, is a strong regulator of apoptosis after DNA damage, acting as a part of an anti-tumor safeguard mechanism, which is crucial for the preservation of cells malignant transformation and for the suppression of tumor formation (Field et al., 1996; Kowalik et al., 1995). The pro-apoptotic activity of E2F1 depends to its marked-box domain that is essential for the induction of both p53 and p73 accumulation (Hallstrom and Nevins, 2003). Apoptosis can be induced by E2F1 in a p53-dependent or p53-independent manner (Stanelle and Pützer, 2006).

Regarding the p53-dependent E2F1-mediated apoptosis, the first mechanism described is the p14ARF/MDM2/p53-dependent pathway. In normal condition, MDM2 (Murine Double Minute 2) negatively controls p53 levels inducing its ubiquitination and proteasomal degradation. In this way, MDM2 maintains low levels of p53 protein in the cells (Haupt et al., 1997; Momand et al., 1992). On the other hand, MDM2 can also interact with p14ARF, which inhibits the ability of MDM2 to target p53 (Stott et al., 1998; Pomerantz et al., 1998; Zhang et al., 1998; Kamijo et al., 1998). Several lines of evidence have shown that E2F1, in turn, can induce an increase of p14ARF levels, leading to p53 stabilization and activation (Bates et al., 1998). The result of this regulation is an accumulation of p53 followed by the activation of its target genes involved in apoptosis (Lv et al., 2014). E2F1 can induce p53-dependent apoptosis also in absence of ARF, as shown in ARF-deficient mouse and cells (Russel et al., 2002; Lindström et al., 2003). Moreover, E2F1 up-regulates ATM at transcriptional level, leading to phosphorylation of p53 on serine 15 and its accumulation (Powers et al., 2004). This mechanism suggests that ATM acts as a functional link between the RB/E2F pathway and p53, in ARF-independent manner (Berkovich and Ginsberg, 2003) (Figure 1.4). E2F1 can also promote apoptosis by directly inducing the expression of four pro-apoptotic cofactors of p53: ASPP1, and ASPP2 (Apoptosis Stimulating Proteins of p53), JMY (Junction Mediating and Regulatory Protein, p53 cofactor) and TP53INP1 (Tumor Protein p53 Inducible Nuclear Protein 1), binding their promoters *in vivo* (Hershko et al., 2005). Furthermore, E2F1 induces p53 phosphorylation on serine 46, suggesting a novel mechanism for the cooperation between E2F1 and p53 in apoptosis (Hershko et al., 2005). In addition, in response to DNA damage E2F1 and p53 directly interact through the Cyclin A-binding domain of E2F1, enhancing the apoptotic function of p53 (Hsieh et al., 2002).

E2F1 also stimulates apoptosis in a p53-independent manner. In this case the p53 homolog p73 plays an important role; E2F1 directly induces the transcription of p73, leading to the transcriptional activation of p53-responsive target genes and apoptosis in absence of p53 (Irwin et al., 2000; Stiewe and Pützer, 2000; Ozono et al., 2012). Moreover, disruption of p73 function shows

an inhibition of E2F-1-induced apoptosis (Irwin et al., 2000). E2F1 can also directly activate the expression of protease-activating factor 1 (Apaf-1), a target gene of E2F1 functionally involved in E2F1-mediated apoptosis (Moroni et al., 2001; Furukawa et al., 2002), which is activated in response to DNA damage. Interestingly, *Apaf-1* is also a direct transcriptional target of p53. Therefore, Apaf-1 might play as a mediator of both p53-dependent and E2F1-dependent apoptosis in a context-dependent manner (Moroni et al., 2001). E2F1 can induce the expression of pro-apoptotic Bcl-2 homology 3 (BH3)-only proteins PUMA, BIM, NOXA, Hrk/DP5 (Hershko et al., 2004; Hao et al., 2007; Bertin-Ciftci et al., 2013) directly binding to their promoters, and of BIK (Real et al., 2006), inducing apoptosis through p53-independent mechanisms. The DIP (Death-Inducing Protein) protein, localized in the mitochondria, also mediates apoptosis induced by E2F1 independently of p53 (Stanelle et al., 2005). Furthermore, E2F1 promotes p53-independent apoptosis via mitochondria inducing also transcriptional activation of the second mitochondrial activator, such as caspase (e.g. CASP3, CASP7, CASP8, CASP9) (Nahle et al., 2002; Bertin-Ciftci et al., 2013) and Second Mitochondria-derived Activator of Caspases/Direct IAP-Binding protein with Low PI (Smac/DIABLO) (Xie et al., 2006) (Figure 1.4). Another death effector protein of E2F1 is SIVA, a pro-apoptotic protein containing a death domain, which is a direct transcriptional target for p53 and E2F1. Indeed, SIVA is directly upregulated by E2F1 and p53 that can activate transcription from SIVA promoter (Fortin et al., 2004). E2F1 is also able to modulate the activity of the p38 MAPK pathway through a transient up-regulation of p38 MAPK phosphorylation. This mechanism involves transcriptional induction of the apoptosis signal-regulating kinase 1 (ASK1) gene, also known as MAP3K5, a member of mitogen-activated protein kinase family, that phosphorylates p38 MKKs (MAPK Kinases) (Hershko et al., 2006). The expression of ASK1 is modulated by E2F1, which binds to its promoter. ASK1 may also favor the p53-independent E2F1 apoptotic activity (Kherrouche et al., 2006). Moreover, E2F1 modulates p38 MAPK signaling through the transcriptional regulation of WIP1, a phosphatase that dephosphorylates and inactivates p38 (Hershko et al., 2006). WIP1 is a modulator of E2F1-dependent apoptosis. Several studies have demonstrated that E2F1 is involved in several different aspects of programmed

cell death depending on the cellular context and the types of stress (Stanelle and Putzer, 2006).

1.3.3 Regulation of E2F1 activity by protein-protein interactions

The activity of E2F transcription factors plays an important role in mammalian cell cycle progression and is controlled by physical association with the pocket proteins. The pocket protein family represents the first type of protein-protein interaction that modulates E2Fs transcriptional activity (Cobrinik, 2005) and includes pRB (Classon et al., 2002), p107 (Zhu et al., 1993) and p130 (Cobrinik et al., 1993). All the pocket proteins are able to regulate cell cycle progression and to arrest cells in G_1 phase when they are overexpressed. The pRB family of proteins can repress the transcription of E2F target genes by recruiting other factors, such as histone deacetylases (HDACs) and histone methyltransferase (SUV39H1) in a complex with the heterochromatin protein 1 (HP1), leading to the transcriptional repression by remodeling the nucleosome (Magnaghi-Jaulin et al., 1998; Nielsen et al., 2001; Vandel et al., 2001; Suryadinata et al., 2011; Takaki et al., 2004). The other pocket proteins, p107 and p130, generally bind to E2F4 and E2F5 (only p130) and modulate their shuttling between nucleus and cytoplasm during different phases of cell cycle (Ginsberg et al., 1994; Moberg et al., 1996; Hijmans et al., 1995; Apostolova et al., 2002; Lindeman et al., 1997).

In response to DNA damage, E2F1 interacts with TopBP1, a DNA topoisomerase II β binding protein 1, which contains eight BRCT (BRCA carboxy-terminal) motifs. The interaction depends on the amino terminus of E2F1 and the sixth BRCT domain of TopBP1, and is induced when ATM-phosphorylates E2F1. Through this interaction, the transcriptional and apoptotic activities of E2F1 are repressed (Liu et al., 2003; Liu et al., 2004). The interaction between TopBP1 and E2F1, and its repression, is specific to E2F1, because is not observed with E2F2, E2F3 nor E2F4 (Liu et al.,2003).

1.3.4 Transcriptional regulation of E2F1

Even if pocket protein interaction is the main mode to regulate E2F1, transcriptional control and post-translational modifications add several important steps in E2F1 regulation. The transcriptional regulation of E2F1 depends, at least in part, on the activity of one or more E2F family members, that bind to the E2F1 promoter (Neuman et al., 1994). The first studies showed that E2F1 promoter contains E2F-responsive sites that are necessary, but not sufficient, for transcriptional regulation of growth (Hsiao et al., 1994). Several lines of evidence show the different transcriptional regulation of E2F-responsive genes (Johnson et al., 1994; Lam et al., 1993). Analysis of E2F1 promoter revealed the presence of two overlapping E2F-binding sites that have distinct roles in the regulation of E2F1 transcription by interacting with different E2F members and cooperating with the contiguous repressor element (Araki et al., 2003).

Another point of transcriptional control is represent to Myc. Activating E2F genes contain Myc binding sites that enhance their transcription at critical cell cycle points (Leone et al., 1997; Leone et al., 2001).

1.3.5 Role of microRNAs in E2F1 regulation

MicroRNA are small non-coding RNAs of 20-24 nucleotide length that negatively regulate eukaryotic gene expression at the post-transcriptional level by binding to the 3'-untranslated region (UTR), coding sequence or 5'-UTR of target messenger RNAs (mRNAs) and mediate their degradation or inhibition of protein translation (Filipowicz et al., 2008; Ambros et al., 2004; Bartel et al., 2004). MiRNAs are predicted to control the activity of approximately 30% of the human protein-coding genes (Filipowicz et al., 2008), and have been shown to participate in the regulation of expression of genes involved in various biological processes, including proliferation, apoptosis, differentiation and metastasis (Filipowicz et al., 2008; Ambros et al., 2004; Bartel et al., 2004). The first evidence that miRNAs are involved in cancer came from the finding

that some miRNAs are downregulated or deleted in most patients with chronic lymphocytic leukemia (CLL) (Calin et al., 2002). miRNAs exhibit differential expression levels in tumors, and can act as oncogenes (oncomirs) or tumor suppressors depending on their target genes.

One of the first studies revealing that expression of E2F1 is negatively regulated by miRNAs was by O'Donnell et al. in 2005 (O'Donnell et al., 2005). Many different types of miRNA clusters regulate the E2F factors. The best characterized miRNA cluster linked to E2F1, includes miRNA-17-92, miRNA-106b-25, miRNA-34, miRNA-330-3p. E2F1 is negatively regulated by two miRNAs of the miRNA-17-92 cluster (Novotny et al., 2007), which play an important role in proliferation and survival and their overexpression promotes high proliferation and undifferentiated phenotype of normal lung cells (Lu et al., 2007). The paralog of miRNA-17-92 cluster, the miRNA-106b-25 cluster, possesses oncogenic properties and can modulate cell proliferation. MiRNA-106b-25, like miRNA-17-92, prevents high expression of E2F1 (Li et al., 2009). In turn, miRNA-17-92 cluster and its paralog cluster miRNA-106b-25 are activated by E2F1, establishing a miRNA-directed negative feedback loop (Sylvestre et al., 2007; Woods et al., 2007; Petrocca et al., 2008; Tan et al., 2014). The E2F1 activity is also regulated by miRNA that act as tumor suppressor, such as miRNA-330-3p cluster, which negatively regulates E2F1 in prostate cancer cells and induces apoptosis in prostate cancer cells through E2F1-mediated suppression of AKT phosphorylation (Lee et al., 2009), and miRNA-34 family of cluster, which decreases E2F1 transcript levels in a p53-dependent manner, suppressing cell proliferation and inducing senescence in human cancer cells (Tazawa et al., 2007).

1.3.6 Regulation of E2F1 by post-translational modifications

Post-translational modifications usually induce conformational changes, which can influence protein stability, enzymatic activity, localization and interaction with other proteins or DNA.

Upon the disruption of the pRB/E2F1 complex, E2F1 is amenable to acetylation on lysine (K) residues at position 117, 120 and 125 near the DNA-binding domain by the acetyltransferase enzyme complex p300/CBP (PCAF). Acetylation has been shown to increase E2F1 stability as well as its DNA-binding activity toward the amino terminus, and to potentiate its activity (Martínez-Balbás et al., 2000). In response to DNA damage, E2F1 acetylation is required for E2F1 recruitment on the *p73* promoter and it is important for the induction of apoptosis (Pediconi et al., 2003). DNA damage also induces E2F1-dependent activation of the deacetylase Sirtuin 1 (SIRT1) expression, which, in turn, binds and negatively regulates E2F1 activity, creating a negative feedback loop (Wang et al., 2006). In addition, SIRT1 interacts with E2F1 and suppresses the induction of its apoptotic target gene p73 by deacetylating E2F1 and/or by deacetylating and inhibiting PCAF (Pediconi et al., 2009).

Several reports have described an increase in E2F1 protein following DNA damage, as a result of E2F1 protein stabilization (Blattner et al., 1999; Höfferer et al., 1999). This stabilization is dependent on ATM (Ataxia Telangiectasia Mutated) /ATR (ATM and Rad3 related), a kinase known to activate p53 in response to DNA damage. The kinase ATM/ATR becomes active in damaged cells and triggers cellular responses phosphorylating E2F1 on serine 31, a site not conserved in the other E2F family members, which increases protein stability (Lin et al., 2001; Jin et al., 2014). The phosphorylation of E2F1 on serine 31 revokes a member of the 14-3-3 family, the 14-3-3 τ, which is a phosphoserine/phosphothreonine-binding protein. 14-3-3 τ binds to phospho-serine 31 of E2F1 and inhibits its ubiquitination during DNA damage response (Wang et al., 2004). E2F1 is also phosphorylated by checkpoint kinase 2 (CHK2), the ATM downstream target, on serine 364 in DNA damage response (Stevens et al., 2003), inducing E2F1 protein stabilization. This site of phosphorylation is not conserved in most other mammals. The mechanism of this stabilization is not clear, but resembles a positive feedback loop, because ATM is also a transcriptional target of E2F1 (Berkovich et al., 2003). Recently it has been reported that E2F1 is phosphorylated on serine 403 in response to

doxorubicin treatment, by an unknown kinase. The phosphorylation on serine 403 leads to an increase of E2F1 transcriptional activity (Real et al., 2010).

E2F1 can also undergo methylation on lysine (K) 185 by the methyltransferase SET domain-containing protein 7 (SETD7, alias Set9), which inhibits the E2F1 transcriptional activity. Lysine-185 is also a substrate for the lysine demethylase 1 (LSD1), which demethylates E2F1, resulting in an increase of its stability and activity (Kontaki and Talianidis, 2010). The molecular mechanism involves a crosstalk between different modifications that influence E2F1 stability: methylation at lysine-185 inhibits acetylation and phosphorylation at distant amino acids (Ser364) and, at the same time, induces ubiquitination and degradation of the protein.

Another important protein modification is arginine methylation that influences a variety of processes including RNA processing, chromatin and transcriptional regulation. E2F1 is methylated on arginine residues 111 and 113 by protein arginine methyltransferase 5 (PRMT5), which regulates E2F1 DNA-binding and transcriptional activity, and influences its stability (Cho et al., 2012). The depletion of PRMT5 causes an accumulation of E2F1, the consequent activation of its pro-apoptotic target genes, and induction of p53-independent apoptosis (Cho et al., 2012).

NEDDylation of E2F1 by the ubiquitin-like enzyme NEDD8 (neural precursor cell-expressed developmentally down-regulated 8) reduces protein stability and regulates its transcriptional activity in a negative manner (Loftus et al., 2012), similar to methylation. The residue K185 is important for an efficient NEDDylation of E2F1. Interestingly, lysine 185 is the same residue that is methylated by Set7/9 (Kontaki et al., 2010); this methylation allows subsequent NEDDylation, which is removed in damage cells, suggesting an interplay between methylation and NEDDylation of E2F1 in regulating apoptosis E2F1-mediated (Loftus et al., 2012; Aoki et al., 2013).

E2F1 activity is strictly controlled through the cell cycle. Following its accumulation in the late G_1 phase of cell cycle, E2F1 is degraded in S/G_2

phase. This event depends on a specific interaction of E2F1 with the F-box protein containing $p45^{SKP2}$ (S-phase kinase-associated protein 2, p45), which is the substrate-recognition subunit of the ubiquitin ligase complex SCF^{SKP2} (Marti et al., 1999). SCF (SKP/cullin/F-box protein complex) complexes belong to E3 ubiquitin ligase family and they are distinguished based on the types of F-box protein associated with the core proteins (Kipreos and Pagano, 2000; Willems et al., 2004). The SCF^{SKP2}-dependent ubiquitination pathway is involved in the down-regulation of E2F1 activity during the S/G_2 phase of cell cycle; indeed, the disruption of E2F1/$p45^{SKP2}$ interaction leads to a reduction of E2F1 ubiquitination, with consequent stabilization and accumulation of transcriptionally active E2F1 protein (Marti et al., 1999). Intriguingly, the human Skp2 promoter is directly activated by E2F1 (Zhang and Wang, 2006), suggesting a novel regulatory loop between Skp2 and E2F1, where they mutually control the expression of each other. E2F1 is a positive regulator of Skp2 expression at G_1/S phase, which, in turn, acts as the negative effector on E2F1 degradation in late S phase. Alteration of this regulatory loop may trigger uncontrolled cell proliferation in human tumor cells. Given this mutual regulation, it remains unclear why the elevated Skp2 expression in tumor cells does not lead to increase of E2F degradation (Zhang and Wang, 2006). The degradation of E2F1 can also take place in the nuclear proteasome through interaction with $p14^{ARF}$ (Martelli et al., 2001), which binds the carboxyl terminus of E2F1, promoting the binding of $p45^{SKP2}$ to the amino terminus of protein (Marti et al., 1999).

1.3.7 E2F1 activity in human cancers

In the past, E2F1 was recognized as an essential regulator of cell cycle progression and apoptosis after DNA damage, but recent evidence shows an implication of this transcription factor in various human malignancies. Several *in vitro* and *in vivo* studies demonstrate that E2F1 can act as either an oncogene (Zhang et al., 2000; Johnson et al., 1994; Olson et al., 2007) or a tumor suppressor (Yamasaki et al., 1996; Field et al., 1996; Costa et al., 2013). The early findings have shown that ectopic expression of E2F1 results in neoplastic

transformation of rodent cells *in vitro* (Xu et al., 1995) and findings from transgenic mice models demonstrate that E2F1 overexpression leads to tumor development in several tissues (Pierce et al., 1999; Conner et al., 2000) in according with oncogenic function of E2F1. In 1990s, *in vivo* studies showed that *E2f1* -/- mice increase incidence of tumors formation, demonstrating the role of E2F1 as tumor suppressor (Yamasaki et al., 1996). In addition, in the majority of human tumor type, the $p16^{INK4}$/RB/E2F1 pathway is often altered. This alteration induces a deregulation and hyperactivation of E2F1 that contributes to the genetic instability associated with the malignant transformation of normal cells to tumor cells (Pickering et al., 2006).

Recent findings indicate that E2F1 aberrant activation has been observed in various cancer cell lines and tumor types (Saito et al., 1995; Eymin et al., 2001), including malignant melanoma (Halaban et al., 2000; Nelson et al., 2006). Although the molecular basis for aberrant melanoma cell phenotype is not well understood the inactivation of pRB is implicated. Indeed, pRB is constitutively inactivated through hyper-phosphorylation or expressed at low levels in melanoma cells (Halaban et al., 1998) and this leads to the release of free E2F1, which plays a crucial role in melanoma development and progression (Halaban et al., 2000). Overexpression of E2F1 is frequently associated with high-grade tumors and poor patients survival prognosis (Han et al., 2003; Salon et al., 2007; Gorgoulis et al., 2002). In addition, amplification of E2F1 is observed in esophageal squamous cell carcinoma (Fujita et al., 2003), human colon cancer (Iwamoto et al., 2004), and in malignant melanoma cell lines and in metastatic lesions (Nelson et al., 2006). The overexpression of E2F1, due to gene amplification, may explain the mechanisms of E2F1/pRB pathway deregulation seeing as the increased levels of E2F1 could abrogate pRB ability to bind E2F1. Moreover, E2F1 overexpression could override growth inhibitory signals and contribute to the growth advantage of melanoma cells (Nelson et al., 2006). In addition, in malignant melanoma cells the deregulation of E2F1 enhances invasion and metastasis formation through direct up-regulation of the epidermal growth factor receptor (EGFR) (Alla et al., 2010). Overexpression of EGFR is often accompanied by activation of the

cytoplasmic Ras/MAPK/ERK and PI3K/AKT signaling cascade (Jiang et al., 2006).

1.3.8 E2F1 and HH/GLI pathway

The first link between HH/GLI pathway and E2F1 was provided by discovery of Regl and collaborators. They showed that overexpression of GLI2, one of the final effectors of HH pathway, stimulates S-phase *in vitro* in human keratinocytes (Regl et al., 2002). Moreover, they demonstrated that GLI2 expression in human epidermal cells leads to a strong increase of mRNA levels of key genes involved in cell cycle progression, including the E2F1 transcription factor. Furthermore, time-course studies show that E2F1 is an early transcriptional target of GLI2 (Regl et al., 2004).

Other evidences that link HH/GLI pathway and E2F1 derive from studies in medulloblastoma and in proliferating primary cerebellum neuron precursor (CGNPs) cells, proposed to be the cells of origin of medulloblastoma. In this tumor, it has been demonstrated that SHH leads to an increase of E2F1 mRNA and protein levels (Bhatia et al., 2011; Bhatia et al.,2012).

All together these findings show that *E2F1* is a possible HH pathway target gene, able to modulate HH pathway-response in neural precursors, in medulloblastoma and in keratinocytes.

1.4 The ASPP family: specific regulators of p53

The apoptotic function of p53 is specifically regulated by members of ASPP family (apoptosis-stimulating protein of p53, or Ankyrin repeats, SH3 domain and proline-rich contain protein) (Liu et al., 2005; Sullivan et al., 2007). The ASPP family includes the activators of p53, ASPP1 and ASPP2 (Samuels-Lev et al., 2001), and iASPP, a negative regulator of p53 (Bergamaschi et al., 2003). ASPP1 and ASPP2 bind to the DNA-binding

domain of p53 and stimulate its transactivation function on the promoters of pro-apoptotic genes, such as BAX and PIG3, but not p21^{WAF1} nor Mdm2 (Samuels-Lev et al., 2001), enhancing p53-mediated apoptosis. ASPP1 and ASPP2 can also interact with p63 and p73 to induce apoptosis (Bergamaschi et al., 2004), and this is important when p53 is inactive. Recently, has been shown that ASPP1 and ASPP2 bind active RAS, and cooperate with this oncogenic protein to increase the apoptosis p53-mediated (Wang et al., 2013). iASPP is the inhibitory member of ASPP family, which inhibits cell apoptosis p53-dependent.

ASPP1, ASPP2 and iASPP share a sequence homology at their C terminus, which contains four ankyrin repeats, and two domains necessary for the interaction with p53, the proline-rich region, and the SH3 domain (Gorina and Pavletich, 1996).

1.4.1 iASPP: a key inhibitor of p53

iASPP (or PPP1R13L) is evolutionary conserved from *C. Elegans* to humans (Bergamaschi et al., 2003). It is the only member of ASPP family that negative regulates p53, inhibiting its normal function, probably by direct binding to DNA-binding domain of p53 (Laska et al., 2010). iASPP is considered an oncoprotein that cooperates with the oncogene Ras, enhancing its transforming activity *in vitro* (Bergamaschi et al., 2003).

Two isoforms have been described for iASPP: iASPP/RAI and iASPP. iASPP/RAI (Rel-associated protein), the first iASPP isoform identified, encodes for a protein of 351 amino acids located exclusively in the nucleus, that originally was found interact with NF-kβ p65 subunit (RelA) (Yang et al., 1999); subsequently it has been shown that endogenous iASPP/RAI interacts with p53 inhibiting its apoptotic function (Bergamaschi et al., 2003). The second isoform identified, iASPP, encodes for a protein of 828 amino acids that shows high identity at C termini to iASPP/RAI, but contains 477 additional amino acids at its N-terminus. The N-terminus is necessary for

iASPP cytoplasmic localization, indeed iASPP can localize both in nucleus and in cytoplasm (Slee et al., 2004), unlike iASPP/RAI, which lacks of the N-terminal region. The longer isoform of iASPP acts as inhibitor of p53-mediated apoptosis, like iASPP/RAI, suggesting that the C-terminus is the responsable of the negative effect on p53, given that this region is common to both (Slee et al., 2004). In 2007 was identify another isoform of iASPP, called iASPP-SV (iASPP splice variant), which encodes a 407 amino acids protein and shares homology with the C-termini of iASPP (Zhang et al., 2007). This novel isoform contains ankyrin repeats domain, proline-rich region and SH3 domain, and share high homology with the other ASPP family members. iASPP-SV shows only a nuclear localization, and like the other isoforms, can binds to p53, and inhibits its activity, reducing p53 transcriptional activity on the promoter of pro-apoptotic genes Bax and p21 (Zhang et al., 2007).

The proline-rich domain of p53 presents a common polymorphism located at codon 72 that encodes either proline (p53Pro72) or arginine (p53Arg72). Bergamaschi and colleagues have demonstrated that iASPP has higher binding affinity to p53Pro72 and regulates its activity more efficiently than p53Arg72, and this implies that p53Arg72 is less sensitive to the inhibitory effects of iASPP (Bergamaschi et al., 2006). p53Pro72 is also more efficient than p53Arg72 in activating p53-dependent DNA-repair target, as shown in a recent study (Siddique et al., 2006).

In 50% of human tumors p53 function is absent or reduced as consequence of mutations in p53 gene (Hollstein et al., 1994). In tumor cells harbouring p53 mutations, the mechanism whereby iASPP regulates apoptosis is not completely defined; probably it interacts with the other two components of p53 family, p63 and p73 and influences their apoptotic function. Recently Chikh et al. have shown a link between p63 and iASPP in the epithelial integrity program, in which iASPP regulates the expression of genes involved in cell adhesion (Chikh et al., 2011). *In vitro* studies have demonstrated that iASPP can interact with p63 and p73 and control their apoptotic activity, negatively regulating their transcriptional activity, but does not influence their protein expression levels. iASPP expression inhibits the DNA-binding functions and

the transcriptional activity of p63 and p73 on the promoters of pro-apoptotic genes, such as BAX and PUMA (Cai et al., 2012).

1.4.2 A link between iASPP and cancer

Several human cancers show overexpression of iASPP, suggesting that iASPP contributes to tumorigenesis by increasing proliferative and anti-apoptotic effects (Zhang et al., 2011; Liu et al., 2011; Lin et al., 2011; Chen et al., 2010). Elevated iASPP expression was found in various types of tumor, including breast cancer (Bergamaschi et al., 2003), acute leukemia (Zhang et al., 2005), hepatocellular carcinomas (Lu et al., 2010), ovarian cancer (Jiiang et al., 2011), head and neck squamous cell carcinoma (Liu Z et al., 2012), oral tongue squamous cell carcinoma (Chen et al., 2014) and melanoma (Lu et al., 2013).

In nearly 80-90% of human melanomas p53 has wild-type sequence, but impaired function. In these melanoma cells, p53 target genes involved in apoptosis are under-expressed (Avery-Kiejda et al., 2011). In a recent study, Lu et al. have demonstrated that cytoplasmic iASPP is mostly detected in primary melanomas, whereas nuclear iASPP is highly expressed in melanoma metastases and associated with poor patient survival, suggesting an association with advanced stages of cancer (Lu et al., 2013). Moreover, they have shown that melanoma cells carrying p53 wild-type, co-express high levels of MDM2, of phosphorylated nuclear iASPP, and of cyclinB1. The complex CyclinB1/CDK1 is responsible of iASPP phosphorylation at S84/S113 residues (Lu et al., 2013) that inhibits iASPP dimerization, and stimulates iASPP nuclear entry as monomer. In the nucleus, iASPP binds to p53 and inhibits p53-mediated transcription on the promoters of its target genes PIG3, BAX, and PUMA. These data suggest that p53 function can be restored by inhibiting both MDM2 and iASPP phosphorylation, for example using inhibitors, such as Nutlin3 specific for MDM2, and JNJ, a CDK1 inhibitor that affects cyclinB1/CDK1 activity and prevents iASPP modifications (Lu et al., 2013).

iASPP plays an important role also in prostate cancer (PCa) progression. The majority of metastatic PC present loss of p63 (Signoretti et al., 2000; Shah et al., 2002), whereas express wild-type p53 (Taylor et al., 2010; Schlomm et al., 2008). Morris et al. have found that nuclear and cytoplasmic iASPP expression is greater in PCa than in benign epithelium, and that nuclear iASPP is enriched in highly metastatic PCa cells. Nuclear iASPP inactivates p53 function and enhances PCa progression (Morris et al., 2014).

Taken together these findings might suggest iASPP as a candidate target gene for cancer therapy. One of the latest discoveries regards a small peptide (A34) derived from p53 linker (289-322) region. A34 was used to investigate its ability to influence the interaction between iASPP and p53, and the possibility to resume the apoptotic activity of p53. A34 can combine with iASPP, release p53 from iASPP, and enhance the transcriptional activity of p53 on the promoters of its target genes (Qui et al., 2015). These data show that the peptide A34 can resume the tumor suppression function of p53.

2. AIMS OF THE STUDY

In human melanoma HH pathway is often activated and it is required for proliferation and survival of melanoma cells, and it drives self-renewal and tumorigenicity of melanoma-initiating cells. Previous data have shown a link between HH pathway and the transcription factor E2F1. This transcription factor is a key modulator of various cellular processes, such as cell cycle progression and apoptosis. E2F1 aberrant activation was found in several types of cancer, including melanoma. Shh can induce E2F1 expression in neural precursors and in medulloblastoma and GLI2, one of the final effectors of HH pathway, enhances its expression in keratinocytes: thus indicating E2F1 as a HH-responsive gene. However, it is not known what are the effects mediated by E2F1, upon HH signalling activation in melanoma. Unlike other types of cancer, more than 80% of melanomas retain wild type p53, but its tumor-suppressor function is impaired by several mechanisms. Recent evidence showed that melanomas with p53 wild type express high levels of phosphorylated iASPP protein, which is frequently upregulated in human cancers and functionally inactivates p53.

This work is articulated in two parts. In the first we show that E2F1 is a direct HH target gene in melanoma, and we identify the effects induced by HH pathway activation that are mediated by E2F1. In the second part, we focus on the p53 inhibitor iASPP, and we evaluate the role of HH/GLI-E2F1 axis in regulating its expression and activation, providing a possible mechanism through which HH signalling pathway might restrain p53 function.

3. MATERIALS AND METHODS

3.1 Cell cultures and patient samples

Human embryonic kidney HEK-293T and commercial A375 melanoma cells were obtained from ATCC (CRL-11268 and CRL-1619). Patient-derived SSM2c and M26c melanoma cells were obtained from human metastatic melanoma samples, as previously described (Santini et al., 2012; Pandolfi et al., 2013). Human melanoma samples were obtained after approved protocols by the local Ethics Committee. Fresh tissue samples were digested enzymatically using 1mg/ml collagenase A and 20 µg/ml DNase I (Roche Diagnostic, Mannheim, Germany) in DMEM/F12 (Euroclone, Milan, Italy) for 1 hour (hr) at 37°C. After dissociation and filtration in 70 µm cell strainers, cells were grown in DMEM/F12 supplemented with 10% fetal bovine serum (FBS), 1% Penicillin-Streptomycin, 1% Glutamine (Lonza, Basel, Switzerland) and epidermal growth factor (EGF) (5ng/ml) (Invitrogen, Carlsband, CA). The identity of melanoma cells was verified by immunocytochemistry using antibodies specific for melanoma: anti-Melan A, anti-S100 and anti-Vimentin antibodies, as previously described (Santini et al., 2012). Mycoplasma was periodically tested by 4',6-diamidino-2-phenylindole (DAPI) inspection and PCR. Direct sequencing revealed that both SSM2c and M26c cells harbour wild-type p53 with codon 72 Proline polymorphism (Pandolfi et al., 2013). Puromycin was used at 2µg/ml to select for transduced cells.

3.2 Plasmids, cloning, mutagenesis and lentiviral vectors

Vectors used for overexpression were: Myc-tagged human GLI1 (kind gift from A. Ruiz I Altaba) (Stecca and Ruiz I Altaba, 2009) and GLI2 (Addgene, Cambridge, MA, USA) (Roessler et al., 2005). Three fragments of E2F1 promoter (-132bp, -269bp and -656bp) were PCR amplified with Platinum Pfx DNA polymerase (Life Technologies, Carlsbad, CA, USA) and cloned in

pGL3Basic vector (Promega, Madison, WI, USA) using NheI-XhoI sites, to generate -132/-269/-656bp-E2F1prom-luc reporters. Primers used were:

Primer name	Sequence (5' to 3')
E2F1prom -132bp FW	ACGCTAGCGCGCGTTAAAGCCAATAGG
E2F1prom -269bp FW	ACGCTAGCATGTTCCGGTGTCCCCAC
E2F1prom -656bp FW	ACGCTAGCACTGGACTGTGAGCTCCTTAGG
E2F1prom RV	ACCTCGAGATCCTTTTTGCCGCGAAA

Mutations of E2F1 prom -269bp reporter were introduced using QuickChange II (Agilent Technologies, Santa Clara, CA, USA) with the following oligos:

Primer name	Sequence (5' to 3')
Mut1, FW	GGTGTCCCCACGCATGCAGCCAGGGGACG
Mut1, RV	CGTCCCCTGGCTGCATGCGTGGGGACACC
Mut2, FW	GCCATTGGCCGTACAGTCCCGCGCCGCCGCC
Mut2, RV	GGCGGCGGCGCGGGACTGTACGGCCAATGGC

Plasmid identity was verified by direct sequencing. All transfections were performed in OptiMEM (Life Technologies) using X-tremeGENE transfection reagent (Roche Diagnostic) according to manufacturer's protocol.

Replication incompetent lentiviruses were produced in HEK-293T cells by co-transfecting cells with the lentiviral of interest, the packaging vector (pCMV-dR8.74) and the envelope vector (pMD2.G) (10, 7.5 and 3 μg, respectively). The supernatant containing the lentiviral particles was harvested after 48hrs and 72hrs 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 (LV-shPTCH1) (targeting sequence 5′-GCACTATGCTCCTTTCCTC-3', exon 18) (Stecca and Ruiz I Altaba, 2009) and pLKO.1-puro-shSMO 64 (LV-shSMO 64) (targeting sequence 5'-GTGGAGAAGATCAACCTGTTT-3', exon 9) (Santini et al., 2012). pLKO.1-puro (LV-c), pLKO.1-puro-shE2F1 50 (LV-shE2F1 50) (targeting sequence 5'-GACCTCTTCGACTGTGACTTT-3', exon 7), pLKO.1-puro-shE2F1 53 (LV-shE2F1 53) (targeting sequence 5'-

ACCTCTTCGACTGTGACTTTG-3', exon 7) were from Open Biosystems (Lafayette, CO, USA). Most experiments were done with LV-shE2F1 50.

3.3 Luciferase reporter assays

To measure the ability of GLI1, GLI2 and E2F1 to activate the E2F1 promoter, luciferase reporters used were: -132/-269/-656bpE2F1prom-luc reporters (described above) and a GLI-responsive luciferase reporter (8x3'GLI-BS, GLI-BS), which contains 8 direct repeats of GLI consensus sequence GACCACCCA cloned upstream the luciferase gene (kind gift from H. Sasaki) (Sasaki et al., 1997). All luciferase reporters were used in a dual-reporter assay in combination with *Renilla* luciferase pRL-TK reporter vector (Promega, Madison, WI, USA), in a ratio 10:1, to normalize luciferase activities; pGL3Basic vector (Promega) was used to equal DNA amounts. Luminescence was measured using the Dual-Glo® Luciferase Assay System (Promega) and the GloMax® 20/20 Luminometer (Promega).

3.4 Protein extraction and western blot

Cells were lysed in ice in RIPA buffer (1% NP-40, 150mM NaCl, 5mM EDTA, 0.25% NaDOC, 50mM Tris-HCl pH 7.5), 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 and visualized on SDS-PAGE (Sodium dodecyl sulphate polycrylamide gel electrophoresis) and transferred onto 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. The following antibodies were used: rabbit anti-GLI1 (Abcam, Cambridge, United Kingdom, Ab49314), rabbit anti-E2F1 (#3742), rabbit anti-BCL2 (#2976) (Cell Signaling Technology, Danvers, MA, USA),

mouse anti-Myc (9E10), mouse anti-HSP90 (F-8), mouse anti-p53 (DO-1), mouse anti-iASPP (2808C5a), rabbit anti-CDK1 (C-19), rabbit anti-CyclinB1 (H-433) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-β-ACTIN (AC-15) (Sigma-Aldrich, St. Louis, MO, USA). After incubation with HRP-conjugated secondary antibody (Cell Signaling Technology, Beverly, MA, USA), bands were visualized by chemiluminescent detection. Blots were imaged using ChemiDoc XRS (Bio-Rad).

3.5 Quantitative Real Time-PCR (qPCR)

Total RNA from adherent cells was isolated with TRIzol Reagent (Life Technologies). Twenty micrograms of total RNA were treated with DNase I (Roche Diagnostics) for 20minutes at 30°C and purified by phenol:cloroform extraction. Three micrograms of DNase I trated RNA was subjected to reverse transcription with High Capacity cDNA Reverse Transcription Kit (Life Technologies). qPCRs were carried out at 60°C using FastStart SYBR Green Master (Roche Diagnostic) in a Rotorgene-Q (Qiagen, Hilden, Germany). Primer sequences are the following:

Primer name	Sequence (5' to 3')
GLI1-FW	CCCAGTACATGCTGGTGGTT
GLI1-RV	GCTTTACTGCAGCCCTCGT
PTCH1-FW	GGCAGCGGTAGTAGTGGTGTTC
PTCH1-RV	TGTAGCGGGTATTGTCGTGTGTG
SMO-FW	GGGAGGCTACTTCCTCATCC
SMO-RV	GGCAGCTGAAGGTAATGAGC
E2F1-FW	GCTGAGCCACTCGGCTGACG
E2F1-RV	CCACTGTGGTGTGGCTGCCC
iASPP-FW	AGCCTTAAAGAGACAGGACGG
iASPP-RV	TGTTTCATGGCCAGCGACT
BCL-XL-FW	GGTAAACTGGGGTCGCATTG
BCL-XL-RV	GCTGCTGCATTGTTCCCATAG
PIG3-FW	CTGAACCGGGCGGACTTAAT
PIG3-RV	GTGTCCCCGATCTTCCAGTG
p53AIP1-FW	CTCGGTGATGCCTCCGAATG
p53AIP1-RV	GGCCTGGAGAGACCTAGACC

Primers for human reference genes are:

Primer name	Sequence (5' to 3')
EIF2α-FW	GGATGGGACCTTGTTTGCCT
EIF2α-RV	CCACGTTGCCAGGACAGTAT
HPRT-FW	GCCAGACTTTGTTGGATTTG
HPRT-RV	CTCTCATCTTAGGCTTTGTATTTTG

3.6 Chromatin immunoprecipitation (ChIP)

Cells were fixed with 1% formaldehyde for 10 min at room temperature (RT) and fixation was stopped by adding glycine to a final concentration of 125mM for 5 min. Cells were harvested and lysed in SDS Lysis Buffer (0.5% SDS, 25mM Tris-HCl pH8) and were incubated for 15min in ice. DNA was sonicated to an average size of 300-500bp using a SONOPULS Mini20 Sonicator (Bandelin, Berlin, Germany) equipped with a cup-horn. The remaining insoluble material was removed by centrifugation at maximum speed for 15min at 4°C and the supernatants were diluted with ChIP Dilution Buffer (1.8% Triton X-100, 2mM EDTA, 300mM NaCl); input material was collected and stored at -20°C. For each samples chromatin was incubated overnight with Dynabeads Protein G (Life Technologies) pre-conjugated with 3μg of anti-GLI1 antibody (N-16) (Santa Cruz Biotechnology), anti-GLI2 (#AF3635) (R&D Systems, Minneapolis, MN, USA), anti-E2F1 (#3742) (Cell Signaling Technology) for 30min at RT for endogenous proteins. 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. Immunocomplexes were then washed 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). All solutions were added with 1X complete Protease Inhibitor Cocktail (Roche Diagnostic). DNA was eluted with 1% SDS at 85°C for 10 min, crosslinks were reversed overnight at

65°C with 200mM NaCl. After treatment with 4μg RNaseA (Life Technologies) at 37°C for 30 min and with 20μg Proteinase K (Roche Diagnostic) at 60°C for 2hrs, the immunoprecipitated DNA (ChIP DNA) and the IM were recovered by using the QIAquick PCR Purification Kit (Qiagen) and eluted in 50μl Of Tris-HCl, pH 8. qPCR were carried out at 60°C using FastStart SYBR Green Master (Roche Diagnostic) in a Rotorgene-Q (Qiagen). For ChIP with exogenous GLI1 and GLI2 cells were transfected with pCS2+MT (negative control) or pCS2+MT-GLI1 or pCS2+MT-GLI2 vectors and harvested 48hrs after transfection and ChIP on exogenous GLI1 and GLI2 was performed.

Primers used were:

Primer name	Sequence (5' to 3')
E2F1prom-FW	CTATAGAAAGGTCAGTGGGATGC
E2F1prom-RV	AGGCTTTGTCCGGATGGTA
PTCH1prom-FW	ACACACTGGGTTGCCTACC
PTCH1prom-RV	CTGTCAGATGGCTTGGGTTT
iASPPprom-FW	GGAGAAATAGGGGCAATCCGT
iASPPprom-RV	GCATGAGACTAAACCCCCGA
CDK1prom-FW	TCGCTCTCCGCTCAATTTCC
CDK1prom-RV	GGGCTACCCGATTGGTGAAT
βACTINprom-FW	TCGAGCCATAAAAGGCAACT
βACTINprom-RV	CTTCCTCAATCTCGCTCTCG

3.7 In vitro growth curves

For growth curve 4500 (SSM2c and M26c) or 3000 (A375) cells/well were plated in 12-well plates and counted on days 3-5-7. For growth assay after JNJ treatment 15000 (SSM2c and M26c) or 10000 (A375) cells/well were plated in 12-well plates and treated with the CDK1 inhibitor JNJ-7706621 (500nM, JNJ) (Merck, Damstadt, Germany) for 72hrs.

3.8 Flow cytometry analysis

For cell cycle distribution analysis, cells were stained with a hypotonic propidium iodide (PI) solution (50μg/ml PI, 0.1% Triton X-100, 0.1% sodium citrate). For proliferation index experiments, cells were labeled with 5μM of CellTrace Violet (Life Technologies), seeded and allowed to proliferate for 72 and 96hrs and analyzed using flow cytometry. CellTrace Violet data were normalized to controls arrested at the parent generation with 1μg/ml mitomycin C (t=0hrs) and proliferation index was calculated using ModFit LT software (Verity Software House, Topsham, ME, USA). For apoptosis analysis, cells were exposed to serum-deprived conditions, and apoptosis was measured after 48hrs using an Annexin V-PE/7-AAD apoptosis kit (Becton Dickinson, Franklin Lakes, NJ, USA). Cells were seeded at 15000 cells/well density in 12-well plates, treated with the CDK1 inhibitor JNJ-7706621 (500nM, JNJ) and apoptosis measured after 48hrs using Annexin V-PE/7-AAD labeling. Cytometric analysis were performed with FACS-Canto II (Becton Dickinson).

3.9 Cell sorting, nude mice and xenografts

M26c melanoma cells were transduced with either pLV-CTH (LV-c) or pLV-CTH-shPTCH1 (LV-shPTCH1) lentiviruses. Cells transduced with LV-c or LV-shPTCH1 also express green fluorescent protein (GFP) and were FACS (fluorescent-activated cell sorter)-sorted with the BD FACS-Aria cell sorter (Becton Dickinson). GFP positive cells were then transduced with either pLKO.1-puro (LV-c) or pLKO.1-puro-shE2F1-1 (LV-shE2F1) lentiviruses, resuspended in Matrigel (Becton Dickinson)/DMEM (1/1) and inoculated subcutaneously in lateral flanks of adult female athymic-nude mice (Foxn1 nu/nu) (Harlan Laboratories, Udine, Italy) (40000 cells/injection). Animals were housed in SPF conditions and monitored daily. Subcutaneous tumor size was measured twice a week with a caliper. Tumor volumes were calculated using the formula: $V=W^2 \times L \times 0.5$, where W and L are, respectively, tumor width and length. The experiment was approved by the Italian Ministry of Health and were in accordance with the Italian guidelines and regulations.

3.10 Statistical analysis

Data represent mean±SEM values and are calculated on at least 3-5 independent experiments. *P*-values were calculated using Student's *t*-test. A two-tailed value of p<0.05 was considered statistically significant.

3.11 Bioinformatic analysis

Publicly available gene expression data for a series of 31 primary and 73 metastatic melanomas were profiled on Affymetrix U133 platform (Gene Expression Omnibus GEO-46517) (Kabbarah et al., 2010). To assess the relationship between *E2F1, PTCH1, GLI1* and *GLI2* expression we performed Pearson's correlation and simple regression analysis using StatGraphics Centurion XV.I software (Statpoint Technologies, Warrenton, VA, USA).

4. RESULTS

4.1 The Hedgehog signaling positively regulates E2F1 expression in melanoma cells

The HH pathway plays a crucial role in controlling proliferation and growth of melanoma cells *in vitro* and *in vivo* (Stecca et al., 2007; Jalili et al., 2013; O'Reilly et al., 2013) and self-renewal and tumorigenicity of human melanoma-initiating cells (Santini et al., 2012). Data from literature showed that Shh induces E2F1 in CGNPs and medulloblastoma (Bhatia et al., 2011) and that GLI2 enhances the expression of E2F1 in keratinocytes (Regl et al., 2004). To investigate whether HH signaling pathway regulates E2F1 mRNA and protein levels in melanoma cells, we inhibited HH signaling by silencing SMO, using a replication incompetent lentivirus expressing a short interference RNA (shRNA) specifically targeting for SMO (LV-shSMO) (Santini et al.,2012), a positive regulator of HH pathway. LV-shSMO strongly reduced mRNA levels of *SMO*, and of *GLI1* and *PTCH1*, two HH target genes, as showed by quantitative real-time PCR (qPCR) analysis. This result confirms that LV-shSMO inhibits HH signaling, as previously shown (Santini et al., 2012) (Fig. 4.1A). Interestingly, *SMO* silencing reduced *E2F1* mRNA, and E2F1 and GLI1 protein levels compared to the control (LV-c) in melanoma cells (Figure 4.1A and B).
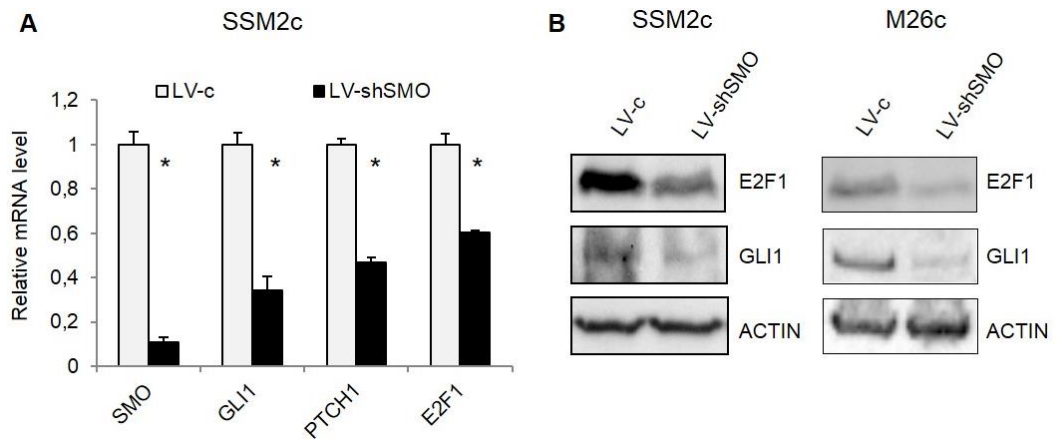
Figure 4.1. *(A) Expression of HH pathway components (SMO, GLI1, PTCH1) and E2F1 in SSM2c cells transduced with LV-c or LV-shSMO lentiviruses, measured by qPCR. The y-axis represents expression ratio of gene/(EIF2α+HPRT average). These data represent mean ±SEM of three independent experiments. *p<0.05. (**B**) Western blot analysis showing the decrease of endogenous GLI1 and E2F1 protein levels upon SMO silencing in SSM2c and M26c cells. β-actin was used as loading control.*

On the other hand, we activated HH pathway by silencing the negative regulator PTCH1. We transduced M26c and SSM2c melanoma cells with a lentivirus expressing a shRNA specifically targeting PTCH1 (LV-shPTCH1) (Stecca and Ruiz I Altaba, 2009). Activation of the HH pathway with LV-shPTCH1 resulted in an increase of *GLI1,* as expected, and induction of *E2F1* mRNA levels in both melanoma cell lines (Figure 4.2A). Similar results were obtained by transfecting melanoma cells with low amount of Myc-tagged GLI1, where an increase of endogenous E2F1 protein levels was observed in both in SSM2c and M26c cells (Figure 4.2B).

Taken together these results indicate that E2F1 expression is positively modulated by HH pathway activation in melanoma cells, suggesting that E2F1 might be a direct HH target.
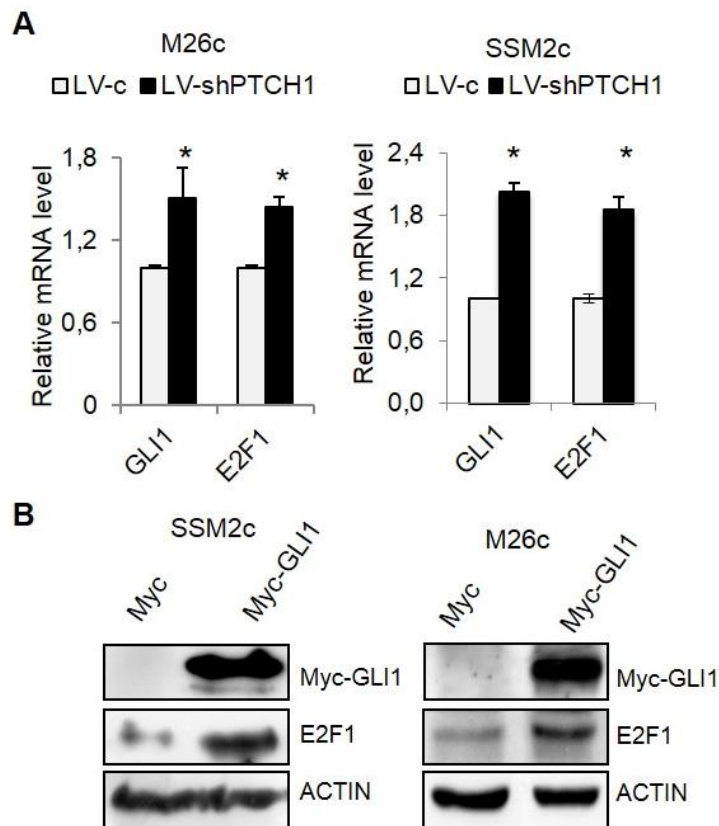
Figure 4.2. *(A) qPCR analysis showing the expression of GLI1 and E2F1 in M26c and SSM2c cells transduced with LV-c or LV-shPTCH1 lentiviruses. The y-axis represents expression ratio of gene/(EIF2α+HPRT average). These data represent mean ±SEM of three independent experiments. *p<0.05. (B) Western blot analysis showing endogenous E2F1 protein upon Myc-tagged GLI1 overexpression in SSM2c and M26c. β-actin was used as loading control.*

To support the importance and the relevance of the modulation of E2F1 expression by HH pathway in melanoma, we analysed a public microarray data set consistent of 31 primary melanomas and 73 metastatic melanomas (GEO-46517) (Kabbarah et al., 2010). We found a significant correlation between *E2F1* and *PTCH1*, *GLI1* and *GLI2* expression in metastatic melanomas (p=0.0351, p=0.0002, p=0.0072 respectively) (Figure 4.3). In primary melanomas we found a correlation only between *E2F1* and *GLI2* (p=0.0025) (not shown). These data suggest the presence of an association between HH pathway activation and E2F1 expression in human melanoma samples, and that this association preferentially occurs in late stages of melanoma progression.
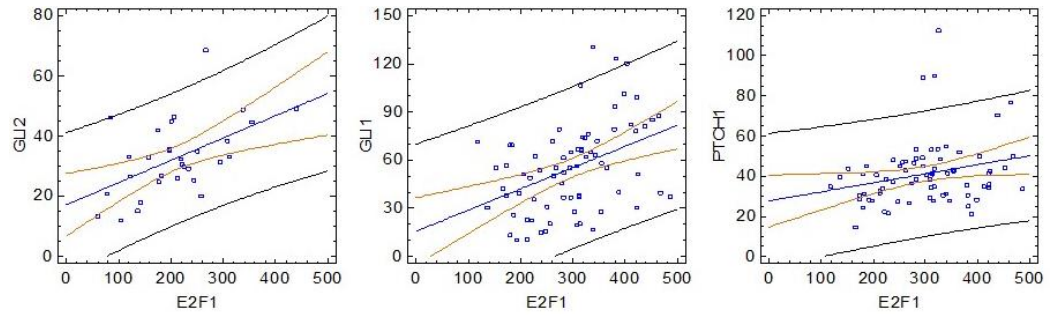
Figure 4.3. *Plots showing the positive correlation between the levels of GLI2 and E2F1 (R^2=0.0972, p=0.0072), GLI1 and E2F1 (R^2=0.1765, p=0.0002), PTCH1 and E2F1 (R^2=0.0611, p=0.0351) in metastatic melanomas.*

4.2 E2F1 is a direct target of GLI1 and GLI2 transcription factors

Our data indicate that HH pathway positively modulates E2F1 expression. To evaluate whether this regulation is directly controlled by GLI1 and GLI2, the downstream effectors of HH signaling, we performed chromatin immunoprecipitation (ChIP) assay in M26c and SSM2c cells by immunoprecipitating endogenous GLI1 and GLI2. qPCR analysis on the immunoprecipitated DNA showed that both GLI1 and GLI2 bound to *E2F1* promoter, as well as to the positive control *PTCH1* promoter (Figure 4.4A). We obtained similar data in M26c and SSM2c cells transfected with Myc-tagged GLI1, Myc-tagged GLI2 or the corresponding empty vector. The DNA was immunoprecipitated by an anti-Myc antibody and it was used to measure the enrichment of *PTCH1*, used as positive control, and of *E2F1* promoter. qPCR analysis on recovered DNA revealed an enrichment for both *E2F1* and *PTCH1* promoters (Figure 4.4B).
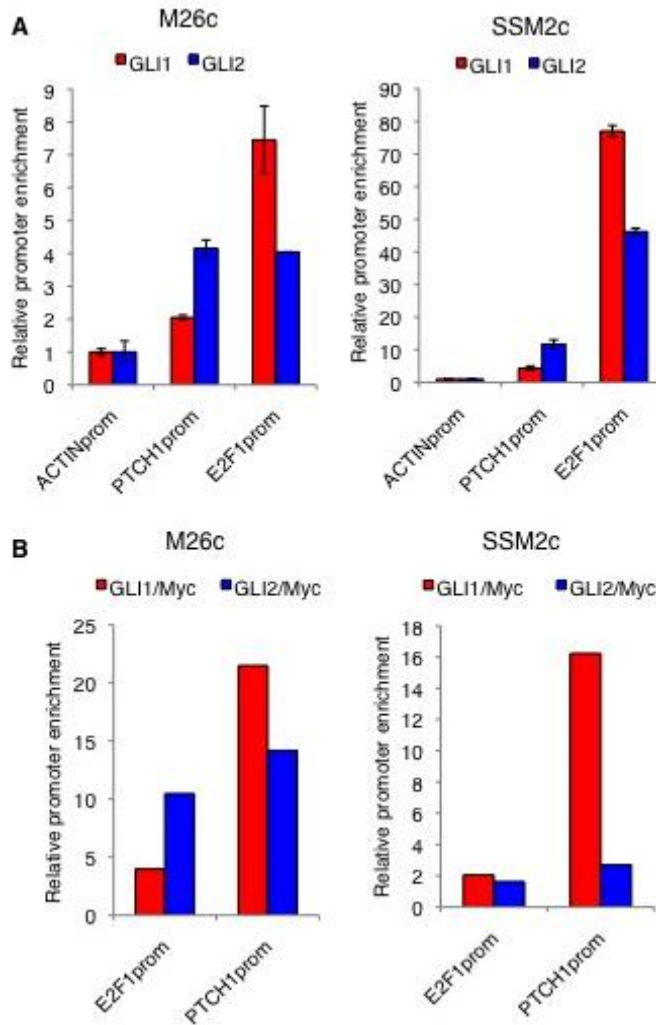
Figure 4.4. (A) Chromatin immunoprecipitation assay reveals the binding of endogenous GLI1 and GLI2 to E2F1 and PTCH1 promoters in M26c and SSM2c cells. PTCH1 promoter was used as a positive control, whereas ACTIN promoter was used as negative control. The y-axis represents the relative promoter enrichment, normalized on input material. (B) Chromatin immunoprecipitation assay showing that both exogenous Myc-tagged GLI1 and Myc-tagged GLI2 bind to PTCH1 and E2F1 promoters in M26c and SSM2c cells. The y-axis represents the relative promoter enrichment expressed as GLI1/Myc or GLI2/Myc ratio.

ChIP experiments indicate that GLI1 and GLI2 directly regulate E2F1 expression by binding to *E2F1* promoter. In order to identify the region of *E2F1* promoter responsible of the modulation through GLI-TFs, we cloned upstream the luciferase gene three different fragments of *E2F1* promoter,

respectively 132 base pairs (bp), 269bp, and 656bp upstream the transcription start site (TSS). We transfected SSM2c and M26c melanoma cells with a vector containing the E2F1 promoter-luciferase reporter along with GLI1, GLI2 or E2F1. The latter was used as positive control because it binds to *E2F1* promoter in a region close to the TSS (Johnson et al., 1994). The luciferase assay showed that both GLI1 and GLI2, although the latter at a lesser extent, were able to transactivate the luciferase reporter driven by the -269bp and -656bp fragments, but not by the -132bp fragment (Figure 4.5). E2F1 transactivated all three fragments, as expected (Figure 4.5). These results show that *E2F1* promoter contains a functional GLI binding site (GLI-BS) located in a region between -132bp and -269bp upstream *E2F1* TSS.
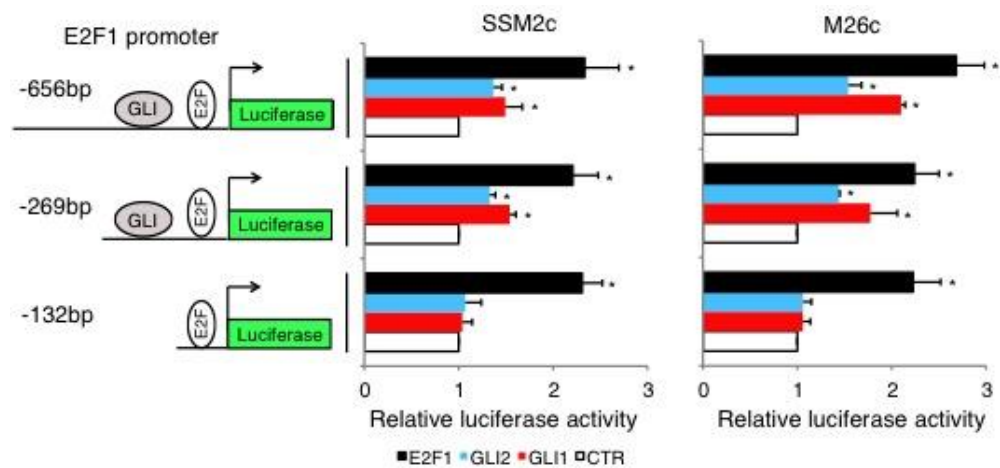

Figure 4.5. *Three fragments of different length, respectively -656bp, -269bp, and -132bp of the E2F1 promoter were used to test the ability of GLI1 and GLI2 to transactivate these promoters. Dual-luciferase report assay in SSM2c and M26c cells showing that GLI1 and GLI2 were able to transactivate E2F1 promoter in a region between -269bp and -132bp from the transcription start site. E2F1 was used as a positive control. Relative luciferase activities were firefly/renilla ratios, with the level induced by control equated to 1. The data represent mean±SEM of at least five independent experiments. p values for -656bp are: CTR versus GLI1, p=0.0214 in SSM2c; p=0.0007 in M26c; CTR versus GLI2, p=0.0013 in SSM2c; p=0.0182 in M26c. p values for -269bp are: CTR versus GLI1, p<0.0001 in SSM2c; p=0.0183 in M26c; CTR versus GLI2,*

p<0.0001 in SSM2c; p=0.0006 in M26c. p values for CTR versus E2F1 are
p<0.04 in -132bp, -269bp, -656bp, in both SSM2c and M26c cells.

Although bioinformatic analysis did not identify any canonical GLI consensus sequence (GACCACCCA) in the *E2F1* promoter, it is known that GLI-TFs are able to bind variant GLI binding site (GLI-BS) with relative low affinity leading to strong transcriptional activation (Winklmayr et al., 2010; Peterson et al., 2012) (Figure 4.6). We thus speculated that the *E2F1* promoter might contain a degenerate GLI consensus sequence, and to further characterized the position of GLI-BS we identified two putative degenerated GLI-BS in E2F1 promoter fragment -269pb (Figure 4.6), called -269Mut1 and -269Mut2, and we mutated each of them in two crucial positions for the efficiency of GLI binding (Winklmayr et al., 2010) (Figure 4.6).
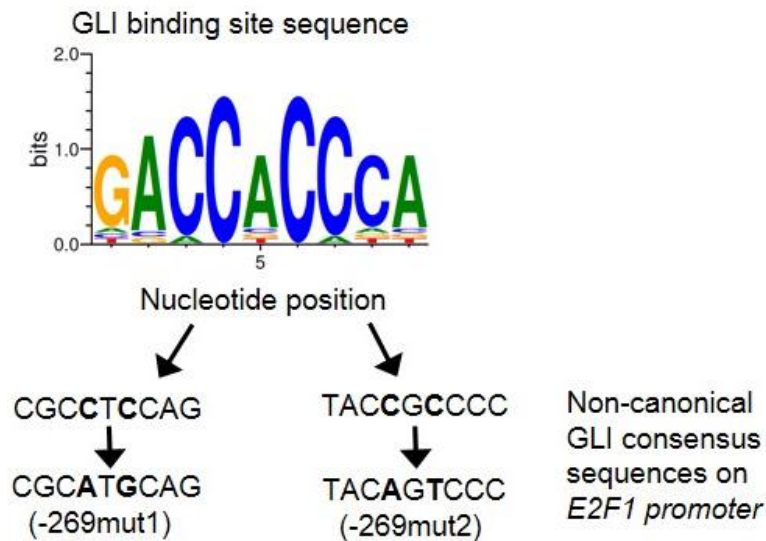

Figure 4.6. *Consensus GLI binding site motif calculated from experimentally validated GLI binding site (Winklmayr* et al., *2010) using WebLogo3 (Crooks* et al., *2004). Cytosines in position 4° and 6° are crucial for DNA binding. We identify putative GLI-binding sites in -269pb fragment of E2F1 promoter that were mutagenized (-269mut1 and -269mut2). Both positions essential for DNA binding (4C and 6C) were mutagenized.*

The disruption of site 1 (-269Mut1) prevented the transactivation of the -269bp fragment of *E2F1* promoter by both GLI1 and GLI2 in M26c and

SSM2c cells, as shown by luciferase reporter assay. Mutation of the site 2 (-269Mut2) did not have any effect (Figure 4.7), suggesting that GLI1 and GLI2 bind to and transactivate site 1 in the *E2F1* promoter.
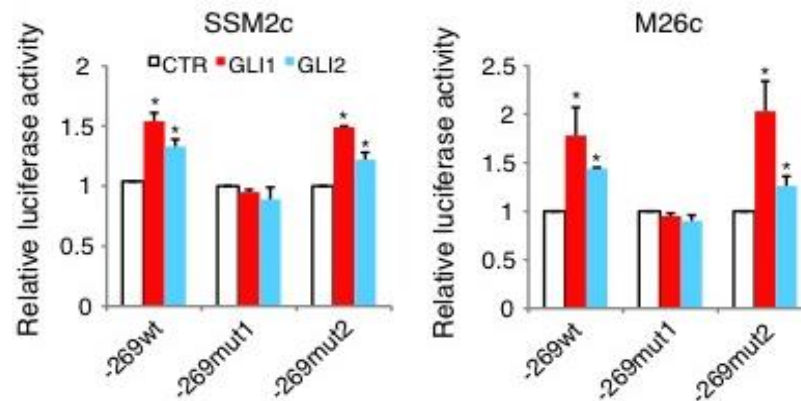

Figure 4.7. *Quantification of dual-luciferase report assay in SSM2c and M26c cells showing that only mutagenesis of -269mut1 prevented GLI1 and GLI2 from transactivating the -269bp fragment of E2F1 promoter. -269mut2 was transactivated by GLI1 and GLI2 similarly to the control (-269wt). Relative luciferase activities were firefly/renilla ratios, with the level induced by control equated to 1. The data represent mean±SEM of at least five independent experiments. *p<0.05*

These results indicate that the expression of E2F1 is directly regulated by the HH pathway and that both the downstream effectors of the HH signaling, GLI1 and GLI2, bind to a non-canonical GLI consensus sequence (CGCCTCCAG) on the *E2F1* promoter, identified by site-directed mutagenesis experiments.

4.3 Silencing of E2F1 in melanoma cells

E2F1 is a transcriptional regulator of proliferation (Müller et al., 2000; Wu et al., 2001). To test the role of E2F1 in melanoma cells, we knocked it down using two lentiviruses expressing shRNA specifically targeting E2F1 (LV-shE2F1-1 and LV-shE2F1-2). qPCR showed a strong reduction of *E2F1*

mRNA expression with both shRNAs, indicating the efficiency of E2F1 silencing (Figure 4.8A). To evaluate the presence of possible compensatory effects upon E2F1 silencing, we also measured the expression levels of other members of the E2F family, the activators E2F2 and E2F3a, and the repressor E2F4. As showed by qPCR analysis, we found a slight increase in E2F2, a minor decrease in E2F3a expression and no changes in E2F4 expression (Figure 4.8B).
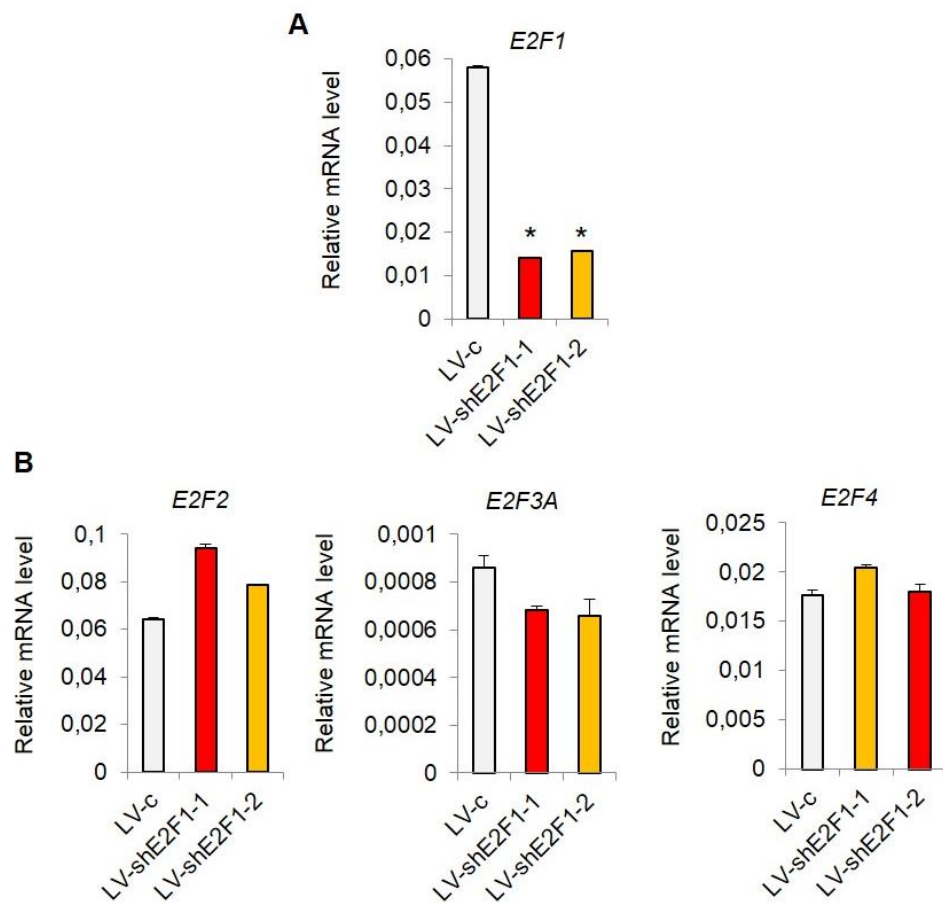

Figure 4.8. *(**A**) Quantitative PCR analysis of E2F1 expression in SSM2c cells transduced with two different shRNA specifically targeting E2F1 (LV-shE2F1-1 and LV-shE2F1-2). The y-axis represents the expression ratio of gene/(EIF2a+HPRT average). *p<0.05 compared to the control. (**B**) Quantitative PCR analysis of the activator E2F2 and E2F3a, and the repressor E2F4 expression in SSM2c cells upon E2F1 silencing with both shRNAs. The y-axis represents the expression ratio of gene/(EIF2a+HPRT average).*

Our results indicate that both LV-shE2F1-1 and LV-shE2F1-2 efficiently silence E2F1 expression in melanoma cells and that upon E2F1 silencing no significant compensatory effects by other members of the E2F family are observed.

4.4 E2F1 is required for melanoma cell growth induced by activation of the HH pathway

E2F1 is directly controlled by HH signaling, as indicated by our data. We and others have previously shown that HH signalling is required for melanoma growth and stemness (Stecca et al., 2007; Jalili et al., 2013; O'Reilly et al., 2013; Santini et al., 2012). To investigate the effects of the HH pathway that are mediated by E2F1 in melanoma, we silenced E2F1 upon HH signalling activation.

To activate the HH pathway we silenced the negative regulator PTCH1, then we transduced melanoma cells with LV-shPTCH1 lentivirus alone or in combination with LV-shE2F1. Western blot and qPCR analysis in M26c transduced cells showed an increase of GLI1 and E2F1 expression upon *PTCH1* silencing, indicating the activation of the HH pathway, and a strong decrease of E2F1 upon *E2F1* silencing (Figure 4.9A e B).
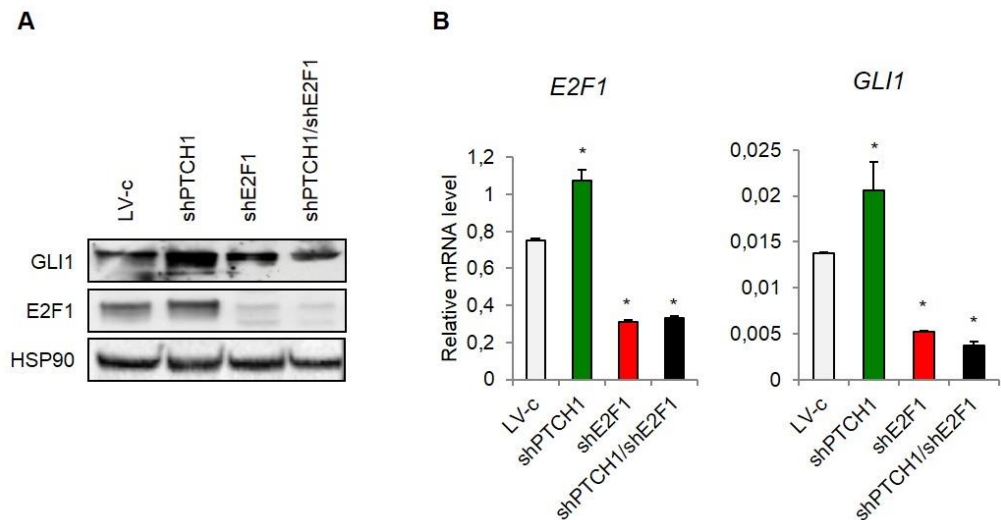
Figure 4.9. *(**A**) Western blot analysis of GLI1 and E2F1 expression in M26c cells transduced with LV-c, LV-shPTCH1, LV-shE2F1 or LV-shPTCH1/LV-shE2F1 lentiviruses. HSP90 was used as loading control. (**B**) Quantitative PCR analysis of E2F1 and GLI1 mRNA levels in M26c cells transduced as described above. The y-axis represents the expression ratio of gene/(EIF2a+HPRT average). *p<0.05 compared to the control.*

To investigate the role of E2F1 in melanoma cell growth induced by HH pathway activation, we transduced M26c, SSM2c and A375 cells with LV-c or LV-shPTCH1 alone or in combination with LV-shE2F1. Cells transduced were seeded and allowed to growth for 7 days. The growth curve showed that in all cell types LV-shPTCH1 increased cell number compared to control (LV-c), whereas LV-shE2F1 reduced it. Interestingly, in cells transduced with both LV-shPTCH1 and LV-shE2F1 lentiviruses we observed a reduction of cell growth stronger than in cells transduced with LV-shE2F1 alone (Figure 4.10). These results identify a critical role of E2F1 in melanoma cell growth induced by activation of the HH pathway.
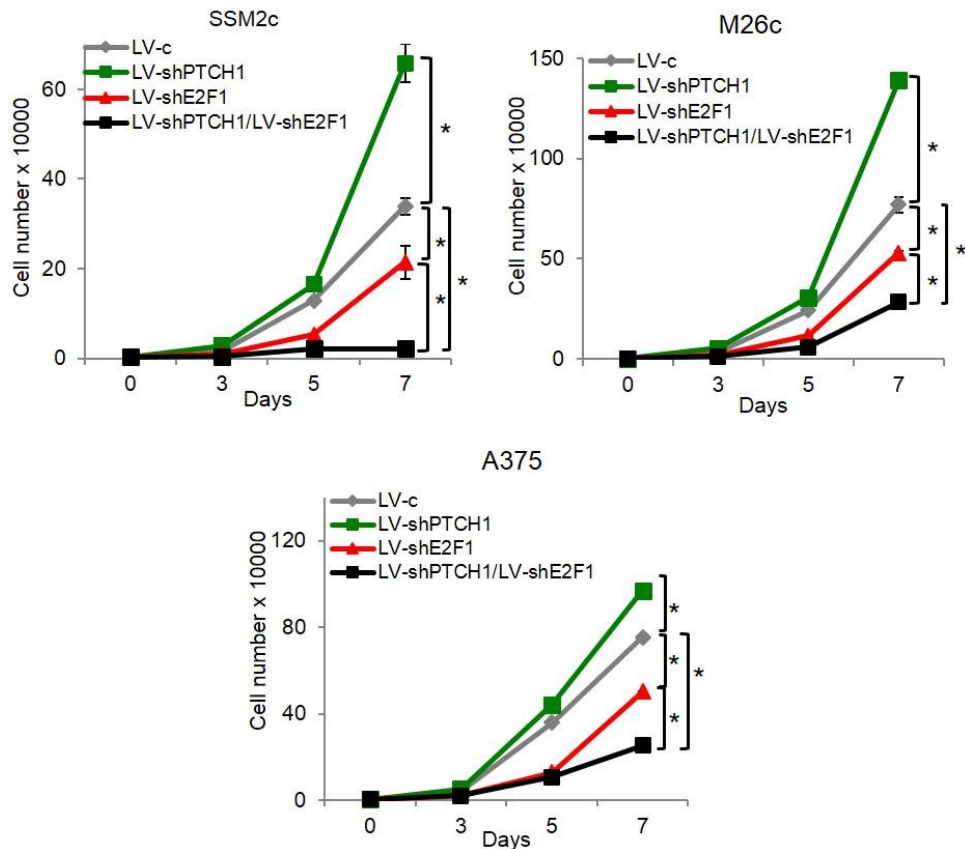
Figure 4.10. *Growth curve in SSM2c, M26c and A375 cells transduced with LV-c, LV-shPTCH1, LV-shE2F1 or LV-shPTCH1/LV-shE2F1 lentiviruses, showing that E2F1 silencing reduced melanoma cell growth and this reduction was stronger in presence of activated HH pathway. Cells transduced with LV-shPTCH1 lentivirus showed an increase of cell growth, consistent with the activation of HH pathway. The data represent mean±SEM of three independent experiments. *p<0.05*

E2F1 is also an important regulator of apoptosis (Pan et al., 1998; Massip et al., 2013). To determine whether the reduction in cell growth observed upon *E2F1* silencing was due to changes in cell proliferation or in cell death, we evaluated the proliferation index by FACS analysis, using CellTrace Violet, in M26c and SSM2c cells transduced with LV-c, LV-shPTCH1, LV-shE2F1 or LV-shPTCH1/LV-shE2F1 lentiviruses. As expected, activation of HH pathway with LV-shPTCH1 led to an increase of cell proliferation, whereas LV-shE2F1 reduced it in both cell lines. In M26c cells transduced with LV-shPTCH and LV-shE2F1 lentiviruses, we did not observe changes in proliferation index,

while in SSM2c cells we observed a slightly reduction in proliferation index compared to LV-shE2F1 transduced cells (Figure 4.11).
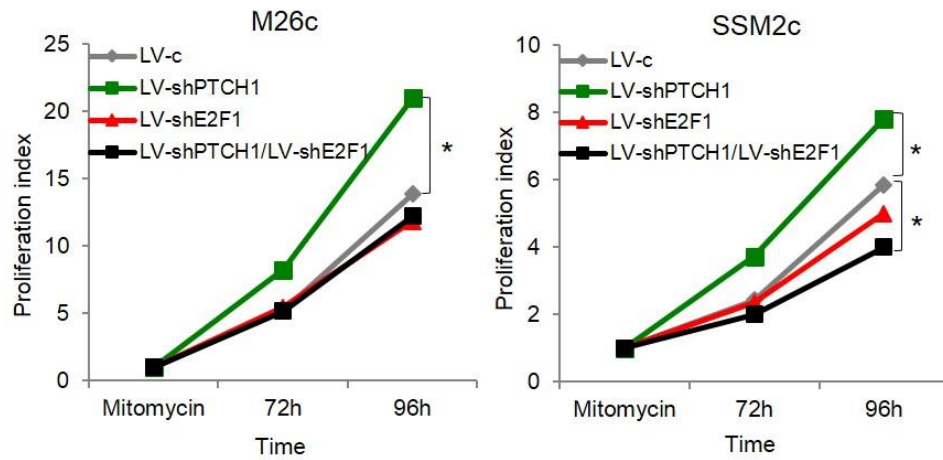

Figure 4.11. *Proliferation index of M26c and SSM2c cells transduced with LV-c, LV-shPTCH1, LV-shE2F1 or LV-shPTCH1/LV-shE2F1 lentiviruses. Proliferation index was evaluated at 72hrs and 96hrs, whereas mitomycin was used to block cell proliferation and was used as control (0hrs), represented on the x-axis. The data represent mean±SEM of three independent experiments. *p<0.05*

We also performed cell cycle analysis using flow cytometry, that showed an increase of percentage of cells in S-phase upon *PTCH1* silencing (Figure 4.12), according to the increase in proliferation observed after HH signalling activation (Santini et al., 2012). In cells where HH pathway was activated in absence of E2F1, cell cycle analysis also showed an increase in the percentage of cells in sub-G_0 phase, corresponding to the apoptotic cellular fraction (Figure 4.12).
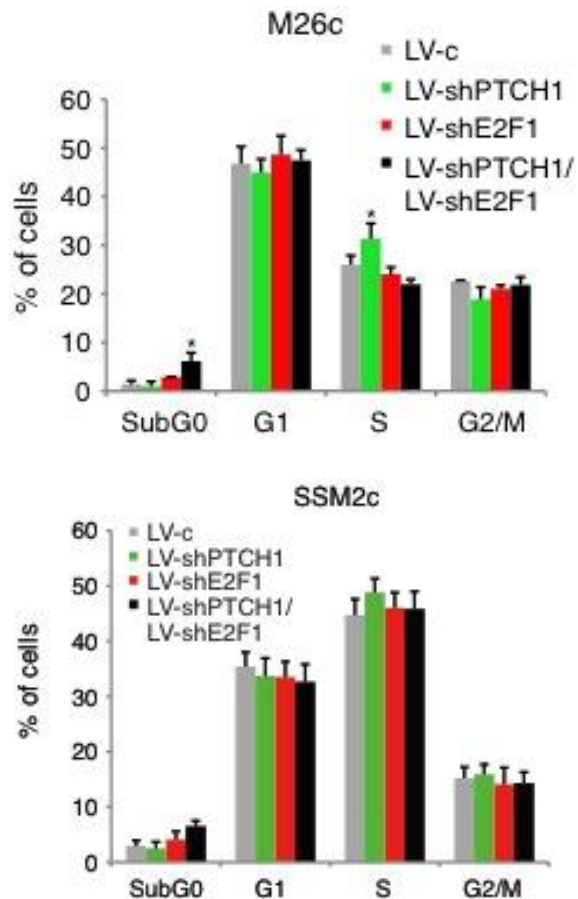
Figure 4.12. *Cell cycle distribution of M26c and SSM2c cells transduced with LV-c, LV-shPTCH1, LV-shE2F1 or LV-shPTCH1/LV-shE2F1 lentiviruses, showing a significant increase of S phase only in M26c cells transduced with LV-shPTCH1. Cells transduced with LV-shPTCH1/LV-shE2F1 lentiviruses show a significant increase in the apoptotic fraction compared to LV-c control. The data represent mean±SEM of three independent experiments. *p<0.05*

The increase of the apoptotic cellular fraction in sub-G_0 phase observed in cell cycle analysis, were confirmed by Annexin V-PE/7-AAD labelling. Cytometric analysis showed an increase of the percentage of cells in late apoptosis in LV-shE2F1 transduced cells, and a bigger increase in cells transduced with LV-shE2F1 in combination with LV-shPTCH1 (Figure 4.13).
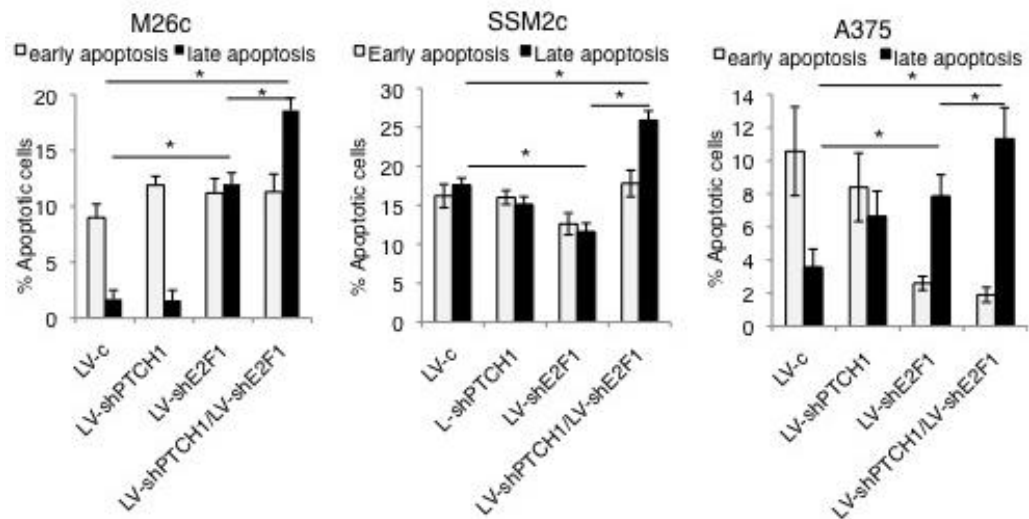
Figure 4.13. *Annexin V-PE/7-AAD labelling of M26c, SSM2c and A375 cells transduced with LV-c, LV-shPTCH1, LV-shE2F1 or LV-shPTCH1/LV-shE2F1 lentiviruses, showing a marked increase of the late apoptotic fraction in M26c and A375 cells transduced with LV-shE2F1 lentivirus, and in SSM2c, M26c and A375 cells transduced with LV-shPTCH1/LV-shE2F1 lentiviruses compared to LV-c control. The data represent mean±SEM of three independent experiments. *p<0.05*

To support these results, we measured the expression levels of genes involved in the apoptotic response. qPCR analysis showed that, upon *E2F1* silencing, the mRNA levels of the anti-apoptotic factor *BCL-XL* decreased, whereas the expression of the pro-apoptotic genes *PIG3* and *p53AIP1*, a p53 target, increased (Figure 4.14A). In line with the data obtained by AnnexinV-PE/7-AAD labelling, only cells with activated HH pathway and with silenced E2F1 (transduced with LV-shPTCH1 and LV-shE2F1 lentiviruses) showed a decrease in the protein level of the anti-apoptotic factor *BCL2* and a slightly increase of p53 (Figure 4.14B).
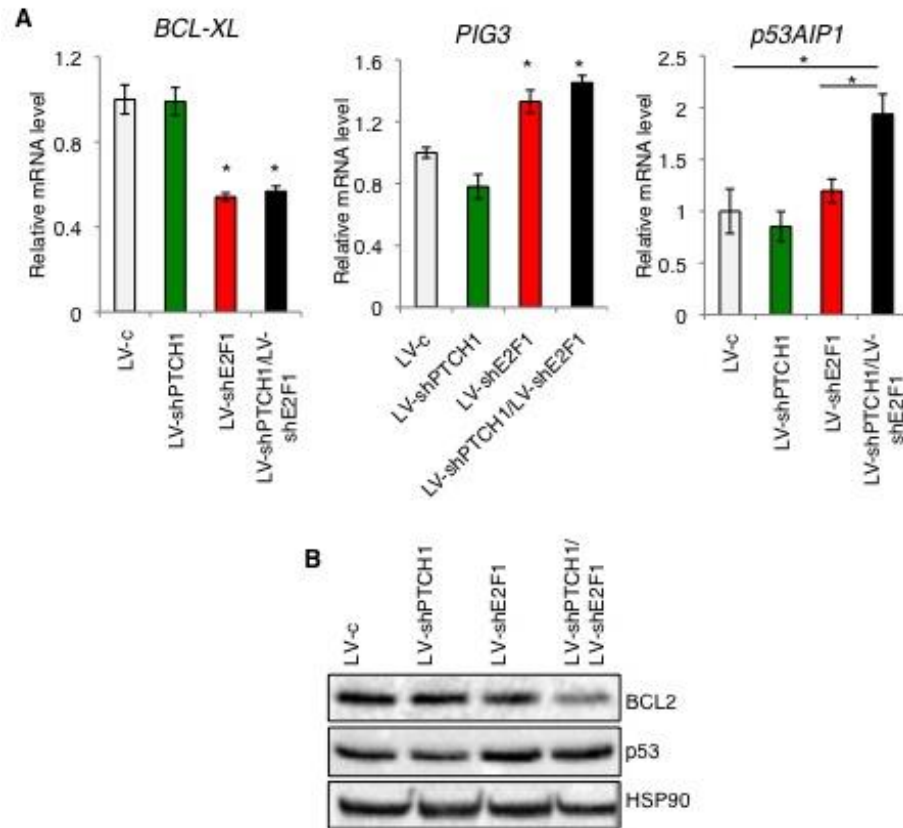
Figure 4.14. *(**A**) Quantitative PCR analysis of BCL-XL, PIG3 and p53AIP1 expression in M26c cells transduced with LV-c, LV-shPTCH1, LV-shE2F1 or LV-shPTCH1/LV-shE2F1 lentiviruses. The y-axis represents the expression ratio of gene/(EIF2a+HPRT average). *p<0.05 compared to the control (**B**) Western blot analysis of BCL2 and total p53 in M26c cells transduced with the indicated lentiviruses. HSP90 was used as loading control.*

These findings indicate that activation of HH pathway in absence of E2F1, rather than increasing cell proliferation, enhances apoptosis, probably as a result of restoration of p53 activity, which is often impaired in melanoma cells (Lu et al., 2013). This result suggests that E2F1 controls the balance between cell proliferation and apoptosis upon the activation of HH signaling; activation of HH signaling in presence of E2F1 increases melanoma cell proliferation, whereas in absence of E2F1 induces apoptosis in melanoma cells.

4.5 HH signaling modulates iASPP expression and activation through E2F1

Recent evidence indicate that iASPP is frequently up-regulated in human cancers (Bergamaschi et al.,2003; Bergamaschi et al., 2006), and that human melanomas harboring wt p53 express high levels of phosphorylated iASPP. Functionally, phosphorylated iASPP binds and inactivates p53 (Lu et al., 2013). To test whether HH pathway modulates iASPP expression, we transduced SSM2c and M26c cells with LV-c or LV-shPTCH1 lentiviruses to activate HH signaling or with LV-c or LV-shSMO lentiviruses to inhibit the pathway. qPCR analysis showed an increase of *GLI1* mRNA levels upon *PTCH1* silencing, confirming the activation of HH pathway. This was associated with an increase of *iASPP* expression (Figure 4.15A). Conversely, *iASPP* mRNA levels were reduced upon inhibition of the HH pathway, that was confirmed by the reduction of *GLI1* expression (Figure 4.15B).

Figure 4.15. *(A) Quantitative PCR analysis of GLI1 and iASPP expression in SSM2c and M26c cells transduced with LV-c or LV-shPTCH1 lentiv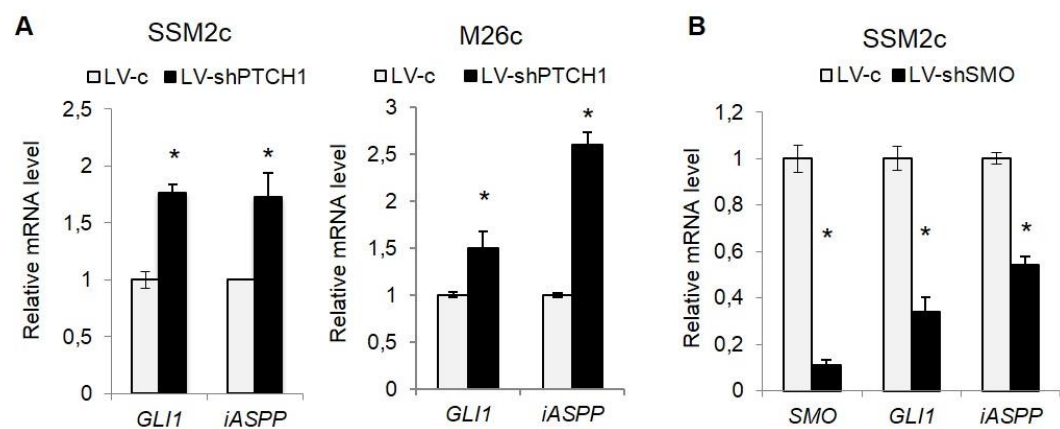iruses. (B) Quantitative PCR analysis of SMO, GLI1 and iASPP expression in SSM2c cells transduced with LV-c or LV-shSMO lentiviruses. The y-axis represents the expression ratio of gene/(EIF2a+HPRT average). *p<0.05 compared to the control.*

These results suggest that HH signaling positively modulates iASPP expression at transcriptional level. To address the mechanism by which E2F1

controls the outcome of the activation of HH signaling in our patient-derived p53wt melanoma cells, we measured iASPP levels in M26c cells transduced with LV-c, LV-shPTCH1 or LV-shE2F1 lentiviruses alone or in combination. Western blot and qPCR analysis showed that iASPP mRNA and protein levels were increased upon *PTCH1* silencing, and that *E2F1* silencing led to a decrease of iASPP protein and mRNA levels (Figure 4.16A and B). In cells transduced with both LV-shPTCH1 and LV-shE2F1, iASPP mRNA and protein levels did not increase and were lower than cells transduced with LV-shE2F1 alone and LV-c control cells (Figure 4.16A and B). These results indicate that HH signalling regulates iASPP expression through E2F1.
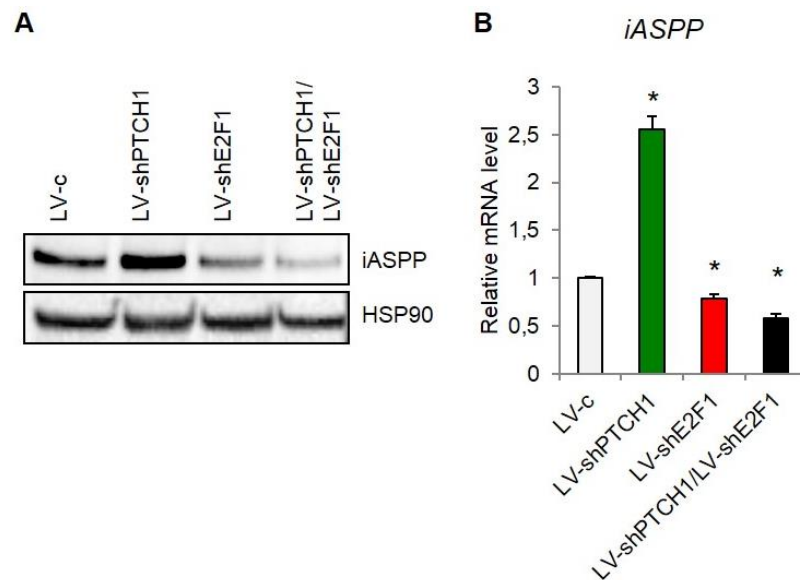
Figure 4.16. *(**A**) Western blot analysis of M26c cells transduced with LV-c, LV-shPTCH1, LV-shE2F1 or LV-shPTCH1/LV-shE2F1 lentiviruses. HSP90 was used as loading control. (**B**) Quantitative PCR analysis of iASPP mRNA levels in M26c cells transduced with the indicated lentiviruses. The y-axis represents the expression ratio of gene/(EIF2a+HPRT average). *p<0.05 compared to the control.*

Bioinformatic analysis with Transfac Matrix Database identified a conserved E2F1 binding site in *iASPP* promoter. We performed chromatin immunoprecipitation (ChIP) assay to confirm this finding. M26c and SSM2c cells were transduced with LV-c or LV-shPTCH1 lentiviruses and we performed ChIP assay by immunoprecipitating endogenous E2F1. qPCR

analysis on recovered DNA showed an enrichment of the *iASPP* promoter that was stronger in cells with activated HH pathway compared to LV-c control cells (Figure 4.17A). These results suggest that E2F1 mediates the transcriptional regulation of iASPP expression induced by activation of HH pathway.

The inhibitory function of iASPP on p53 results from its phosphorylation status that depends on the CDK1/Cyclin B1 complex (Lu et al., 2013). Thus, we evaluated whether HH pathway might modulate CDK1 and Cyclin B1 levels. Western blot analysis showed that CDK1 protein levels were completely abolished upon *E2F1* silencing both in M26c cells transduced with LV-shE2F1 and LV-shPTCH1/LV-shE2F1 (Figure 4.17B), accordingly with a previous study indicating that E2F1 regulates CDK1 expression (Wu et al., 2012). ChIP assay in SSM2c cells transduced with LV-c or LV-shPTCH1 lentiviruses showed that E2F1 bound to *CDK1* promoter (Figure 4.17C), confirming a direct regulation (Konishi et al., 2003). Notably, the enrichment of CDK1 promoter was greater in LV-shPTCH1 transduced cells compared to LV-c control cells, in line with E2F1 induction. Cyclin B1 expression increases after HH pathway activation, as shown by western blot analysis of M26c transduced cells (Figure 4.17B), confirming previous reports (Eichberger et al., 2006; Locker et al., 2006). The increase in Cyclin B1 protein levels was independent of E2F1, because it occurred also in cells transduced with LV-shPTCH1/LV-shE2F1 lentiviruses (Figure 4.17B).

Figure 4.17. *(**A**) Chromatin immunoprecipitation assay. Endogenous E2F1 was immunoprecipitated from M26c and SSM2c cells transduced with LV-c or LV-PTCH1 lentiviruses. Quantitative PCR shows the enrichment of iASPP promoter in cells with activated HH pathway (LV-shPTCH1). The y-axis represents the relative promoter enrichment, normalized on input material. ACTIN promoter was used as negative control. (**B**) Western blot analysis of CDK1 and Cyclin B1 in M26c cells transduced with LV-c, LV-shPTCH1, LV-shE2F1 or LV-shPTCH1/LV-shE2F1 lentiviruses. HSP90 was used as loading control. (**C**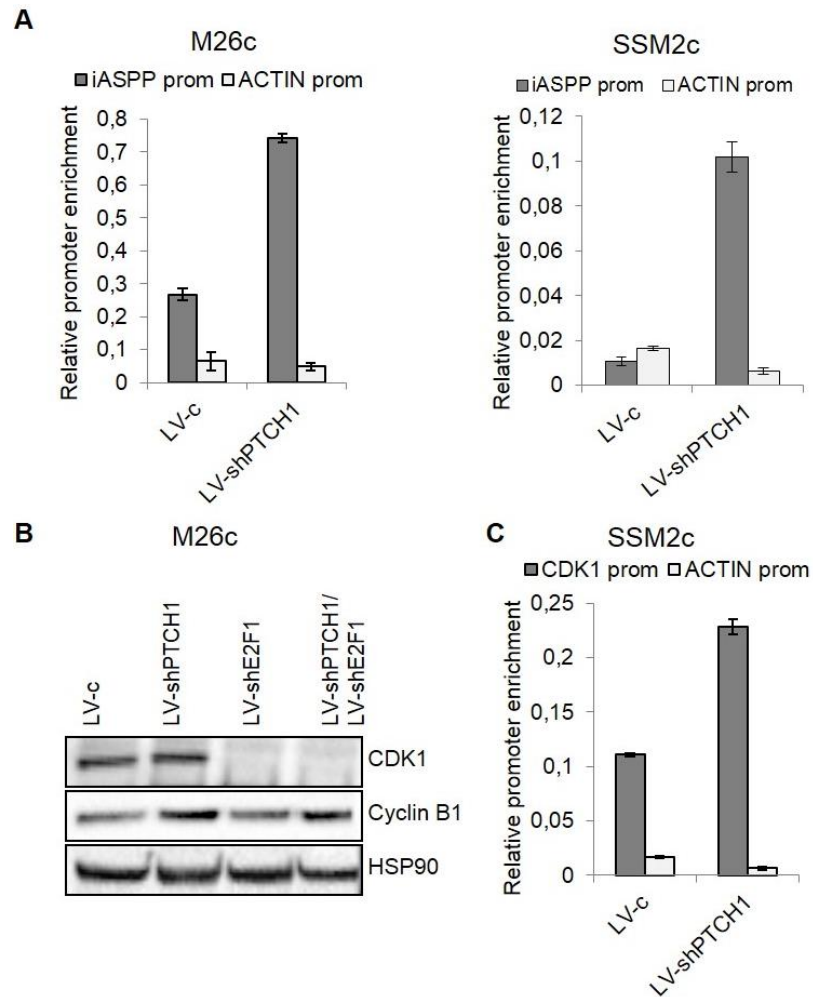) Chromatin immunoprecipitation assay. Endogenous E2F1 was immunoprecipitated from SSM2c cells transduced with LV-c or LV-shPTCH1. Quantitative PCR shows the enrichment of CDK1 promoter in cells with activated HH pathway (LV-PTCH1). The y-axis represents the relative promoter enrichment, normalized on input material. ACTIN promoter was used as negative control.*

These results indicate that HH signaling positively modulates iASPP at multiple levels. First, HH pathway contributes to induction of iASPP expression through E2F1, which directly binds to *iASPP* promoter. HH signaling also contributes to iASPP activation through E2F1-dependent regulation of CDK1 and through modulation of Cyclin B1 in an E2F1-independent manner.

To confirm that the activation of HH signaling controls iASPP function through E2F1, we treated A375, SSM2c, and M26c cells transduced with LV-c or LV-shPTCH1 lentiviruses with the CDK1 inhibitor JNJ-7706621 (JNJ), which at the dose of 500nM specifically inhibits CDK1 (Emanuel et al., 2005). JNJ-7706621 inhibitor was recently shown to block iASPP phosphorylation (Lu et al., 2013) (Figure 4.18A). Growth assay showed a reduction in cell number in melanoma cells treated with JNJ compared to untreated cells, and this reduction was stronger in cells transduced with LV-shPTCH1 (with activated HH pathway) compared to cells transduced with LV-c control cells (Figure 4.18B). These data show that cells with activated HH pathway are more sensitive to CDK1 inhibition, paralleling the results obtained by *E2F1* silencing (Figure 4.10).
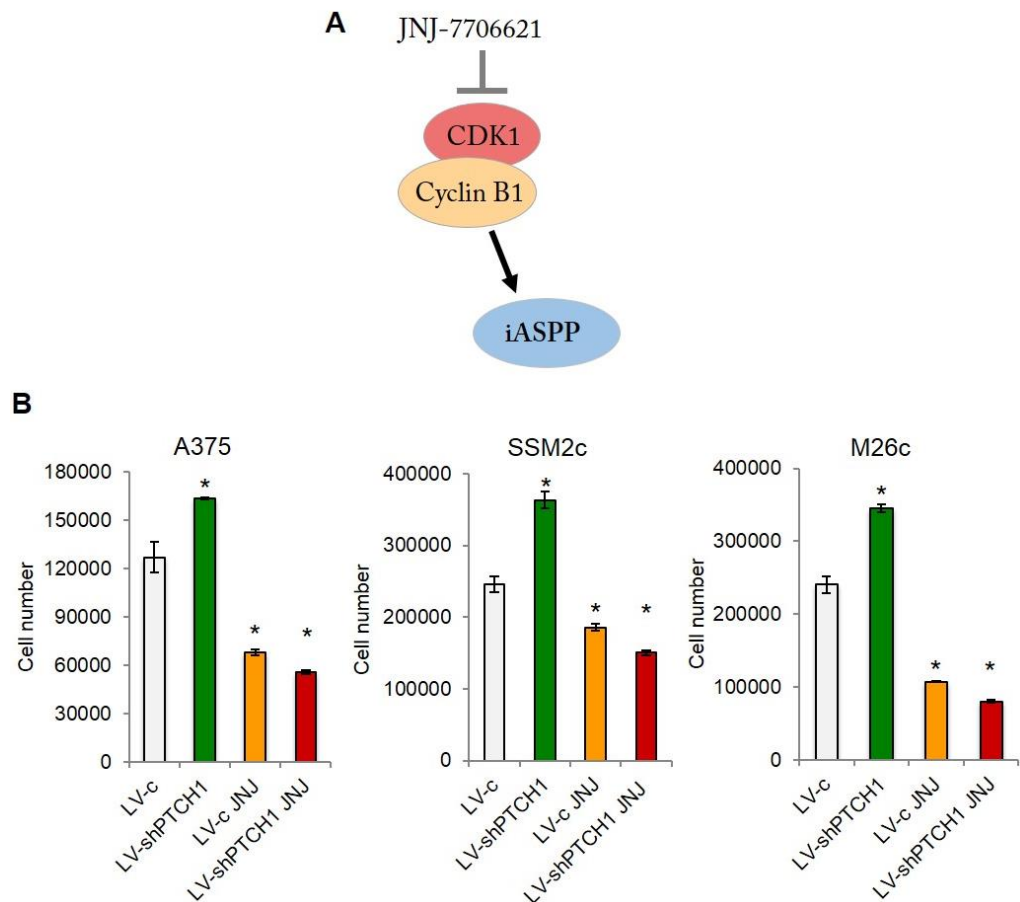
Figure 4.18. (A) *Schematic representation of iASPP phosphorylation by the CyclinB1/CDK1 complex. This phosphorylation is reduced when cells are treated with the CDK1 inhibitor JNJ-7706621.* (B) *Cell growth assay in A375, SSM2c and M26c cells transduced with LV-c or LV-shPTCH1 lentiviruses after treatment with 500nM JNJ-7706621 for 72hrs. The data represent mean±SEM of three independent experiments. *p<0.05*

We also evaluated the percentage of apoptotic cells by cytometric analysis after Annexin-V/7AAD labelling in SSM2c, M26c and A375 cells transduced as described above. We observed that cells with activated HH signaling were more sensitive to JNJ treatment than control cells. We found an increase of cells in late apoptosis upon treatment with the CDK1 inhibitor, with a stronger increase in cells transduced with LV-shPTCH1 (Figure 4.19).
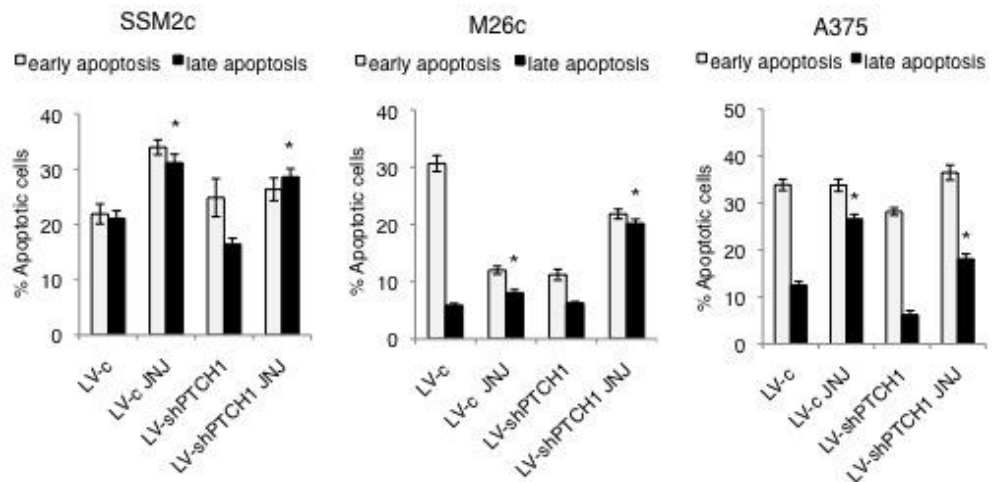
Figure 4.19. *Evaluation of cellular apoptosis by AnnexinV/7AAD labelling in SSM2c and M26c cells transduced with LV-c and LV-shPTCH1 lentiviruses after treatment with JNJ-7706621 500nM for 48hrs. These data represent mean±SEM of three independent experiments. *p<0.05 compared to LV-c and LV-shPTCH1 respectively.*

Altogether, these findings indicate that E2F1 determines the outcome of HH pathway activation by regulating the expression and the activation of iASPP.

4.6 E2F1 mediates HH-induced melanoma xenograft growth

To investigate the role of E2F1 in regulating growth of melanoma xenografts *in vivo* induced by HH pathway activation, we injected $4x10^4$ M26c cells stably transduced with LV-c, LV-shPTCH1 and/or LV-shE2F1 lentiviruses subcutaneously into athymic nude mice (Figure 4.20A). M26c cells transduced with LV-shPTCH1 resulted in 2-fold larger xenografts than LV-c control cells (Figure 4.20B), confirming that the activation of HH pathway increases melanoma cell growth *in vivo* (Santini et al., 2012; Pandolfi et al., 2013). *E2F1* silencing reduced of nearly 50% the size of melanoma xenografts compared to LV-c transduced cells, and strongly reduced the increase of tumor growth due to *PTCH1* silencing (Figure 4.20B). Compared to *in vitro* studies, the difference in xenografts growth between cells transduced with LV-shE2F1 alone or in combination with LV-shPTCH1 was not significant (Figure 4.20B).
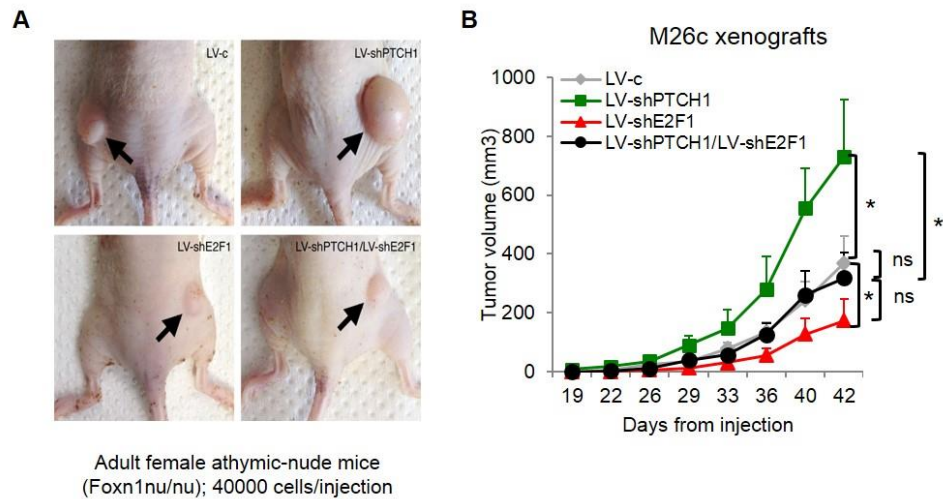
Figure 4.20. (A) Representative images of M26c xenografts into athymic nude mice. (B) M26c cells transduced with LV-c, LV-shPTCH1, LV-shE2F1 or LV-shPTCH1/LV-shE2F1 lentiviruses were injected subcutaneously into athymic-nude mice. Quantification of tumor volume (n=12 for each group), showing that E2F1 silencing prevented the increase of tumor growth induced by activation of HH pathway. P-values are as follows: LV-c versus LV-shPTCH1, p=0.047; LV-c versus LV-shE2F1, p=0.049; LV-shPTCH1 versus LV-shPTCH1/LV-shE2F1, p=0.043.

Tumors were dissected 42 days after injection. Western blot analysis of GLI1, E2F1, iASPP, and CDK1 confirmed the reduction of E2F1 protein levels upon *E2F1* silencing, and its increase following HH pathway activation (Figure 4.20), consistently with tumor growth curve. Western blot analysis showed protein levels of iASPP, E2F1, and CDK1 (Figure 4.21) with the same pattern obtained *in vitro* (Figure 4.9A, Figure 4.16A, and 4.17B).
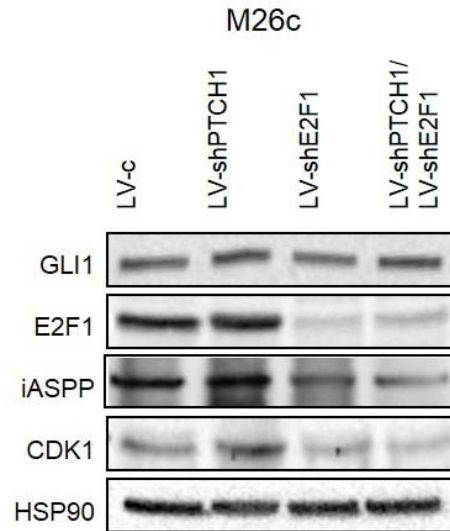
Figure 4.21. *Western blot analysis of GLI1, E2F1, iASPP, and CDK1 in tumors derived from M26c xenografts. HSP90 was used as loading control.*

Taken together these data suggest that E2F1 plays a crucial role as mediator of HH signaling pathway in promoting growth of melanoma xenografts.

5. DISCUSSION

In this study, we identify the transcription factor E2F1 as direct target of the GLI1 and GLI2, and a crucial mediator of the effects induced by HH pathway activation. Interestingly, we show that HH/GLI-E2F1 axis positively modulates the oncoprotein iASPP, a key inhibitor of p53, at multiple levels. First, HH activation induces iASPP expression through E2F1, which directly binds to iASPP promoter. Second, HH pathway also contributes to iASPP activation by induction of Cyclin B1 and by E2F1-dependent regulation of CDK1. Our data together suggest a novel HH/GLI-E2F1-iASPP axis that is involved in controlling melanoma growth and provide an additional mechanism through which HH signaling restrains p53 function during tumorigenesis (Figure 5.1).

The Hedgehog-GLI (HH-GLI) signaling is a conserved pathway, which is an important mediator of embryonic development. In adult tissue, HH signaling is critical for maintaining tissue polarity in vertebrate and invertebrate embryos. HH-GLI signaling is also involved in regulating growth and survival of human melanomas both *in vitro* and *in vivo* (Stecca et al., 2007) and it drives self-renewal and tumorigenicity of melanoma-initiating cells (Santini et al., 2012). The first evidence of an interplay between HH-GLI pathway and the transcription factor E2F1 was provided by discovery that the transcription factor GLI2, one of the final effectors of HH pathway, enhances E2F1 expression in keratinocytes (Regl et al., 2004). Additional findings showed that SHH stimulation induces E2F1 expression in neural precursors and in medulloblastoma (Bahtia et al., 2011). These data indicate that E2F1 is a HH-responsive gene, however, there is no evidence that indicate a direct transcriptional regulation of E2F1 by HH pathway. Here we show that E2F1 expression is directly regulated by the HH signaling effectors, GLI1 and GLI2, in melanoma cells and that both GLI1 and GLI2 are able to bind and to transactivate *E2F1* promoter. Although bioinformatics analysis did not identify a GLI canonical consensus sequence on *E2F1* promoter, we identified two

putative non-canonical GLI consensus sequences: site 1 (CGCCTCCAG) and site 2 (TACCGCCCC), located between -132bp and -269bp upstream the *E2F1* transcription start site. Mutation of site 1 prevented the transactivation of the reporter by GLI1 and GLI2, whereas mutation of site 2 did not have any effect, suggesting that GLI1 and GLI2 directly regulate the expression of E2F1 by binding to the non-canonical GLI consensus sequence CGCCTCCAG in *E2F1* promoter. This degenerate binding site contains 5 base pair substitutions respect to the canonical consensus sequence (GAC**CA**CCCA), but it maintains the two cytosines in 4th and 6th position flanking the central adenine in the consensus sequence (GAC**CA**CCCA), which are essential for binding as suggested by previous studies (Winklmayr et al., 2010; Peterson et al.,2012).

Sequence-specific DNA binding to the cis-regulatory region of a GLI target gene mainly involves zinc fingers 4 and 5, which make extensive base contact within the 9-mer binding sequence, while fingers 2 and 3 mainly establish contacts with the phosphate backbone (Pavletich et al., 1993). Although global ChIP analyses and *in vitro* GLI-DNA binding screenings confirmed the consensus sequence as dominant binding site for the GLIs, the importance of GLI binding sequences with some degree of base pair substitutions is underappreciated. Certainly, variations of the consensus sequence might have significant impact on the transcriptional output in response to defined GLI activator levels. For instance, substitution of the cytosine at position 7th for adenine results in a GLI binding site with enhanced transcriptional response compared to the canonical consensus motif (Winklmayr et al., 2010). The degenerate consensus sequence we identify in our study retains a strong transcriptional response for both GLI1 and GLI2, although GLI1 induces a stronger response than GLI2. This is predictable because, although all GLI proteins bind the 9-mer consensus sequence with comparable affinity, repressor and activator forms bind the same sites, and different GLI proteins affect the same target genes differently. Also differential epigenetic modifications of the cis-regulatory regions of GLI targets affects GLI-DNA binding affinity. Cell-type specific histone acetylation of methylations and/or CpG methylation patterns of GLI target gene promoters are thus likely to modulate both the qualitative and quantitative response to

GLI. Therefore, it will be interesting to test epigenetic modifications of the E2F1 promoter in the future. Moreover, it should be noted that our ChIP experiments were performed with endogenous GLI1 and GLI2. Most of the global ChIP approaches were performed with epitope tagged and overexpressed GLI (Vokes et al., 2007; Vokes et al., 2008; Lee et al., 2010), which may not fully mimic endogenous GLI function.

The biological relevance of the direct regulation of E2F1 by GLI1 and GLI2 is supported by our expression data in human melanoma samples, that indicate a significant correlation between HH pathway components (PTCH1, GLI1 and GLI2) and E2F1 expression in human metastatic melanomas. This might suggest that during melanoma progression activation of the HH signaling leads to a strong induction of E2F1 expression.

The transcription factor E2F1 is a crucial modulator of different cellular processes, including cell cycle progression and apoptosis (O'Connor et al., 2000). Several lines of evidence indicate that E2F1 is often aberrantly activated in various types of cancer, including melanoma (Halaban et al., 2000; Nelson et al., 2006) and are frequently associated with poor patient survival. E2F1 is an HH-responsive gene, but it is not known what are the E2F1-mediated effects inducing by HH signaling activation. In this study, we identify a functional role of E2F1 in regulating melanoma cell growth upon the activation of HH pathway. In particular, our data show that the silencing of E2F1 reduces melanoma cell growth *in vitro* and increases apoptotic response. Interestingly, these effects are stronger when E2F1 depletion is couple with activated HH signaling. These data suggest the presence of a HH/GLI-E2F1 axis that might contribute to melanoma progression, and indicate that the effects of activated HH pathway in absence of E2F1, rather than increasing cell proliferation, enhances apoptosis response, probably as a result of restoration of p53 activity.

The main tumor-suppressor p53 is frequently mutated in human cancers; nevertheless more than 80% of melanomas retain wild type p53, but often it is inactive and its function impaired by several mechanisms, and p53 target genes involved in apoptosis are under-expressed (Avery-Kiejda et al., 2011).

Previous evidence have shown that HH signaling contributes to reduce the tumor-suppressor function of p53 by increasing the levels of MDM2, a known negative modulator of p53 (Abe et al., 2008; Stecca and Ruiz I Altaba, 2009). In turn, GLI1 transcriptional activity is negative regulated by p53, promoting GLI1 ubiquitination and degradation (Stecca and Ruiz I Altaba, 2009). Recently, another mechanism of p53 inactivation has been proposed, in which the inhibitor of p53 iASPP might mediate this mechanism. The p53 inhibitor iASPP is frequently over-expressed in human cancers (Zhang et al., 2005; Saebø et al., 2006; Liu et al., 2012; Chen et al., 2010; Cao et al., 2013), including melanoma, suggesting that iASPP contributes to tumorigenesis by inducing proliferative and anti-apoptotic effects. However, little is known about the regulation of iASPP expression. Here we find that HH-GLI pathway activation positively modulates iASPP levels in melanoma cells, enhancing its expression upon HH signaling activation. In addition, we show that E2F1 directly mediates this induction binding to *iASPP* promoter. Previous data have shown that iASPP activation is controlled by Cyclin B1/CDK1 complex that phosphorylates iASPP protein (Lu et al., 2013). Consistently, our findings indicate that HH pathway activation controls the levels of both Cyclin B1 and CDK1, thus contributing to iASPP activation. Moreover, we find that the expression of Cyclin B1 increases upon HH pathway activation, in according to literature (Eichberger et al., 2006; Locker et al., 2006), and this regulation do not require E2F1. Conversely, we find that CDK1 expression is modulated by HH signaling through E2F1 (Wu et al., 2012), which binds to *CDK1* promoter.

In this study, we provide evidence that E2F1 is a mediator of HH signaling also *in vivo*. Previous data showed that the HH-GLI pathway is active and required for melanoma cells proliferation and xenografts growth *in vivo* (Stecca et al., 2007). Although HH pathway activation induces xenografts growth, we find that the silencing of E2F1 in presence of HH signaling activation counteracts this increase. In this case, we do not find significant difference in tumor size between LV-shE2F1 and LV-shPTCH1/LV-shE2F1 xenografts, as describe by *in vitro* results, that show a greater reduction of cell growth upon E2F1 silencing coupled with HH signaling activation. This discrepancy between *in vitro* and *in vivo* results probably is due to other mediators induced

by activated HH signaling, such as for instance stemness factors. These factors *in vivo* can promote cancer stem cell self-renewal and induce tumor growth, independently of E2F1. However, western blot analysis on xenografts shows the same pattern of iASPP and CDK1 expression obtained *in vitro*.

The reactivation of wt p53 function is the major challenge in cancer, and it has been proposed as a novel therapeutic approach to suppress melanoma growth, in combination with target therapy (Jochemsen et al., 2014; Lu et al., 2014). Lu and colleagues have shown that p53 function can be restored using a CDK1 inhibitor that affects cyclinB1/CDK1 activity preventing iASPP phosphorylation (Lu et al., 2013). Our findings indicate that the treatment with CDK1 inhibitor (JNJ-7706621), which prevents iASPP phosphorylation and activation, induces the same effects of E2F1 depletion. Cells treated with CDK1 inhibitor show a reduced cell proliferation and an increased apoptosis; moreover, we observe that cells with activated HH pathway are more sensitive to JNJ-7706621 treatment compared to the control. Thus, the inhibition of iASPP phosphorylation through E2F1 silencing or JNJ-7706621 treatment might be used as a therapeutic approach to resume the p53 tumor-suppressor function that leads, lastly, to an increase of the apoptotic response, suggesting the activation of p53.

In conclusion, we propose a novel HH/GLI-E2F1-iASPP axis, which is involved in regulation of melanoma cell growth, and provides a possible novel mechanism through which activation of HH signaling impairs p53 function. In addition, we provide a further strategy for therapeutic treatment of melanomas and, probably other tumors with activated HH pathway and wt p53.
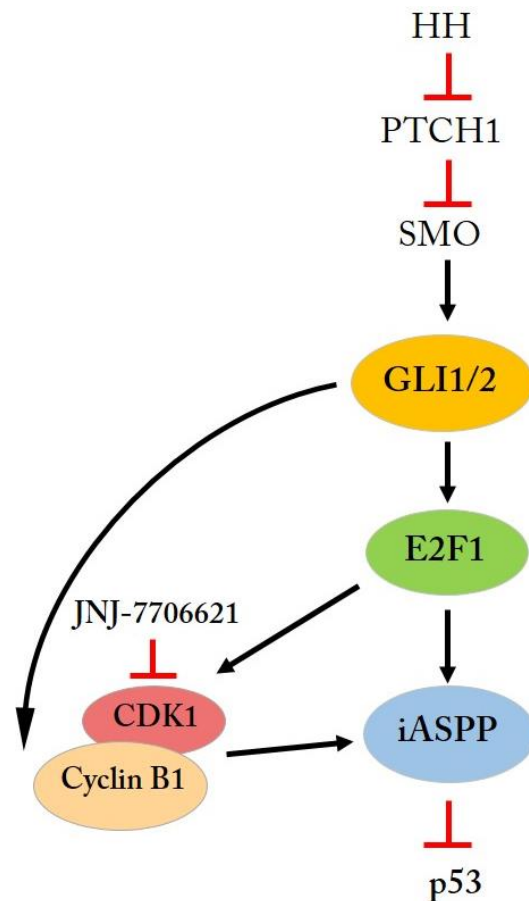
Figure 5.1: *Model of the HH/GLI-E2F1-iASPP axis. E2F1 is a direct target of the transcription factors GLI1 and GLI2. E2F1 acts downstream of HH signaling, modulating the expression and the activation of the p53 inhibitor iASPP. E2F1 regulates the expression of iASPP and CDK1. The final effectors of HH pathway, GLI1 and GLI2, modulate Cyclin B1 expression independently of E2F1. Cyclin B1/CDK1 complex phosphorylates iASPP leading to an increase of its ability to impair p53 function. JNJ-7706621 is a CDK1 inhibitor.*

6. REFERENCES

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